ADJUVANTS IN LABORATORY ANIMALS

evaluation of immunostimulating properties and side effects
of Freund's complete adjuvant and alternative adjuvants
in immunization procedures

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ADJUVANTIA IN PROEFDIEREN

evaluatie van immunostimulerende eigenschappen en bijwerkingen
van Freund's complete adjuvant en alternatieve adjuvantia
in immunisatie procedures

PROEFSCHRIFT

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Contents

List of abbreviations

AP	alkaline phosphatase
APC	antigen-presenting cells
BSA	bovine serum albumin
CD40L	CD40 ligand
Cl_2MDP	dichloromethylene-diphosphonate
CWS	cell wall skeleton
DDA	dimethyldioctadecylammonium bromide
d.f.p.	dorsal foot pad
ELISA	enzyme linked immunosorbent assay
FA	Freund's adjuvant
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
HRP	horseradish peroxidase
i.d.	intradermal(ly)
IDC	interdigitating cells
IFN-γ	interferon-gamma
Ig	immunoglobulin
IL	interleukin
i.m.	intramuscular(ly)
i.p.	intraperitoneal(ly)
iscom	immune-stimulating complex
kDa	kilo dalton
LN	lymph node
LPS	lipopolysaccharide
MΦ	macrophage(s)
mAb	monoclonal antibodies
MBP	myelin basic protein
MPL	monophosphoryl lipid A
NBP	nonionic block polymers
OD	optical density
PBS	phosphate buffered saline
PLN	popliteal lymph node
POT	primary observation test
RV-ISCOMs	rabies virus-ISCOMs
s.c.	subcutaneous(ly)
SD	standard deviation
SP	synthetic peptide
TDM	trehalose dicorynomycolate
Th	T helper
TNP-KLH	2,4,6-trinitrophenyl-keyhole limpet haemocyanin
w/o	water-in-oil

Chapter 1

Introduction

Introduction

The body has a variety of defense mechanisms to protect it against invasion of potentially harmful agents such as viruses, bacteria, and parasites. The first line of defense is a more physical barrier e.g. skin and mucosa. When organisms succeed in passing this first line of defense and enter the body, the immune system is activated and the ensuing immune response generally leads to elimination of the invaders and often results in resistance to the organism. The immune response involves the specific recognition of the organism which leads to humoral and/or cellular immunity and memory. Through the formation of memory cells the immune system is capable of responding to the same organism more rapid and effective upon subsequent encounter. In the humoral immune response, antibody producing B cells play a central role. Antibodies are secreted in body fluids and can bind to organisms with high specificity. Cellular immunity is primarily exerted by T cells, consisting of cytotoxic T cells, which kill virus-infected and tumor cells, or helper T cells which regulate the function of other cells via secreted cytokines.

For different reasons, immune responses can be evoked in humans or animals via injection of an antigen (= substance to which an immune response is induced). For example, for vaccination purposes, humans and animals are injected with antigen to induce protection against harmful pathogens. Animals can be injected with antigen to generate specific antibodies applicable in research, diagnosis and therapy. Injection of an antigen is not always sufficient to induce an effective immune response. When this is the case, the immune system needs help. To augment an effective immune response, adjuvants (Latin: *adjuvare*, to help) may be used. Adjuvants are substances (e.g. mineral salts, oil emulsions, bacterial products, saponins) which upon administration lead to non-specific immuno-stimulation. The non-specific immunostimulation can be used to enhance the specific immune response to the antigen. Adjuvants can influence the magnitude as well as the quality (e.g. isotype, specificity and avidity of antibodies and cytokine profiles of T cells) of the induced immune response. Besides the intended stimulation of the immune response, adjuvants can lead to unintended stimulation of different reactions. This unintended stimulation, induced by adjuvants, can result in unwanted side effects like fever, granulomatous inflammation, neurological disorders and carcinogenicity. When selecting an adjuvant, the immunostimulating and side effects of the adjuvant have to be evaluated. The intended immunostimulating effects of adjuvants are determined by the goal of the immunization. When the adjuvant is used in a vaccine, the goal is to induce immune responses and memory sufficient to protect against a harmful organism while for the production of antibodies the main goal is to elicit the highest specific antibody response achievable. The acceptability of side effects is determined by the species in which the adjuvant is applied. Two categories for adjuvant application can be distinguished: (1) applications for which

the efficacy aspect is more important than the safety aspect (e.g. laboratory and production animals) and (2) applications where safety is more important than efficacy (e.g. companion animals and humans). This thesis emphasizes the use of adjuvants for the production of antibodies in laboratory animals. The requirements of adjuvants for this purpose are: high antibody levels to a broad range of antigens, commercial availability, ease of use, and low price. Freund's complete adjuvant is often used for production of antibodies in laboratory animals because this adjuvant meets all these requirements. In 1993, before the 'Code of Practice for immunization of laboratory animals' (Veterinary Public Health Inspectorate, 1993) was issued in the Netherlands, 64% of the researchers used Freund's adjuvant for routine immunizations and in 1995 two years after publication, still 52%, albeit more selective and sometimes in adapted protocols. FCA consists of a non-metabolizable mineral (paraffin) oil and heat-killed *Mycobacterium butyricum* or *Mycobacterium tuberculosis* and can induce, besides effective immune responses, severe side effects. These side effects include fibrous adhesions and sterile peritonitis after intraperitoneal injection and ulceration, sterile abscesses, and focal necrosis after subcutaneous injection.

Growing public concern about the use of animals for research, is leading to new and stricter regulations; the use of FCA is or will be restricted in several countries because of its severe side effects. In the Netherlands, restrictions on the use of FCA were given in the 'Code of Practice for immunization of laboratory animals' (Veterinary Public Health Inspectorate, 1993). Selection of less toxic adjuvants became necessary. Various adjuvants, which might serve as an alternative, are available. However, adequate selection of an alternative to FCA is difficult because sufficient combined information on antibody responses and side- effects of these alternatives is usually lacking.

Aim of the study

The aim of the studies presented in this thesis was to compare FCA and several commercially available adjuvants on immunopotentiating properties and side effects, providing a basis for recommendations on the use of adjuvants in immunization procedures and ultimately reduce pain and distress in immunized animals.

Introduction to the chapters

In this study, emphasis is put on the evaluation of adjuvants to reduce pain and distress in immunized laboratory animals. Besides the adjuvant, other factors contribute to, or even determine, efficacy and side effects of an immunization. Such factors include: antigen type and dose, site of injection, scheduling of immunizations in time, genetic make-up and animal species. In Chapter 2, factors influencing the final result of an immunization are described. Chapter 2 may serve as a toolkit to

optimize *in vivo* immunizations of laboratory animals to generate antigen specific antibodies.

Adequate selection of an adjuvant is not possible from the literature overview in Chapter 2. Consequently, we conducted a comparative study in rabbits (Chapter 3) to obtain information on efficacy and side effects of commercially available adjuvants. FCA ('gold standard') and three commercially available adjuvants (Specol, TiterMax, RIBI) were evaluated in one study for their efficacy and side effects after injection in combination with three weak immunogens. Efficacy was evaluated based on specific antibody production and side effects on clinical, behavioural and pathological changes in those rabbits.

Rabbits are used for the production of polyclonal antibodies, whereas mice are the prime donors of B cells for preparation of monoclonal antibodies. Therefore we also wanted to study efficacy and side effects of adjuvants in mice. Special attention is paid to our observation in Chapter 3, that minimal clinical signs of pain were observed while pathological lesions were severe. In Chapter 4, behavioural studies were performed in mice to be able to detect possible painful side effects. Besides FCA, Specol was also studied in mice since pathological changes in rabbits were minimal and antibody responses were comparable to those observed after immunization with FCA. In this study, also two experimental adjuvants (*Lactobacillus* strains and ISCOMs) were tested.

In view of the surprising results that immunization with effective adjuvants did not lead to severe pain (note that pathological changes are found), we looked at the side effects of adjuvants in a more detailed study (Chapter 5). Additional behavioural and pathological studies were performed to assess adjuvant-induced side effects.

Besides specific antibody production, other parameters can be used to analyse adjuvant efficacy. To study the efficacy of adjuvants in mice in more detail, we compared FCA with four commercially available adjuvants (Specol, TiterMax, RIBI, Montanide ISA50) with respect to the quantity and the quality (isotype distribution and cross-reactivity with native protein) of the induced antibodies and to cytokine production (Chapter 6). Also side effects were evaluated in this study. Isotype distribution of antibodies and cytokine profiles after adjuvant/antigen injection were compared to study their correlation.

Since cytokines are thought to be the primary regulators of the immune response, cytokine profiles generated directly after immunization may give information on the outcome of an immunization. By selecting an adjuvant based on cytokine profiles rapidly after immunization, the duration of potential pain and the number of animals used for immunization, can be decreased. In Chapter 7, the possibilities to predict the result of an immunization, based on early (within hours) cytokine production, is described.

Knowledge of the mode of action of adjuvants can help in predicting the outcome of an immunization and therefore help in selecting the most appropriate adjuvant for

a specific purpose. The mode of action of many adjuvants is not yet completely elucidated. It is generally thought that macrophages, which take up particulate matter, are important in the enhancement of the immune response by adjuvants. By making use of a well-established macrophage elimination technique, we showed that macrophages indeed play a role in the induction of the immune response with water-in-oil emulsions, but that this can be a suppressive role (Chapter 8). This suppressive role of macrophages was confusing in the light of presumed macrophage involvement in adjuvant efficacy of water-in-oil emulsions. Consequently, we developed a new method (Chapter 9) to label the oil phase of water-in-oil emulsions with a lipophilic fluorescent dye (DiI) and studied oil distribution. After injection of water-in-labeled-oil emulsion in mice, localization of emulsion in lymphoid organs, with emphasis on macrophage uptake, was studied. We could show that only a fraction of antigen containing oil emulsion ends up in the (suppressive) macrophages, thereby possibly explaining the efficacy of water-in-oil emulsions i.e. by keeping the antigen away from macrophages which would degrade it and take it away from the immune system.

Chapter 10 presents a general discussion on the main points emerging from the experimental work. Antibody responses and side effects induced by FCA and alternative adjuvants are assessed. Finally, recommendations are given on the use of adjuvants for antibody production in rabbits and mice to support guidelines for immunization of laboratory animals.

Chapter 2

Antigens and antigen presentation

P.P.A.M. Leenaars, E. Claassen, W.J.A. Boersma.
In: *Immunology Methods Manual*, I. Lefkovits (Ed.), Academic Press Ltd., London, 1997, pp. 989-1013.

Introduction

Antigens are those structures which the immune system recognizes as non-self. To achieve recognition and subsequent immune responsiveness the antigen has to be presented to the immune system in such a way that it is recognized in a form most suitable for elimination of the antigen. An antigen in this context is any substance that is recognized by antigen-specific structures of the immune system: antibodies, T cell receptors. Antibodies can bind to unmodified antigens as well as to fragments and degradation products thereof. For recognition by T cells, antigens have to be degraded to peptides, which complexed with MHC molecules are recognized by T cell receptors. Recognition by the immune system is a prerequisite for an antigen to elicit a response. Molecular structures that can elicit an immune response are called immunogens. Although almost any substance can be recognized as an antigen, the immunogenicity of a substance is determined by the intrinsic properties of the antigen: source, nature, structure, complexity, and size (Table 1).

Table 1. General features influencing immunogenicity of antigens

Parameter	*Immunogenicity*
Source	xenogeneic > allogeneic > syngeneic > autologous
Chemical nature	protein > polysaccharide > lipid
Size	high mol wt > low mol wt
Complexity[1]	complex > simple

[1]multiple antigenic/immunogenic molecules

Nevertheless, exactly what determines the capacity of a structure to induce an immune response (immunogenicity) and to be recognized by an antibody is a subject of discussion (Hopp, 1986; Van Regenmortel, 1986, 1989; Boersma *et al.*, 1993).

Here we will discuss antigens and their properties in *in vivo* immunization for the generation of immunodiagnostic reagents and in vaccine applications, with emphasis on induction of humoral immunity.

The recipient organism

In immunization and vaccination procedures most attention is usually focused on the properties of the antigen; however, the immune status of the immunized individual is also a decisive factor. The slow development of the immune system in young individuals, as the decline of the immune system in old individuals, determines the quality of an immune response. In newborns circulating maternal antibodies which form complexes with the antigen may have enhancing as well as inhibiting effects on

the immune responses dependent on antibody/antigen ratios. In young individuals, in general the response to (lipo)polysaccharides develops more slowly than responses to proteins; in a sense young individuals (mouse, man) are partly immunodeficient with respect to these antigens. In addition, protocols for intentional immunodeficiency induction (transplantation, malignancies), natural immunodeficiency by superinfection (HIV) have a strong negative influence on immune responses.

Furthermore, genetic factors as sex, MHC haplotypes, and intrinsic low or high respondership may play a role in the outcome of an immunization procedure.

Antigen presentation

On the surface of cells, MHC molecules are expressed of which the function is to present auto-antigens and foreign antigens to potential responder cells with an immune surveillance function. Immunological antigen presentation is effected via two distinct routes. (1) For endogenous antigen presentation, intracellular proteins and therefore also viral proteins, for example, are expressed and processed in an active turnover process. As a consequence, peptides and fragments generated in this process are brought to the cell surface mainly bound to class I MHC molecules. There the complexed fragments are recognized by class I-responsive T cells (mainly CD8 T cells; cytolysis, suppression). (2) Exogenous antigens can be taken up by complex sequences of events leading to transfer of antigen to the cytoplasma (e.g. phagocytosis, pinocytosis). Alternatively antigens which are recognized by surface immunoglobulins can be taken up directly in B cells. Antigens which are complexed with circulating antibodies can be bound via Fc receptors. In all cases an antigen processing pathway, different from that for endogenous molecules, is followed for digestion of antigens. After antigen processing, the antigen fragments are presented to the immune system in the context of MHC class II surface molecules. This form of presentation elicits a response mainly of CD4 T cells (T cell help, delayed type hypersensitivity (DTH)). So-called professional antigen-presenting cells (APC) apart from B cells include dendritic cells and macrophages.

The main functions of activated T cells are: (1) cell mediated responses which are mainly direct cytotoxic responses to cells expressing specific antigens (in MHC bound form) and can be performed by CD4 and CD8 type T cells, (2) T cell help (Th) for B cells. Th1 and Th2 cells are each able to generate different sets of cytokines to modulate immune responsiveness of other cells. T and B cells interact not only via recognition of antigen by antigen receptors but also via mutual recognition of series of activation markers, i.e. receptors and ligands expressed on the surface, as discussed for CD40 and CD40 ligand by Laman and de Boer (1997). As a result, some antigens upon recognition will lead to production of other antibody isotypes than others. Also, the response of monocytes and macrophages in a DTH response is orchestrated by Th cells.

Primary immunization and memory

Primary immunization and vaccination aim at introduction of antigens so as to evoke an optimal immune response and to generate memory formation for secondary responsiveness. For antibody responses, priming generally leads to strong responses of IgM isotype and low levels of antibodies for which class switching is required (IgG, IgA, IgE). For class switching and memory formation, the activation of either Th1 or Th2 cells is necessary.

In general, expression of isotypes other than IgM in a primary immune response is low. After a second encounter with the same antigen, the isotype will be expressed mainly which is related to antigen and immunization conditions as discussed below. In the secondary or booster reaction both the quantity (level) as well as the quality of the response is enhanced (higher affinity, specific isotype).

Specific antigenic properties may lead to specific isotype expression in immune responses (Table 2). In general, IgG will be elicited with (glyco)proteins, when the antigen is presented to the peripheral immune system. In human and mouse, the major isotype produced in response to proteins is IgG1. IgG3, apart from bacterial infections where polysaccharide play a role, in general is low in mice and in man. In man, in addition, IgG4 is relatively low. Isotype induction is dependent on nature of the antigen, route of introduction, and the formulation in which the antigen is introduced. When similar antigens are presented to the immune system via the mucosal surface, the resulting isotype will be IgA. IgE is mainly induced by parasitic (surface) antigens which locally may act like allergens. Allergenic responses to other harmless antigens are also of the IgE isotype. Antigen-IgE complexes lead to activation of effector cells.

DTH reactions are in general developed to all protein antigens when introduced in low concentrations in the skin, where specific antigen-presenting cells (Langerhans cells, interdigitating cells) will take them up and migrate to the draining lymph nodes to activate specific T cells. The recruited effector cells are mainly monocytes and macrophages. DTH reactions can also be elicited with more complex and particulate antigens (such as xenogeneic erythrocytes, dead micro-organisms).

Memory T cells play a pivotal role in the elicitation of secondary responses. In secondary responses mainly that isotype is expressed for which T help (Th1/Th2) is provided. Memory T cells as well as memory B cells formed as a result of the priming immunization allow the immune system to respond more vigorously and more rapidly in a secondary or booster response.

T- independent antigens

Binding of antigen fragments to class I or class II MHC molecules is observed for proteins. Other antigens, such as polysaccharides and lipids, do not follow the antigen presentation pathways as described above. As a result they generally do not activate T cells and are therefore called T-independent antigens. The consequence

is that these antigens in general do not lead to immune responses of other isotypes than IgM and in part to IgG3 (mouse) or IgG2 (man). T-independent activation of B cells in general occurs with polymeric antigens that are cross-linking surface immunoglobulins (for review see Laman and Claassen, 1995; Van den Eertwegh *et al.*, 1992). Memory formation, affinity maturation as well as germinal center formation, is absent. To elicit IgG responses to this category of antigens, the conjugation to T-dependent antigens (proteins) is necessary.

Table 2. General antigen categories and induced response

Antigens		*MHC I pathway*	*MHC II pathway*	*Isotype*
Soluble				
Protein		-	+	IgG1 > IgG2(a,b) >> IgG3 > (IgG4), IgE
Polysaccharide		-	+/-	IgM/IgG3 > IgG2a,b (mice) IgG2 (human)
Lipid		-	-	
Particulate/complex				
Dead	Complex[1]	-	+	antigen dependent
	Micro-organism	-	+	antigen dependent
Live	Intracellular			
	virus	+	+	IgG1, (mice) IgG1, IgG3 (human)
	parasite	(+)	+, DTH	IgG, IgE (mice) IgG1, IgG3, IgG4, IgE (human)
	bacteria	(+)	+	IgG1/IgG3 (mice) IgG1/IgG2 (human)
	Extracellular			
	virus	-	+	IgG1 (mice) IgG1, IgG3 (human)
	parasite	-	+	IgG, IgE (mice) IgG, IgE (human)
	bacteria	-	+	IgM, IgG2a/IgG3 (mice) IgM, IgG1/IgG2 (human)

[1]See Table 1.

Isotypes of antibody and Fc receptors

Antibodies of different isotypes have specific roles in protective immune responses. Binding to Fc receptors may facilitate the opsonization of micro-organisms and mediates antibody-dependent cellular cytolysis. Complement binding to the Fc part is of importance for activation of the complement system. Fc parts of IgG2a and IgG2b (in mouse) and IgG1 and IgG3 (human) have complement binding sites. These isotypes are therefore most effective in protection against viruses and other pathogens.

Antigens: source, chemical nature, size

The immune system of an organism reacts against any foreign compound (antigen) which crosses the defense lines of epithelia and skin and in general is tolerant (unable to react) towards its own body components, which may be immunogenic in other organisms. The intensity of an immune response is directly related to the degree of foreignness. The greater the phylogenetic distance between donor animal and the animal to be immunized, the better the immune response that it evokes (see Table 1). It is likely that in those cases peptides will bind to MHC molecules which are distinguishable from self peptides.

Antigens include substances as structurally diverse as proteins, polysaccharides, lipids, and nucleic acids. Most effective as immunogens are molecules which display diverse chemical and structural characteristics. Natural immune responses are directed mainly to proteins, glycoproteins, lipoproteins, lipopolysaccharides, and carbohydrates as part of the defense to infectious or invasive agents.

Proteins are synthesized as long, flexible polypeptide chains that assume a unique three-dimensional shape. Proteins are either rod-like fibrous (structural) proteins or globular proteins. Both structures are unfolded or denatured by extremes of pH and temperature and by high concentrations of dissociating agents (e.g. urea, guanidine, and sodium dodecyl sulphate), whereas more subtle alterations in conformation can be engendered by suitable modification of the amino acid side-chains. In general, these alterations in conformation, especially for structure-related determinants, are associated with changes in the antigenic reactivity.

Antigens with a low molecular weight are in general not immunogenic. These so-called haptens may be small organic structures or short synthetic peptides which consist of a B cell epitope only. They may evoke immune response only when coupled to potent immunogens, e.g. proteins which provide the necessary T cell epitopes. The minimal molecular size required for immunogenicity can be debated. In principle the antigen needs at minimum one T cell epitope to be included, which may be expected in proteins/peptides which are of a size over 2-5 kDa.

(Glyco)proteins and (lipo)proteins dependent on the route of introduction and the adjuvant applied lead to T cell responses directed towards the protein backbone as well as antibody formation of isotypes other than IgM.

Polysaccharides exist in the capsules of bacteria, or may occur as lipopolysaccharides as part of the cell walls of Gram-negative bacteria. Purified polysaccharide antigens can serve as immunogens in certain species (e.g. mice and humans) but not in others (e.g. rabbits and guinea-pigs). Microbial polysaccharides are located on the cell surface and are, therefore, of importance in recognition and immune responses of a higher organism to microbacterial infection. Complex lipopolysaccharide antigens are found in a large variety of micro-organisms, notably in Gram-negative bacteria. As they are largely T-independent, responses to (lipo)polysaccharides are mainly of the IgM isotype. Dependent on local antigen presentation, specific IgG isotypes (IgG3 in mouse) and IgA may be elicited. Pure lipids are not immunogenic. Coupled to proteins, they may act like haptens. Also, glycolipids such as cardiolipin may become immunogenic after binding to a carrier.

Role of physicochemical properties of antigens

In antibody responses the influence of physical parameters of the antigens is predicted in part based on common-sense reasoning. Antigenic sites of intact proteins are accessible to large molecules, like antibodies, only when exposed on the outside of a protein. Hydrophilic sequences readily soluble in aqueous solutions are thus most likely to be antigenic. Flexibility might enhance the fitting of antigen and antibody (Westhof *et al.*, 1984; Karplus and Schulz, 1985; Fieser *et al.*, 1987) and hence segmental mobility of the epitope may enhance antigenicity (Novotny *et al.*, 1986). Loops of the protein may stick out from the globular form of a protein and as a result they may be more readily be engaged in binding to a circulating antibody (Kyte and Doolittle, 1982). Synthetic peptides forced into a loop were shown to have an enhanced affinity for preselected antibodies (Jemmerson and Hutchinson, 1990). Methods have been developed to describe the tendency to form secondary structures: α-helix or β-sheet or β-turns (Chou and Fasman, 1978; Garnier *et al.*, 1978; Hopp and Woods, 1981; Emini *et al.*, 1985). A relative value is attributed to each amino acid which represents its ranking in terms of hydrophilic behaviour (Hopp and Woods, 1981; Kyte and Doolittle, 1982). The 'surface seeking' tendency or affinity for the membrane interior was computed by Eisenberg *et al.* (1984a, b). Normalized hydrophobicity values have been specially developed and adapted for the prediction of transmembrane sections of proteins which function as membrane-spanning transport proteins (Eisenberg *et al.*, 1984a,b). The mean value for a number of these physicochemical parameters, combined in an antigenic index, gives an estimate of the antigenic properties (index) of a protein segment (Wolf *et al.*, 1988). Amino acids exert mutual influences over short distances (Bangham, 1988). In addition, amino acid sequences which, on a linear scale, are at great distance

may interact by forming backfolding loops. It is of great importance to take this into account when choosing the length of peptides used for immunization (Jacob *et al.*, 1986; Horiuchi *et al.*, 1987).

Antigen complexity

Antigens may be divided into two groups: particulate complex multi-antigens and single antigens. The group of particulate complex antigens includes intact micro-organisms like bacteria, viruses, parasites, protozoans, and mammalian cells, but also artificial particles (e.g. poly(lactic acid) derivatives). Antigen presentation of particles generally follows the class II MHC pathway in specialized phagocytic cells. Polyclonal responses are directed to all constituents. Antigens which consist of one molecular species which include proteins, peptides, polysaccharides, glycolipids, oligosaccharides, haptens, and nucleic acids, evoke polyclonal responses to the various intra-antigen epitopes present. Immunodominance of specific determinants may lead to overrepresentation of specific responses.

Responses to dead micro-organisms follow in general the profile for particulate antigens. For live micro-organisms, antigen presentation is dependent on the character of the organism. Invasive micro-organisms may become intracellular parasites to which in general DTH responses are directed (class II). More recently the involvement of γ/δ CD8 cells together with α/β CD4 Th1 and Th2 cells has been substantiated (Kaufmann, 1995). Viruses which infect cells are processed as class I MHC restricted antigens.

Isotypes of antibody responses to micro-organisms are dependent on the structure of surface molecules and other constituents; e.g. for bacterium *Brucella abortus*, IgG2a, IgG3 (Snapper and Mond, 1993). For nematode parasites, IgG1 and IgE restricted responses were observed (Snapper and Mond, 1993).
When antigens are presented to the mucosal immune system, the uptake of soluble antigens takes place mainly via the endothelial cells of the mucosa, which then produce cytokines to enhance the specific immune response. Particulate antigens are generally taken up by the microfold cells (M-cells) of the Peyer's patches and are presented there to the resting naive cells. Toxins and micro-organisms may have specific receptors on cells along the mucosal lining. These mostly pathogenic agents do encounter the immune system each in their specific way.

Antigen construction and preparation

The specificity of the immune response obtained (antiserum, T cell response) is dependent on the purity of the antigen applied. Minute impurities (<1%) may prove to be immunodominant, as is the case with many bacterial antigens. This is a problem encountered with any antigen produced via recombinant DNA techniques and not marked with special tags for identification. In that case the selection of specific monoclonal antibodies may lead to specific reagents. Alternatively, the

antigen may be synthesized. This, however, is only sensible for small peptides (<50 amino acids) and other low-molecular-weight substances.

Synthetic antigenic determinants have a wide variety of applications. The actual role of a determinant can best be judged from the results of immunizations, e.g. the antibody responses in sera, the recognition of antigenic determinants by antibodies in various immunoassays, each with its typical micro-environmental conditions, and the specificity of the mAbs selected. Certainly for short peptides and haptens, polyclonal antibodies tend to be determinant-specific (monospecific) since they recognize one or a few more extensively overlapping determinants in a short sequence. Such pAbs may differ slightly in epitope specificity and thus may show an in-assay behaviour similar to that of mAbs.

Haptens in general are small rigid molecules which are recognized independent of the conditions of assays. In general, short peptides tend to elicit antibodies which recognize the denatured form of the protein only (Boersma *et al.* 1988a). Application of short peptides therefore may be of advantage for the elicitation of antibodies to be used *in assays* where the antigen is encountered in denatured form e.g. in SDS-PAGE, fixed tissue specimens etc. (Van Denderen *et al.*, 1989, 1990). In contrast, Dyson *et al.* (1985) showed that an immunogenic nona-peptide which showed a preferential conformation in aqueous solutions, elicited anti-native antibodies to heamagglutinin of influenza virus. Ultrastructural analysis showed that longer peptides indeed tend to mimic the native structure of a protein much better since these peptides maintain a specific space filling structure (Horiuchi *et al.*, 1987).

An important clue to the successful use of haptens and peptides (SP) as immunogens is the mode of presentation of the selected antigens to the immune system. Using these small antigens, the construct applied is usually an assembly consisting of a carrier protein, a hapten or peptide and bridging coupling reagents (for review see Boersma *et al.*, 1993).

Antigen carrier

Antigens which in themselves do not contain elements for T cell activation (small peptides or polysaccharides) need a protein-carrier which provides these elements (T cell epitopes).

The properties of a carrier protein determine to a large extent the outcome of immunizations with the conjugated antigenic determinant, e.g. haptens and small peptides. Larger proteins (>60 kDa) are preferred as carriers because they contain a sufficient number of $-NH_2$, -SH, arginine, etc., groups for coupling to generate satisfactory determinant/protein ratios. In principle most proteins will suffice as carriers if derived from a non-self source. However, the more genetically distant a protein is from the animal to be used for immunization experiments, the better the chances for potent immunogenicity. Keyhole limpet haemocyanin (KLH), a large aggregated protein from a gastropod, is therefore often used as a carrier protein in

mice, rabbits, goats, and sheep. Other widely used carriers are of bacterial origin: tetanus toxoid (TT), purified protein derivative of tuberculin (PPD), and diphtheria toxoid. Less efficient in mammals are ovalbumin (OVA) and bovine serum albumin (BSA) (Geerligs *et al.*, 1989), probably because of tolerance to these highly conserved proteins. Chicken gamma-globulin (CGG) is rather immunogenic in mice.

However, coupling of relatively large numbers of determinants, depending on the protein, can have a negative influence on the properties of a conjugate. Overloading may lead to precipitation and decreased immunogenicity (Peeters *et al.*, 1989). Also, cross-linking agents such as diazo compounds and glutaraldehyde may drastically decrease the solubility and immunogenicity of a carrier-determinant conjugate.

For non-immunogenic peptides, (e.g. B cell epitopes only) and haptens, coupling to a carrier protein to provide T cell help is required. This is not a matter of molecular mass since immunogenicity of peptides is in general not increased by homopolymerization with, for example, glutaraldehyde (Boersma, unpublished results). Polymerization using carbodiimides led to encouraging results, though in part based on the coupling of T-determinants (Borras-Cuesta *et al.*, 1988). Straightforward covalent elongation with a selected T cell determinant is an alternative option (Hackett *et al.*, 1985; Francis *et al.*, 1987; Zegers *et al.*, 1993).

A method has been developed for the synthesis of branched peptides or multiantigen peptides (MAP) (Tam and Zavala, 1989). This multiple antigen peptide method was developed for sensitive detection of anti-peptide antibodies, but in addition the multimeric peptides show enhanced immunogenicity compared to free peptides, peptide conjugates, or peptides still attached to their solid support (McLean *et al.*, 1991). MAP which include a specific T cell epitope may function as efficient immunogens.

Admixture of T cell epitopes and B cell epitopes under specific conditions may be sufficient for T cell and B cell activation. Such conditions can be found in water-in-oil emulsions and in liposomes. There the orientation of epitopes, dependent on their amphipathic character, may be such that they are presented so that T-B cell interaction is possible (Sarobe *et al.*, 1991; Partidos *et al.*, 1992; Prieto *et al.*, 1995).

Immunization procedures

Besides the immunogenicity of the antigen, a large number of parameters influence the result of each immunization: (1) the site at which the antigen is introduced; (2) the dose of antigen; (3) the scheduling of immunizations over time; (4) the type of adjuvant applied; and (5) the genetic make-up of the recipient (discussed above). These parameters determine which antigen-presenting cells the antigen meets first, how many and which cells will be involved in the response, as well as the quantity of the response (Tables 2 and 3).

The location at which the antigen is deposited in part determines the lymphoid organs activated and the isotype of the antibody response. For detailed description

based on histocytochemical analyses, see Laman and Claassen (1995). Routine immunizations, intraperitoneal (in mice) and subcutaneous (in rabbits and mice), rapidly deposit antigen mainly in spleen and peripheral lymph nodes. Soluble antigens injected intravenously (in the tail vein or retro-orbitally) also rapidly end up in the spleen.

Oral application in general leads to induction of tolerance. For effective immune responses (IgA) antigen presentation requires a vehicle which may either be a micro-organism, a particle of specific size (1-10 μm), or a toxin. Application of antigen via the lungs (intratracheally) will in general follow the same rules as for oral application. Nasal application may efficiently lead to IgA induction.

Deposition of antigen at subcutaneous, intracutaneous, or intramuscular sites will in general lead to slow release of the antigen into the immune system. This slow release generally leads to continuous stimulation and strong immune responses. Leenaars *et al.* (1994) studied the effect of s.c. injection of a volume of FCA/antigen (0.5 ml/rabbit) in one injection site compared to the same volume spread over 2 or 4 injection sites. Similar antibody responses were observed, for both immunization procedures, suggesting that dividing antigen over more sites does not increase the antibody response.

How the route of antigen administration controls the nature and intensity of the response is not known. Antigen-presenting cells (APC), different lymphoid tissues and the special characteristics of the regional immune system play an important role in the induction of the response. De Becker *et al.* (1994) showed that the isotype and the amplitude of the B cell response can be regulated by the nature of the APC. The antigen presented by dendritic cells induces the production of Th1-dependent isotypes (IgG2a), whereas an antigen pulsed on peritoneal macrophages seems to induce a Th2-associated response (IgG1, IgE). The route of injection determines which APC comes in contact with the antigen. The route of injection is limited by the form of the antigen. Insoluble antigens cannot be immunized intravenously. In order to waste little of the antigen in the periphery, the antigen can be directly applied locally in the spleen either free or attached to a vehicle (nitrocellulose, sepharose beads). Owing to reduction of antigen loss in the circulation, doses 50-100x lower than normal could be used on intrasplenic immunization (Spitz, 1986; Hong *et al.*, 1989). Certain immunogens, when injected parenterally, lead to the production of circulating antibody; when given intradermally, the same immunogen in relatively low doses may provoke delayed-type hypersensitivity in the absence of circulating antibody.

Peptides which appear to be low immunogenic upon routine i.p. or i.v. immunization may be immunized locally to sensitize peripheral lymph node cells. In the mouse, immunization in the footpad or subcutaneously in the back may very efficiently activate regional lymph nodes when strong adjuvants are applied (Freund's adjuvant).

Table 3. Immunomodulation by adjuvants

Function	*Effect on antigen presentation*	*Adjuvant*	*support MHC I presentation*	*support MHC II presentation*	*Cytokines*	*Isotypes (mice)*
Vehicle	localization,	- inert beads	-	+		IgG1/IgE
	enhanced uptake	- mineral salts[1]	-	+, poor DTH	IL-3, IL-4, IL-5, IL-6[9]	IgG1/IgE
		- oil emulsions[2]	-	+, DTH	IL4	IgG1/IgE
Depot	slow release	- mineral salts	-	+, poor DTH	IL-3, IL-4, IL-5, IL-6	IgG1/IgE
		- oil emulsions	-	+, DTH	IL-4	IgG1/IgE
Micelle induction	enhanced uptake	- liposomes[3]	+	+		IgG1/IgG2a,2b
		- ISCOMs[4]	+	+		IgG1/IgG2a,2b
Macrophage activation	enhanced uptake	- NBP[5]	-	+, DTH		IgG2a,2b
		- DDA[6]	-	+, DTH		IgG2a,2b
		- microbial products[7]	-	+, DTH	IFN-γ, IL-4	IgG2a,2b
		- oil emulsions		+, DTH	IL-4	IgG1/IgE
Direct B cell activation		- microbial products	-	+, DTH	IFN-γ, IL-4	IgG2a,2b
Growth/differentiation of B cells		- cytokines[8]				
		IL-1				
		IL-2			IL-2	IgM
		IFN-γ		+, DTH	IFN-γ	IgG2a

[1]Nicklas, 1992; [2]Boersma *et al.*, 1992 [3]Buiting *et al.*, 1992; [4]Claassen and Osterhaus, 1992; [5]Hilgers and Snippe, 1992; [6]Verheul and Snippe, 1992; [7]Warren *et al.*, 1986; [8]Heath and Playfair, 1992; [9]Valensi *et al.*, 1994.

Targeting of antigens to certain molecular structures on cells is a form of specific routing of antigen. For peptides, enhanced responsiveness has been shown to occur by targeting of these peptides to surface immunoglobulin or class I molecules (Casten, 1988). For BSA, Lees *et al.* (1990) showed that especially targeting with anti-IgD led to strong potentiation of antibody responses. Immunological targeting to, for example, MHC molecules acts as a replacement of adjuvant in that it targets the antigen and at the same time acts to activate the antigen-presenting cells. To this end, MHC-specific antibodies have been applied (Carayanniotis *et al.*, 1988; Carayanniotis and Barber, 1990).

Dose of antigen

Immune responses are antigen dose-dependent. For most antigens an optimum is found in the dose-response relationship. A low dose induces the formation of small amounts of antibodies with relatively high affinity and specificity. Weak responses may be enhanced by coadministration of adjuvants (see below). The order of magnitude of optimal doses do not differ among various animals. For vaccination in humans, for most antigens 1-50 µg of antigen is administered. Similar optimal doses are found in cattle and small laboratory animals. For T-independent antigens, about 20 µg intravenously, or intraperitoneally is sufficient in mice (Laman and Claassen, 1995).

Studies of immunologic tolerance illustrate that a very low or a very high dose of a foreign material can inhibit future responses to subsequent injection of an otherwise immunogenic dose.

Proteins with specific routes of entry into the immune system (e.g. toxins) may elicit immune responses in extremely low doses: 10^{-14} g for endotoxin in rodents. In general, doses of a few µg are sufficient for an efficient priming reaction. This shows that low antigen doses may efficiently stimulate T cells and that immunological memory may be induced by doses that do not produce detectable antibody responses. DTH reactions to specific antigens are evoked at lower doses than are needed for antibody responses to the same antigen.

Functions of adjuvants

When antigens are poorly immunogenic the immune system needs a stimulus to evoke a response. Adjuvants are used for this purpose. Adjuvants are simple or complex admixtures (natural or synthetic) of compounds which upon administration to individuals lead to an aspecific immune stimulation (Tables 3 and 4). The route of administration determines the localization. In addition, adjuvants often also function as the vehicle which takes care of localization of the antigen. Specific localization (intraperitoneal, intra- or subcutaneous, intrasplenal) of the antigen can be obtained by administration of antigen in water-in-oil or oil-in-water emulsions, antigen bound to or enclosed in liposomes, antigen bound or absorbed on to amorphous or

crystalline material as in alum hydroxide-phosphate gels, or with nitrocellulose or solid beads of various compositions.

These immunizations have in common that the administration of antigen results in local immune responses. The surface area of the vehicle to which the antigen is attached determines the load and density of the antigen during antigen presentation. Vehicles convert soluble antigens into particulate material which is more readily ingested by antigen-presenting cells such as macrophages. Some vehicles such as alum and oil emulsions have immunostimulating (adjuvant) properties, while others, such as sepharose beads and nitrocellulose are relatively inert in an immunological sense.

Table 4. Mode of action of adjuvants

Category	*Examples*	*Mode of action*
Mineral salts	$Al(OH)_3$, $AlPO_4$[1]	- vehicle - depot-effect
Oil emulsions	FIA, Montanide, Specol[2]	- vehicle - depot-effect - activation of macrophages
Microbacterial products	LPS, MDP, MPL,TDM[3]	- direct stimulation of B cells (LPS) - activation of macrophages - stimulation of T cells - activation of complement - enhanced antigen uptake
Saponins	Quil A[4]	- facilitate cell-cell interaction - aggregation of antigen
Synthetic products	DDA[5]	- activation of macrophages - activation of complement
	ISCOMs[6]	- facilitate cell-cell interaction - stimulation of T cells - aggregation of antigen
	Liposomes[7]	- vehicle - enhanced antigen uptake
	Nonionic block polymer (NBP)[8]	- activation of macrophages - activation of complement
Cytokines	IL-2, IL-1ß, IFN-γ[9]	- growth/differentiation T and B cells - enhanced antigen-presentation

[1]Nicklas, 1992; [2]Boersma *et al.*, 1992; [3]Warren *et al.*, 1986; [4]Campbell and Peerbaye, 1992; [5]Hilgers and Snippe, 1992; [6] Claassen and Osterhaus, 1992; [7]Buiting *et al.*, 1992; [8]Verheul and Snippe, 1992; [9]Heath and Playfair, 1992.

Various adjuvants also offer a depot function. When deposited at a given site the antigen leaks slowly from the adjuvant compound and gradually becomes available for stimulation of the immune system.

Adjuvants are foreign to the body and therefore depending on the nature of the constituents, may in themselves be antigenic. If appropriately administered they lead to sterile inflammations that attract the various cells of the aspecific defense system -macrophages- which in turn produce immunomodulating factors that increase the level of immune surveillance. Macrophages as well as other antigen-presenting cells therefore play an important role in the generation of the adjuvant effect. Most adjuvants directly or indirectly stimulate the generation of interleukin-1 and also other factors that support growth and differentiation.

In summary: adjuvants contribute to improved antigen presentation and immunomodulation; this is the true adjuvant function. The choice of the adjuvant is dependent on the type of antigen and on the desired immune response (cellular versus humoral). In vaccines adjuvants should preferentially enhance the immune response of all cell types involved in the process of generating protection. To this end, the stimulation of the antigen presentation, the T regulator cells (Th1 and Th2), the B cell, and effector T cells is most important. For the generation of antibodies as immunoreagents, the requirements of immune stimulation are less stringent.

Properties of various adjuvants

The exact way in which adjuvants stimulate the immune system is not always known. The properties of various adjuvant products are summarized in Tables 3, 4.

Mineral salts

Mineral salts consist of hydrated gels of aluminium hydroxide, aluminium phosphate, or calcium phosphate which form efficient vehicles to which proteins readily adsorb dependent on ionic strength and pH. The depot function is most prominent, whereas the immune stimulative capacity is weak. Mineral salts have the major drawback that they do not support cell-mediated immunity very well. The irritation caused suggests that these adjuvant function by local production of cytokines to activate antigen-presenting cells. Their relatively harmless character led to approval of these adjuvants in humans by the US Food and Drug Administration and the World Health Organization. Local (e.g. vaginal) application led to humoral response induction without harmful side effects seen with other adjuvants. The effective depot function has led to combined use of mineral salts with other adjuvants such as micro-organisms (*Bordetella pertussis*; Bomford, 1980). Aluminium compounds may be suited for antigens which are highly immunogenic and available in large amount, but they are not recommended when only weak immunogens (e.g. peptides, subunit vaccines) are used or when there is limited amount of antigen available. Compared

to other adjuvants, mineral salts are rarely used for experimental immunization of laboratory animals.

Upon administration of aluminium salts in mice, Valensi *et al.* (1994) detected IL-3, IL-4, IL-5, and IL-6 in serum 3-24 hours after i.m. injection, while IL-1α, IL-2, IFN-γ, and IL-10 were measured but not detectable in serum.

After primary immunization of mice with BSA in $Al(OH)_3$, Bomford (1980) found only antibodies of the IgG1 isotype, whereas during the secondary response an IgG1 and IgG2a response was stimulated by $Al(OH)_3$. This was confirmed by Byars *et al.* (1991) who found, in contrast to several other adjuvants, that predominantly the IgG1 isotype and only little IgG2a and IgG2b was produced. Haaijman *et al.* (1988) also found mainly IgG1 responses in BALB/c mice. It is suggested that this is of special importance when protective immunity is required, as antibodies of the IgG2a and IgG2b isotype act synergistically with complement and antibody-dependent effector cells. Aluminium adjuvants are very efficient in enhancing the synthesis of IgE antibodies in rabbits and rodents. $AlPO_4$ does not induce IgE antibodies (Allison and Byars, 1986).

Micro-organisms and microbial products

Micro-organisms and microbial products can exhibit strong adjuvant activity. In general the bacteria are applied in heat-killed form and coadministered with the immunogen under study. Mycobacteria cause severe side effects (e.g. sterile inflammations and granulomas; Claassen *et al.*, 1992). Mycobacterial cell wall products have been studied to obtain fractions with similar immune-stimulating properties but without severe side effects. Trehalose dimycolate (TDM, cord factor) was identified and found to have immunostimulating properties. The smallest subunit of the mycobacterial cell wall that still results in adjuvant activity is N-acetylmuramyl-L-alanyl-D-isoglutamine, abbreviated as muramyl dipeptide (MDP) (Azuma *et al.*, 1976).

Lipopolysaccharide (LPS) is the main surface structure of Gram-negative bacteria. The adjuvant activity of LPS was first described in 1956 by Johnson *et al.* (1956). Its high toxicity precludes its clinical use. Low toxicity analogues of LPS have been developed which retain their immunostimulatory properties. The lipid A moiety has received most attention in this respect, it can be prepared by chemical treatment of natural lipid A (Alving *et al.*, 1984) or by chemical synthesis of lipid A (Yasuda *et al.*, 1982). Monophosphoryl lipid A (MPL) is an example of a lipid A derivative of LPS which retains all the immunostimulating properties of LPS (Gustafson and Rhodos, 1992).

Microbial products may lead to mitogenic activation of B cells (LPS), and activation of macrophages, T cells and the complement system (reviewed by Warren *et al.*, 1986). Lipid A, as well as lipopolysaccharide, is hydrophobic, while lipid A is also amphiphilic. Administration of this adjuvant separately or together with antigen

leads to generation of cytokines. Most prominent is the production of IFN-γ by natural killer (NK) cells. This cytokine in turn activates antigen-presenting cells such as macrophages. Activated macrophages generate tumor necrosis factor-α. The activation of antigen presentation and production of cytokines following the administration of this adjuvant switches the humoral immune response in the mouse to IgG2a (Takayama *et al.*, 1991). In the promotion of protective immunity against bacteria and viruses antibodies of the IgG2a isotype are considered to be superior owing to their complement fixation (Gustafson and Rhodes, 1992). Only few non-pathogens can serve as adjuvant at the mucosal surfaces. Live *Lactobacillus* of various strains (*casei, plantarum*), can serve as adjuvants after intraperitoneal but also after oral administration (Boersma *et al.*, 1994). Their efficacy using peptide antigens is relatively low. Peptides need to be coupled or produced at the surface by *Lactobacillus* to generate acceptable levels of immune responsiveness (Claassen *et al.*, 1994, 1995a; Boersma *et al.*, 1994).

Combination of lipid A or lipopolysaccharide with nonionic block polymers or alum leads to a shift to IgG1 antibodies. The same effect can be obtained with recombinant IFN-γ, which is much more expensive than LPS or lipid A and in addition cannot be applied to the sites where the local immune response is generated. LPS also stimulates cell-mediated immunity as measured by DTH.

Preparations consisting of a mixture of components from a diversity of micro-organisms have been shown to influence severely the traffic of lymphocytes within the mucosal immune system, thereby enabling immunization on site to support responses in other mucosal locations also (Ruedl *et al.*, 1993).

The adjuvanticity of microbial products can be enhanced by mixing them with depot preparations of the immunogen (e.g. mineral salts, oil emulsions). The best-known example is Freund's complete adjuvant (FCA). The side effects of FCA led to its being banned in various countries (see Claassen and Boersma, 1992; Claassen *et al.*, 1992).

Toxins have been widely applied as immunological carriers with a high immune-stimulating potential. Heat-labile toxin of *E.coli*, tetanus toxoid as well as cholera toxin are the best-known examples (Vajdy and Lycke, 1992). Simple coupling of peptides to these protein carriers leads to efficient immune response generation (Arnon, 1989). Cholera toxin is most investigated for its mucosal immune-stimulating character. Cholera toxin was shown to enhance the IL-1 production by macrophages. However, it inhibits T cell IL-2 production but enhances proliferation. *In vitro* enhanced IgA synthesis in lipopolysaccharide-stimulated B cells was demonstrated (Lycke *et al.*, 1989).

Other microbial products which have been tested as adjuvants include the water-soluble adjuvants from *Nocardia* species, yeast glucans, various peptidoglycans, and certain bacterial exotoxins (Munoz, 1964; White, 1976).

Water-in-oil emulsions

Water-in-oil, reversed, and double emulsions with vaccines have been in use for a considerable time. Freund (1956) applied refined paraffin oil (Freund's incomplete adjuvant, FIA) with vaccines and since then this oil has been used as an adjuvant mainly for experimental purposes. Oil emulsions have a depot function, they can stimulate macrophages, and they promote uptake of antigen (Waksman, 1979). The depot function of oil emulsions is enhanced by intraperitoneal, subcutaneous, intradermal, or intramuscular administration. The most potent adjuvant known for stimulating both humoral and cellular immunity is a combination of an emulsion of mineral oil and killed mycobacteria (FCA). Adjuvants like FCA stimulate the immune response very effectively but can have dramatic side effects. As a result not all adjuvants are permitted for human use or for application in veterinary medicine. In addition in many countries guidelines on animal experimentation limit the use of the most potent adjuvants for ethical reasons.

From the results of cytokine production, no skewing in either Th1 or Th2 response direction can be determined for this type of adjuvant. The mode of action relies on the enhancement of hydrophobicity of antigens, targeting to antigen-presenting cells, and on the presentation of antigens on a large surface area. The oil microspheres carry antigen to the surface of antigen-presenting cells. Endocytosis and presentation in the context of MHC class II is therefore expected (Boersma *et al.*, 1992).

The antigen to which an immune response has to be elicited is generally taken up into the hydrophilic phase. (Bokhout *et al.*, 1981; Woodard and Jasman, 1985; McKercher, 1986; Woodard, 1990).

Bokhout *et al.* (1981) first described Specol, a selected water-in-oil emulsion; its application has been evaluated in the prevention of future diseases by vaccination as well as in the enhancement of natural *in vivo* immune responses. This adjuvant was developed from their observation that vaccinated pigs were protected against pathogens not present in the vaccine. Specol certainly has advantageous properties with respect to animal care, ease and reproducibility of preparation and administration, and the use of constituents which generally are regarded as safe for use in animals.

Although proliferative T cell responses and DTH reactions could be demonstrated with use of Specol, formal proof of the support of a cytolytic T cell response has yet to be produced. In contrast to most adjuvant admixtures, Specol can be prepared easily and highly reproducibly since all constituents can be obtained from the suppliers in quality-controlled batches. The low viscosity of Specol compared to many blends (FIA) facilitates administration by injection (Boersma *et al.*, 1992).

Water-in-oil emulsions activate macrophages, suggesting that the cytokine involved is most likely IL-1 (for review see Boersma *et al.*, 1992). Recent experiments suggest induction of IL-2, IL-4, and IFN-γ together with expression of

the T cell activation marker gp39 (Zegers *et al.* unpublished results). Most water-in-oil emulsion evoke the production of IgG1 responses (Allison and Byars, 1991). With Specol in BALB/c mice, IgG1 responses were frequently observed. In the mouse, IL-4 stimulates the generation of IgG1 and IgE while IFN-γ is involved in the IgG2a responses (Finkelman *et al.*, 1988a,b).

Liposomes

Liposome adjuvanticity is influenced by the charge, composition and the method of preparation. *In vivo,* liposomes act by facilitation of antigen uptake, ingestion, fragmentation, processing, and presentation, mainly by macrophages (for review see Claassen, 1991). The antigen is encapsulated in the water or lipid phase or can be coupled to the surface (for review see Buiting *et al.*, 1992). Van Rooijen and Van Nieuwmegen (1980) showed that surface exposition of antigens is most efficient in antigen presentation. For peptides, the elongation with a hydrophobic tail (lipopeptides) enhances the uptake and correct surface orientation of the determinants of interest in a given antigen. As such, a liposome can be envisaged as a hapten-vehicle with a large surface. The repetitive presence of the determinant may lead to B cell activation (IgM responses). T cell dependent responses are found only when a T cell determinant is included in the antigen. The fusion of liposomal and endosomal membranes delivers the antigen essentially in the MHC class II processing pathway (Germain, 1986; Bevan, 1987). Liposomes seem to support primarily CD4-dependent T cell responses (DTH, B cell help). Recently, activation of CD8 cells in cytolysis (class I dependent) after application of antigen with pH-sensitive liposomes has been documented. These liposomes introduce antigen in the cytoplasmic phase of antigen processing. However, questions with respect to the quantitative contribution of this route of processing based on liposomal delivery of antigen remain to be clarified.

The potency of liposomes can be further increased by the inclusion of other immune-stimulating agents such as bacterial lipopolysaccharide, lipid A, MDP, and saponins. Targeting of liposomes using antibodies anchored in the membrane is controversial (Buiting *et al.*, 1992). Liposome-associated proteins elicited IgG2a and IgG2b antibodies in the mouse (Phillips and Emili, 1992). Whereas MDP preferentially stimulated IgG1 in their experiments, with a combination of liposomes and MDP a shift to IgG2 was observed. After oral application of pH-stable liposomes, Jackson *et al.* (1990) observed secretory humoral immunity (IgA). Humoral responses elicited with antigens presented in liposomes in general show response profiles similar to responses elicited with water-in-oil emulsions.

Saponins

Saponins are plant-derived, rather complex, glycoside-containing compounds which are amphiphilic in nature and tend to form micelles. The best-known example of a saponin adjuvant is derived from the bark of *Quillaja saponaria Molina*. Quillaja

saponin is a mixture of potent adjuvants. Crude saponin extracts are unreliable. Quil A, one of the purified derivatives, is used as the basis for the formation of immune-stimulating complexes (iscoms) (Dalsgaard, 1978; Morein, 1990). Together with cholesterol (mostly also phospholipids) Quil A tends to form particulate matrix structures with high adjuvanticity (ISCOM matrix). Though hydrophobic interaction, proteins can strongly complex with these structures to form immune-stimulating complexes, so-called ISCOMs (Lövgren and Morein, 1988).

Saponins and ISCOMs promote a strong immunogenic enhancement most probably by direct interaction with cellular membranes. The role of macrophages in the effectiveness of ISCOMs has been described by Claassen *et al.* (1995b). Apart from humoral responses, saponins also seem to support CD8 (class I restricted) mediated cellular cytotoxicity (Newman *et al.*, 1992; Campbell and Peerbaye, 1992). CD8 T cell effected cytolysis has been demonstrated which might be dependent on IFN-γ production. The generation of IgG2a humoral responses in mice is indicative for this mode of action. It is not known how the induction of IFN-γ is established. Saponins are toxic, and local depot-like administration may lead to necrosis. Oral immunizations of animals with ISCOMs have been only partly successful. Relatively high doses of antigen (100 µg) were needed, but class I restricted responses were also demonstrated (with the antigen ovalbumin). The support of mucosal immune responses has been documented upon intranasal application of antigens/vaccines (Jones *et al.*, 1988). Intravaginal responses have also been demonstrated but these required relatively high amounts of antigen (Thapar *et al.*, 1991). For application via the oral route the induction of IFN-γ is thought to be most important. This is in agreement with the antibody isotypes observed (Finkelman *et al.*, 1988a). In mice, saponins stimulate production of IgG1, IgG2a, and IgG2b. The major part of the response is formed by IgG2a antibodies. IgE was not observed (Kensil *et al.*, 1991). Quil A elicited mainly IgG1 in mice (Kenney *et al.*, 1989), whereas IgE responses have also been reported (Allison and Byars, 1992). For those responses where elicitation of IgE has to be avoided (vaccination), the choice of adjuvant should be made carefully.

With MHC class II targeting, enhanced responsiveness in humoral immunity was mainly restricted to IgG1-restricted isotype (70%), whereas about 25% was of the IgG2a isotype in mouse (Skea and Barber, 1993).

Synthetic adjuvants

Synthetic adjuvants are rather heterogeneous. The mode of action of *nonionic block polymers* (NBP) is not fully understood but the activation of the complement system as well as the activation of macrophages seems to play an important role. NBP have a low intrinsic toxicity. They are build from repetitive combinations of poly-oxy-ethylenes and poly-oxy-propylenes. NBP usually have an amphipathic character. They stabilize water-in-oil emulsions and thereby may stabilize the tertiary structure

of antigens as well. In contrast to most adjuvants studied, NBP have a strong adjuvant effect for poly- and oligosaccharides in particular, whereas for most proteins they are not as potent as many other adjuvants. The mode of action may be similar to that of lipid A since the predominant antibody response is IgG2a in the mouse, which is enhanced by IFN-γ production. The isotype distribution is influenced by the balance between the proportions of hydrophobic and hydrophilic moieties in the NBP composition. CTL responses have not been documented, but it has been demonstrated that DTH responses are supported (for review see Verheul and Snippe, 1992). Owing to their partly hydrophilic and partly hydrophobic nature, NBP are readily usable with liposomes and water-in-oil emulsions. With SAF, the combination adjuvant which includes a muramyldipeptide analogue, a nonionic block polymer in a water-in-oil emulsion (Byars *et al.*, 1990), a skewing of the response to IgG2a antibodies was demonstrated in mice which was attributed to the induction of IFN-γ production (Th1 responses) (Kenney *et al.*, 1989; Byars, 1990; Byars *et al.*, 1991).

Dimethyldioctadecylammonium bromide (DDA) is a representative of a specific category of adjuvants, the quaternary amines. The lipophilic character of DDA, which forms liposome-like structures in aqueous solutions, might be responsible for its capacity to enhance T cell responses. However, most responses documented are DTH responses, which is a CD4 T cell function, and CTL responses have not yet been described in detail. Adjuvant and antigen need to be administered together, following the same route, which suggests that in general binding of antigen and adjuvant is required to generate optimal responses. Binding of antigen to DDA serves the purpose of increasing the hydrophobicity of the antigen and neutralization of negatively charged moieties, but, DDA is not an optimal adjuvant to elicit antibody responses. For a sufficient antibody response combination with alum or water-in-oil emulsions, for example, is required. The mild side effects of DDA and some other quaternary amines make them promising adjuvants for veterinary application. For application in man, new less irritative analogues need to be developed (for review see Hilgers and Snippe, 1992).

Lipopeptides are nontoxic and non-immunogenic analogues of bacterial cell walls of Gram-negative bacteria (Wiesmüller *et al.*, 1983). They present a novel intrinsic adjuvant model for peptide immunization. Lipopeptides can not only serve efficiently to immunize peptides, in addition they can serve as a carrier with adjuvanticity (for review see Bessler and Jung, 1992). They are amphipathic in nature, which seems to be a favourable property for adjuvanticity. Lipopeptides coupled to low-molecular-weight haptens are able to elicit high antigen-specific antibody responses in mice and rabbits. *In vivo* as well as *in vitro* their ability to generate CTL responses even to single CTL epitopes has been demonstrated (Martinon *et al.*, 1992; Nair *et al.*, 1992). Conjugation as well as simultaneous and separate administration of adjuvant and antigen elicits immune responses. A drawback is that relatively high doses of

antigen (10 times the dose used for regular immunization to generate antibody formation with adjuvant) are needed in most cases, but then high antibody titres have been obtained. In a number of cases valuable characteristics of lipopeptides were demonstrated. The toxicity of toxins decreased after conjugation without loss of immunogenicity. Other non- or poorly immunogenic substances showed enhanced immunogenicity (Bessler and Jung, 1992). Lipopeptides are very easily applied and are commercially available. Recently the use of virus particles (HbsAg) as a carrier for peptides with a hydrophobic tail that dissolves in the outermembrane has been proposed as an alternative for both carrier and adjuvant (Neurath *et al.*, 1989).

As has been summarized above, most adjuvants which support cell-mediated immune responses (CMI), especially CTL responses, require an increase in the hydrophobicity of the antigen. It remains unclear what the mode of action might be. The chemical nature of most adjuvants which support CTL responses (micelles, specific liposomes, and ISCOMs) suggests that binding or association with membranes of antigen-presenting cells is facilitated. With IFN-γ the conjugation of antigen enhanced the immune response (Heath and Playfair, 1990).

Natural adjuvants

Cytokines are pleiotropic immunomodulatory agents which together form a partly degenerate system. The mode of action of various adjuvants has been unraveled to the level of immunomodulatory substances, among which are cytokines (Table 3). Cytokines mutually influence each other's function. Since cytokines are the primary immune response regulators, the analysis of adjuvant effects, apart from antibody production, DTH responses, and cellular cytolysis is based on demonstration of cytokine production. Cytokines may mimic the actions that are generated with adjuvants. As a consequence immunomodulation is aimed at mimicking of adjuvant effects by administration of the cytokines themselves. These body-derived or physiological adjuvants can be administered or produced locally as a secondary effect of an immunization procedure using various other adjuvants (micro-organisms, microbial membranes). Some cytokines have been evaluated for their adjuvant activity: IL-1, IL-2, and IFN-γ. These cytokines influence growth and differentiation of T and B cells as well as antigen presentation by various cells. However, because the mode of action of cytokines is complex, cytokine subunits with specific response-enhancing modalities were developed (IL-1). Fragments of cytokines have been selected which induce immune responsiveness without expressing the adverse side effects. The application of the active component together with antigen has led to promising results for thymus-dependent as well as for thymus-independent antigens. Various modes of administration have been investigated; experimentally, intraperioneal administration is most effective (Staruch and Wood, 1983; Boraschi *et al.*, 1988; Tagliabue and Boraschi, 1993).

Scheduling of immunization

T cell proliferation and production of response modifiers (cytokines) precedes the development of T-dependent antibody responses (class switch). For T cell responses *in vitro* in general the cells are isolated from lymphoid organs between 5 and 10 days after *in vivo* priming.

For optimal B cell responses it is helpful to allow resting periods of more than 4 weeks between the first and subsequent immunizations. The effective response is probably based on synchronization of resting B memory cells. Whether the decrease of primary antibody titres is a prerequisite for the second immunization to be given is a matter of debate. On one hand, the circulation of specific antibodies may lead to immune complex formation which may enhance antigen presentation; on the other hand, the reduction in antigen load by circulating antibodies, dependent on the ratio of antigen over antibody, determines whether immune complexes may lead either to stimulation (antigen excess) or to possibly inhibition of responsiveness (antibody excess). For hybridoma production, a prefusion booster immunization was given after a long interval (a few months). The frequency of specific antibody-forming clones exceeded the level expected on the basis of the previous serum antibody responses. Repeated immunization with relatively low doses of antigen leads to stimulation and selection of clones that produce relatively high-affinity antibodies. This affinity maturation is used in the generation of strong polyclonal antisera.

Immunizations with peptide-carrier conjugates are given at 4-week intervals. In general, the maximum titres are obtained after two to three subsequent immunizations. The best results following booster immunizations are obtained when priming titres have decreased. However, using KLH, a large aggregated protein which itself has adjuvant properties, anti-carrier titres will remain high for months. After priming with KLH as a carrier protein in mice serum antibodies (IgG + IgM) increased gradually up to at least 21 days. Nevertheless, 4-week immunization schedules can be completed.

The timing of the immunization preceding spleen cell fusion depends on the routing: soluble antigens (conjugates) can be introduced intravenously three days before fusion. Less-soluble antigens are given intraperitoneally with FIA, as a water-in-oil emulsion or precipitated on aluminium hydroxide gel 4 days before an intended spleen cellfusion. Although we prefer spleen cells for fusion, for fusion of lymph nodes regional nodes are activated by immunization with adjuvant (as for i.p. immunization). Mirza *et al.* (1987) performed fusion of lymph node cells at day 14 after primary immunization for the production of anti-insulin mAbs.

ISCOMs are generally applied intramuscularly or subcutaneously. In contrast to many other adjuvants, the administration of ISCOMs-related antigen along these routes is most effective when the interval between two administrations is 6 to 8 weeks. Short intervals give low antibody titres (Lövgren *et al.*, 1990).

The timing of immunizations and responses is dependent on the mode of antigen presentation; the antigen preparation and formulation is important in this. Slow release of antigen from emulsions or crystalline deposits may introduce a delay.

Side effects and safety of immunization procedures

In a Dutch Code of Practice (Veterinary Public Health Inspectorate, 1993) the side effects of immunization procedures are scaled in arbitrary units of injury from I (low) to III (severe). Intravenous antigen application (when possible) and intraperitoneal and subcutaneous injections score injury level I; intradermal and intramuscular application score injury level II, whereas intrasplenal immunization and immunization in the foot are level III.

The degree of injury is determined not only by routing but also by the nature of the adjuvant. Freund's complete adjuvant and ISCOMs by various routes (e.g. intraperitoneally) cause a high degree of injury and therefore are either better not used (FCA) or applied via routes that cause only a low degree of harm.

Snippe *et al.* (1992) distinguish two categories of adjuvants applications: (1) includes adjuvant applications for which the efficacy aspect is more important than the safety/toxicology aspect and (2) applications where safety is more important than efficacy.

Group (1) consists of applications of adjuvants in experimental and production animals. All adjuvants seem to be applied in the former category and only four — water-in-oil and oil-in-water emulsions as well as aluminium salts and liposomes — in the latter category. For production animals, only FCA is considered not-allowed, whereas some doubts are expressed with respect to application of most other adjuvants. In this category the application of an adjuvant may be considered based on the presently available safety records of the adjuvants. However, sufficient alternatives are available to eliminate FCA from animal experimentation in most fields of biomedical research.

In group (2) applications of adjuvants in companion animals and humans are distinguished. For humans, only aluminium salts are allowed. Most other adjuvants are not considered for application except for specific purposes. Water-in-oil and oil-in-water as well as FCA are considered not to be applicable in humans at all. For companion animals, ISCOMs are thought to be sufficiently safe enough for application. Water-in-oil emulsions as well as FCA are considered not to be applicable in companion animals. All other adjuvants are worth considering for application on the basis of the presently available safety records.

For application in humans, production animals and pets the development of safe adjuvant formulations is still of great importance. The efficacy of present adjuvants seems in most cases to be in opposition to safety. For many vaccines an appropriate level of adjuvanticity is required to reach a sufficient level of vaccine

efficacy. The ability to provide formulations which combine efficacy with safety is a challenge (see also Gupta *et al.*, 1993).

Conclusions and suggestions

Immunization, which aims at optimal antigen presentation, is a complex multivariate issue. The properties of the antigen, of the route of introduction, of the formulation, and of the recipient all contribute to the final result. Therefore, finding the optimum immunization results is a matter of experimentation and experience.

The tables included in this chapter may provide a guide to the choices to be made when specific immune responses to antigens have to be elicited.

Purity of an antigen determines the specificity of the responses. Subunits require better adjuvant than intact complex antigen. T cell determinants in general are MHC class restricted, however promiscuous epitopes are frequently encountered. Use of the latter epitopes with haptens produces efficient immunogens. Single epitopes (haptens) conjugated to an efficient carrier form efficient immunogens. The use of a 'foreign' protein as a carrier (KLH in mammals) is more efficient than a related carrier protein (BSA in mammals) (Boersma *et al.*, 1993) See also Table 1.

When generation of antibody for use in detection is the main aim of immunizations, there is a rather broad choice of routes of introduction depending on the solubility of the antigen. Care should be taken to immunize more than once to obtain sera with relatively low titres of IgM antibodies, which in many immunoassays lead to background and crossreactivity. Water-in-oil emulsions are simple to use, and need no complex laboratory equipment. subcutaneous or intraperitoneal application is easy to perform with a low injury index.

For production of monoclonal antibodies, cellfusion is best performed after a booster immunization (intravenously if possible) without adjuvant so as to avoid depot effects, and if necessary with alum-antigen precipitates as a vehicle (intraperitoneally)(Table 4).

When expression of specific isotypes is needed (complement fixation), the response can be skewed in that direction by the choice of adjuvant (lipopolysaccharides, liposomes, ISCOMs, NBP) (Tables 2 and 3).

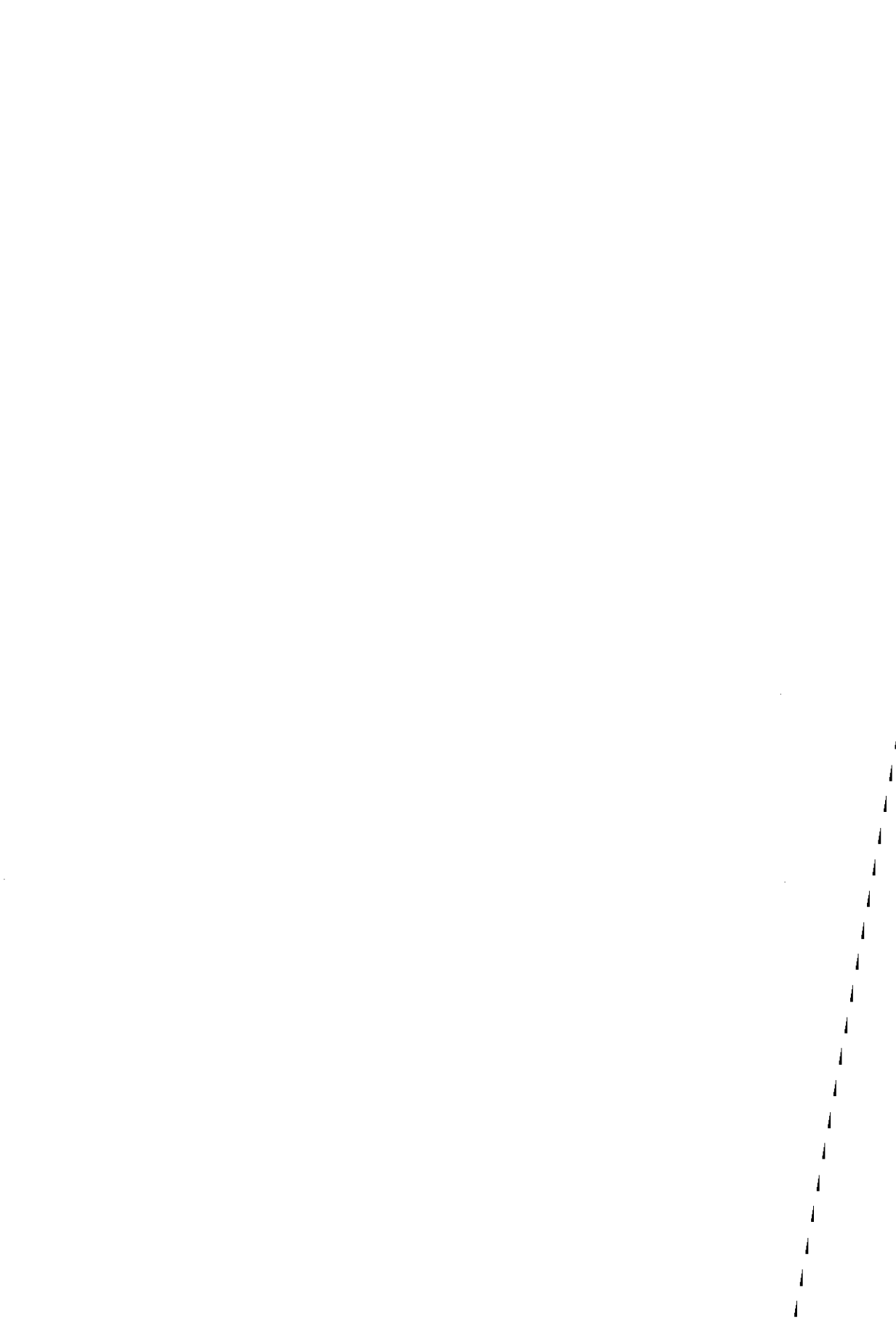
For DTH reactions the choices of adjuvants for specific antigens depend on the antigen properties. Mineral salts are not efficient. Water-in-oil emulsions, liposomes, various synthetic antigens, and ISCOMs are very efficient (Tables 2- 4).

When cell-mediated immunity on the level of class I restricted responses is required for protection, then the choice of adjuvants is highly restricted. In fact, only ISCOMs and to a lesser extent liposomes seem to be very efficient in elicitation of class I restricted responses. However, this type of construction is needed only when we use selected fragments of antigens (isolated viral proteins, cell surface molecules) for the induction. When whole live viruses or cells are applied for immunization, class I restricted responses are easily induced.

We have given above an outline of choices to be made with many variables. Although most of the data are from mouse and man, in general the picture is similar in other animals. For these other animals, however, the distribution of isotypes in immunoglobulins, and the balance between Th1 and Th2 may be somewhat different. We hope that our guidelines will lead to successful immunizations.

New and exiting developments are moving rapidly from preliminary evidence to clinical application. Production and purification are difficult for many antigens. Recombinant DNA expression systems have therefore been developed for many antigens. An alternative route of influence over antigen presentation is the direct introduction of naked DNA (plasmids, vectors) into cells which bring the introduced sequence to expression. The antigens have proper glycosylation. This technique leads to antigen presentation via class I and possibly class II MHC molecules such that an immune response results. Not all easily accessible cells (skin, muscle) may be good antigen-presenting cells. However, when vectors are developed which, apart from sequences coding for antigens, in addition code for cytokines which induce the right molecular structures for antigen presentation it seems that most problems can be solved.

It is envisaged that this method of immunization will open new ways to treat malignancies (idiotypic vaccines) as well as chronic infections. The new 'DNA-vaccines' will be easily produced, purified, stored, and administered. The development of this alternative approach leads the immunization from expression products to the 'source' (genetic material). However, it will take some time before all regular vaccinations will follow this approach.



Chapter 3

Evaluation of several adjuvants as alternatives to the use of Freund's adjuvant in rabbits

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Abstract

In three experiments we evaluated several types of adjuvants as an alternative to Freund's adjuvant (FA). In the first experiment three adjuvant preparations (a water-in-oil emulsion (Specol), a combination preparation of monophosphoryl lipid A + trehalose dimycolate + cell wall skeleton (RIBI) and a nonionic block polymer surfactant (TiterMax)) were evaluated. The adjuvants were combined with three different types of (weak immunogenic) antigens (synthetic peptide, glycolipid and particulate antigen) and administered following the intramuscular or subcutaneous route. The evaluation was based on clinical, pathological and immunological parameters. The animals did not appear to be severely or chronically impaired by the experiment. After injection of the RIBI adjuvant, the severity of the side effects was similar to or exceeded those induced after FA injection, while low antibody titres were produced. TiterMax caused little side effects, while antibody responses were very low. In comparing Specol and FA; Specol had far less adverse effects than FA. However, Specol had immunostimulating properties of the same level as FA. In the second experiment, the effect of injected volume of FA on side effects and antibody titre was studied. Immunization of rabbits with a total of 0.5 ml FA at different sites does not seem to increase the immune response when compared with the immune response seen after injection of 0.5 ml FA at one site. However, side effects were seen in all the animals. In the third experiment, the side effects of intradermal (i.d.) injection of the adjuvants were studied. After i.d. injection of FA or RIBI, undesirable effects were found. No side effects occurred after i.d. injection of Specol or TiterMax. From the studies it is concluded that Specol is an alternative to FA for hyperactivation of the immune response in rabbits.

Introduction

In experimental animals, the induction of effective B and T cell responses to antigens usually requires that the antigen is administered with a potent adjuvant. For this purpose Freund's adjuvant (FA) has been used in laboratory animals for decades. Administration of Freund's complete adjuvant (FCA) and to a lesser extent of Freund's incomplete adjuvant (FIA) induces local and systemic lesions (Broderson, 1989). The side effects following administration of FA suggest that FA has a great potential to cause pain and distress in animals (Morton and Griffiths, 1985). For this reason, guidelines in Canada, the USA and the Netherlands restrict researchers in using FA (Claassen *et al.*, 1992).

Experimental studies in animals have revealed an array of diverse products which could function as an adjuvant and probably be used as an alternative to FA. An ideal alternative adjuvant should have the effectiveness (with different types of antigen) of FA but does not induce adverse effects. In this study, the induction of effective B cell responses in rabbits was investigated, as this species is commonly used for the production of hyperimmune serum. In the 44th Forum in Immunology an overview of most of the adjuvants presently available is given (Claassen and Boersma, 1992). Many types of water-in-oil (w/o) emulsions are used as adjuvants. An example of a w/o emulsion is Specol (Bokhout *et al.*, 1981; Boersma *et al.*, 1992). Since 1981, the immunomodulating properties of a group of synthetic materials, the nonionic block polymer surfactants (NBP), have been studied (Hunter and Bennett, 1984). At the moment, two adjuvant formulations are commercially available in which NBP are an essential component: TiterMax (CytRx Corporation, Atlanta, GA) and Syntex adjuvant formulation (SAF-M: Syntex Research, Palo Alto, CA). Other types of synthetic adjuvant are muramyl dipeptide (MDP), trehalose dimycolate (TDM), dimethyldioctadecylammonium bromide (DDA) and avridine (Woodard, 1990). Among the surface active adjuvants, the Saponin adjuvants like Quil A and ISCOM (immune-stimulating complexes) (Claassen and Osterhaus, 1992) are commonly used. Commercial combination preparations, such as RIBI (MPL+TDM+CWS (monophosphoryl lipid A and trehalose dimycolate and cell wall skeleton) emulsion) are available.

This study was designed to evaluate several types of adjuvants, with a different mode of action, as an alternative to FA. The evaluation was based on clinical, immunological and pathological parameters. The adjuvants selected were one step mixing products and included: FA, Specol, RIBI and TiterMax. The antigens used were a synthetic peptide (SPek15a), a glycolipid (galactocerebroside) and a particulate antigen (*Mycoplasma pneumoniae*). The effect of an injected volume of FA on side effects and antibody titre and the side effects of intradermal (i.d.) injection of the adjuvants were also studied.

Animals, materials and methods

Animals

Seventy eight (surplus) New Zealand White rabbits (*Oryctolagus cuniculus*) of both sexes were obtained from the specific pathogen free (SPF) breeding centre of the RIVM. The rabbits were 6 months and weighed between 3.5 and 4.0 kg initially. They were housed individually in standard stainless-steel cages at a room temperature of 20-21°C under a 12 h day-night light cycle. The relative humidity was between 45-60%. The rabbits were fed rabbit chow (80 g day^{-1}, Hope Farms BV, Woerden, the Netherlands) and tap water was available *ad libitum*.

Materials

The following materials were used in the experiments described below:
Freund's complete adjuvant (FCA; Difco Laboratories, Detroit, MI), Freund's incomplete adjuvant (FIA; Difco Laboratories), Specol (water-in-mineral oil emulsion described by Bokhout *et al.* (1981); ID-DLO, Lelystad, the Netherlands), RIBI (MPL+TDM+CWS emulsion; Sanbio BV, Uden, the Netherlands) and TiterMax (NBP; CytRx Corporation, Atlanta, GA).

Antigens

Three different types of antigen were used; synthetic peptide (SPek15a, complexing B and T cell epitope (Zegers *et al.*, 1992; MBL-TNO, Rijswijk, the Netherlands), glycolipid (galactocerebroside; MBL-TNO, Rijswijk, the Netherlands) and particulate antigen (*Mycoplasma pneumoniae*, whole antigen inactivated 30 min. at 57°C; RIVM, the Netherlands). In order to discriminate between poor and potent adjuvants, the antigens used were selected for their low immunogenicity and they were not coupled to a carrier protein. The SPek15a and *Mycoplasma pneumoniae* antigen were diluted in PBS (phosphate-buffered saline), galactocerebroside antigen was diluted in DMSO ((dimethyl sulphoxide) : PBS=1 : 1). The amount of antigen injected per rabbit was 200 µg SPek15a, 200 µg galactocerebroside or 87 µg *Mycoplasma pneumoniae* in primary and secondary immunization.

Adjuvant/antigen preparation

Freund's adjuvant (FCA and FIA) was emulsified with an equal volume of antigen in solution using two syringes and a double hub connector. The antigen in solution was added to FA via the connector and mixed for 1 min. Specol adjuvant and antigen in solution was emulsified 11 : 9. In the first experiment, Specol was mixed as described for the FA mixtures. In a second experiment, Specol was mixed on a vortex and the antigen in solution added dropwise while vortexing. RIBI adjuvant was prepared according to the manufacturer's instructions by reconstituting a final

volume of 2 ml with antigen/solution mixture in the RIBI vial. TiterMax was emulsified in the same way as FA. However, the antigen in solution was added to the adjuvant in two steps.

Immunization experiments

Experiment 1

The adjuvants to be studied were combined with the three different types of antigens. On day 0 the animals were immunized (two rabbits by subcutaneous (s.c.) route and two intramuscularly (i.m.)) with either 0.5 ml FCA/antigen mixture, 0.5 ml Specol/antigen mixture, 2 x 0.5 ml RIBI/antigen mixture or 0.08 ml of a TiterMax/antigen combination. The injected volumes of the RIBI and TiterMax adjuvants followed the specifications of the manufacturer. The control group was injected (two animals subcutaneously and two intramuscularly) with 0.5 ml antigen in solution mixture. On day 28 a secondary injection was given. The secondary immunization contained the same mixture as the primary immunization with the exception of FCA being replaced by FIA. As shown previously, FCA and FIA, when used for secondary immunization, have the same effect on the immune response (De Greeve *et al.*, 1982). However, the side effects after secondary immunization with FIA are less severe than after secondary immunization with FCA (Broderson, 1989).

On day 0 the animals were injected at the left side of the body, while the secondary injection (day 28) was given at the right side. The site of injection was shaved and cleaned with ethanol (70%). The s.c. injections were made dorsally just behind the scapula. The i.m. injections were given in the muscles of the posterior thigh. The second i.m. injection of the RIBI injected animals was given in the dorsal muscles in the lumbar region. Sterile 20-gauge needles were used for s.c. and i.m. injections.

Experiment 2

The effect of volume of injection on adverse effects and antibody titre was studied by immunizing rabbits subcutaneously with 1 ml, 0.5 ml, 2 x 0.25 ml and 4 x 0.125 ml FA emulsion. SPek15a was used as antigen. Two rabbits were used per volume. Animals immunized with 2 x 0.25 ml were injected in two sites, animals immunized with 4 x 0.125 ml received the mixture at four sites. Two control animals were injected with 1 ml antigen in PBS. As in Experiment 1, the animals were injected in the left site on day 0, while the secondary injection (day 28) was given in the right site. On day 0, FCA was injected, on day 28, FCA was substituted by FIA. The animals injected with 1 and 0.5 ml, were injected in the same way as the s.c. injected animals in Experiment 1. The 2 x 0.25 ml injected animals received one injection 5 cm caudal from the scapula and 5 cm from the median line, the second 5 cm cranial from the crista iliaca and 5 cm from the median line. The four s.c. injections of 0.125 ml per site were given in a zigzag formation to each flank.

Experiment 3

The effect of i.d. inoculation of the adjuvants was evaluated in a third experiment. The animals were injected i.d. on day 0 receiving two inoculations of 50 µl adjuvant solution (FCA, Specol, RIBI or TiterMax) in different sites (one animal per adjuvant). No antigen was administered. The adjuvants were mixed as described above, with PBS as aqueous solution. The site of injection was shaved and depilated and cleaned with ethanol (70%). Sterile 25-gauge needles were used for injections. The i.d. inoculations were given at comparable sites as the 0.25 ml injections described in Experiment 2.

Clinical observation

The animals were observed every 2 days for clinical signs of disease. Body weight was recorded once each week. The body temperature of the *Mycoplasma pneumoniae* injected animals was recorded twice a week. The rabbits were placed in a rabbit box; after an initial acclimatization of 30 min., the rectal temperature was recorded four times in 30 min. The average of these four temperatures was taken as the body temperature. Locomotor activity was observed twice during the first week after primary and secondary immunization.

Bleeding samples

Blood (5 ml) was sampled immediately prior to the first injection (prebleed) and then at 7-day intervals. After sedation with Hypnorm 0.2 ml per animal (Janssen Pharmaceutica BV, Tilburg, the Netherlands) blood was obtained from the marginal vein of the rabbit's ear. The blood was centrifuged at 2000 *g* for 20 min. and the sera were stored at -20°C. Blood samples were taken for antibody titration.

Immunoassays

Antibody responses in the serum were evaluated using a direct enzyme-linked immunosorbent assay (ELISA) (in which the antigen was coated directly to the plate by passive adsorption) and immunofluorescence (only determined for the *Mycoplasma pneumoniae* group).

Serum antibody titrations for the SPek15a and galactocerebroside immunized animals were performed using the ELISA method described by Zegers *et al.* (1991). The antibody responses to α_1-antitrypsin (T cell epitope; MBL-TNO, Rijswijk, the Netherlands) were performed in the sera of animals immunized with SPek15a using an ELISA procedure. The plates were coated overnight at 4°C with 50 µl PBS containing 10 µg/ml SPek15a or with 50 µl ethanol/PBS (1 : 1) containing 80 µg/ml galactocerebroside or with 50 µl PBS containing 5 µg/ml α_1-antitrypsin. Swine anti-rabbit serum conjugated to alkaline phosphatase, diluted 1 : 200, was used. After 30 min. the absorbance was read at 405 nm in a Titertek Multiskan reader (Flow Laboratories, Irvine, UK). The sera were diluted using serial 2-fold dilutions from 1 : 20

to 1 : 40960. The titre in the ELISA was chosen as the dilution at which the absorbance was half of the maximum absorbance.

The ELISA procedure for the *Mycoplasma pneumoniae* antibodies was performed as described by Cassell and Brown (1983). To obtain an ELISA antigen from *Mycoplasma pneumoniae*, the Tween 20 solubilization method was used. The supernatant fluid was filtered through a 0.45 µm pore diameter membrane. Horse radish peroxidase conjugated donkey anti-rabbit serum (Amersham, Buckinghamshire, UK) was used. The sera were diluted using 2-fold dilution from 1 : 20 to 1 : 2560.

The antibody responses to *Mycoplasma pneumoniae* were determined by immunofluorescence in accordance with Rosendal and Black (1972). Cultivations of *Mycoplasma pneumoniae* on Chanock agar and horse anti-rabbit serum conjugated with fluorescein isothiocyanate (FITC) were used. Microscopical observation was done at a magnification of 100X by using an Orthoplan Leitz microscope with an incident illumination of 490 nm wavelength.

Pathology

After 42 days animals were killed by intravenous injection of 2.5 ml euthanasia solution (Nembutal; Sanofi BV, Maassluis, the Netherlands). Necropsy consisted of dissection and careful examination of injection sites and draining lymphnodes, supplemented by examination of major organs in selected cases. For histology, tissues were fixed in 4% neutral buffered formaldehyde. Lesion sites were embedded in glycol methacrylate and sectioned at 1 µm while lymph nodes were embedded in paraplast and sectioned at 5 µm. All sections were stained with haematoxylin and eosin.

Results

Immune response

Immunization of rabbits with the different adjuvant and antigen combinations resulted in primary and secondary antibody responses depending on type of adjuvant and antigen. Data are presented separately for Experiments 1, 2 and 3.

Experiment 1

Animals immunized with the synthetic peptide SPek15a (Fig. 1 and 2) produced the highest antibody titres when Specol or FA was used as adjuvant, both by the s.c. and i.m. route of immunization. A marked increase in antibody production was seen after secondary injection in week 4. Antibody levels in animals immunized with TiterMax were very low and only detectable from week 5 s.c. and from week 4 i.m. RIBI animals produced a detectable titre only in week 6 (i.m. immunized animals). Antibody responses were undetectable in our study when SPek15a without adjuvant was injected.

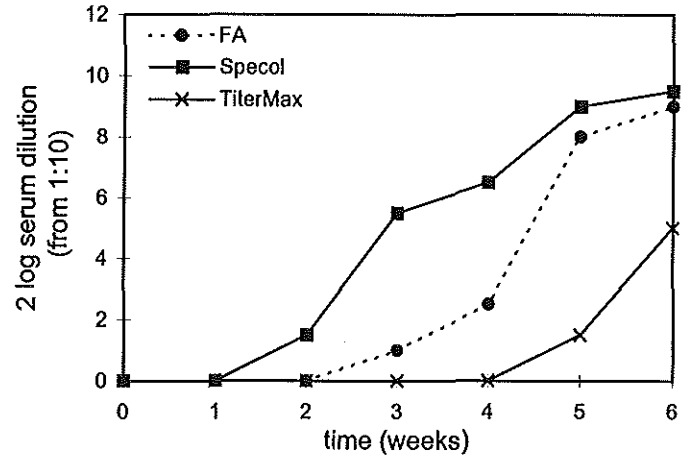

Figure 1 ELISA antibody titres in rabbits s.c. immunized and boosted with FA-, Specol-, RIBI-, TiterMax- or PBS/SPek15a mixtures. Sera of animals immunized with RIBI or PBS-mixtures did not show any response. The initial serum dilution was 1 : 10 (point 0). Results are shown as the mean value for two rabbits.

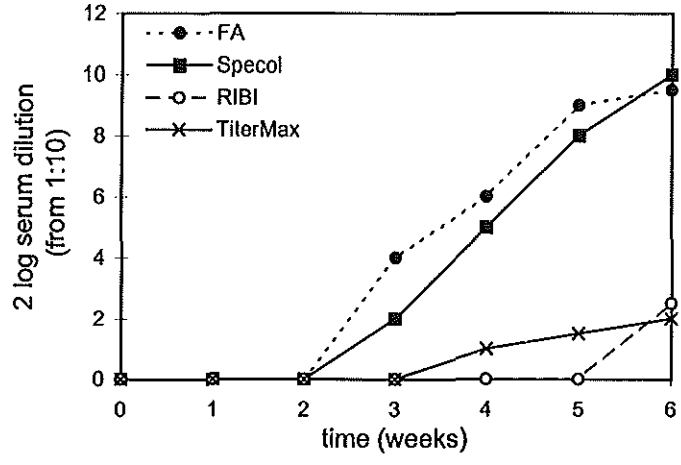

Figure 2 ELISA antibody titres in rabbits i.m. immunized and boosted with FA-, Specol-, RIBI-, TiterMax- or PBS/SPek15a mixtures. Sera of animals immunized with PBS-mixtures did not show any response. The initial serum dilution was 1 : 10 (point 0). Results are shown as the mean value for two rabbits.

When *Mycoplasma pneumoniae* was used as antigen (Fig. 3 and 4) a similar picture emerged for the antibody responses of animals immunized with FA or Specol. Because of the marked clinical findings in the animals s.c. immunized with Specol/*Mycoplasma pneumoniae* (see clinical observations and pathology) s.c. immunization was repeated with Specol and FA. In comparison with the first experiment ELISA antibody titres were somewhat higher for FA immunized animals and comparable for Specol immunized animals. Antibody levels in animals immunized with RIBI and TiterMax .were low. It should be noted that ELISA antibody titres decreased from week 5 for the RIBI and TiterMax immunized animals. For the RIBI adjuvant titres in i.m. immunized animals were higher than in s.c. immunized animals. On the contrary, TiterMax produced higher ELISA titres s.c. than i.m.

In the determination of the titres of *Mycoplasma pneumoniae* group by immunofluorescence only the rabbits treated with Specol showed fluorescence three weeks after the first injection at the dilution 1 : 20. One week after the administration of the booster injection all rabbits showed a fluorescent reaction at dilution 1 : 80 with exception of those animals treated subcutaneously with FA and RIBI, which did not react at all. One week later the situation was the same except for the animals injected subcutaneously with RIBI where reaction was observed at dilution 1 : 20.

None of the animals immunized with galactocerebroside produced a detectable antibody response during the period of immunization.

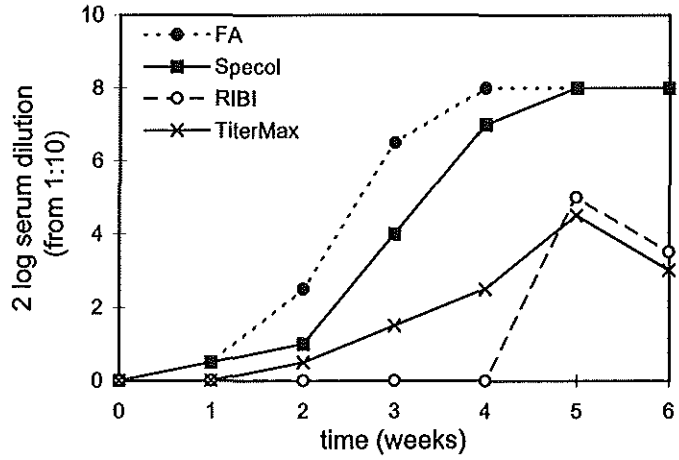

Figure 3 ELISA antibody titres in rabbits s.c. immunized and boosted with FA-, Specol-, RIBI-, TiterMax- or PBS/*Mycoplasma pneumoniae* mixtures. Sera of animals immunized with PBS-mixtures did not show any response. The initial serum dilution was 1 : 10 (point 0). Results are shown as the mean value for two rabbits.

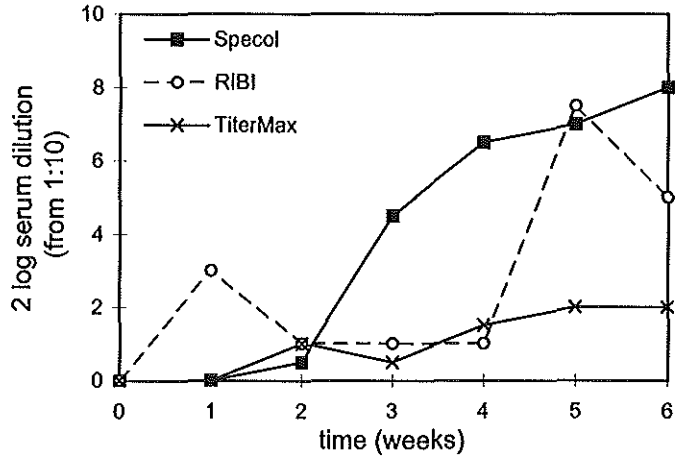

Figure 4 ELISA antibody titres in rabbits i.m. immunized and boosted with FA-, Specol-, RIBI-, TiterMax- or PBS/*Mycoplasma pneumoniae* mixtures. Sera of animals immunized with PBS-mixtures did not show any response. ELISA not performed on sera of FA-immunized rabbits. The initial serum dilution was 1 : 10 (point 0). Results are shown as the mean value for two rabbits.

Experiment 2

The antibody titres after injection of different volumes of FA/SPek15a are shown in Fig. 5. After primary immunization the antibody responses were low. On day 21, the antibody response was detectable in all the sera (except for the control group). The titres increased until day 35 and reached a plateau on day 42. The antibody responses did not differ significantly between the animals injected with different volumes of FA.

Clinical observations and pathology

Locomotor activity was not affected by immunizations. Body weight and body temperature were within the normal limits for the post-immunization period for all types of adjuvant. In none of the samples taken for bacteriology growth could be demonstrated. Data of gross pathology are specified separately for Experiment 1, 2 and 3.

Experiment 1

The adjuvants (FA, Specol, RIBI and TiterMax) were combined with the three different types of antigens in this experiment. On clinical observation, soft tissue swelling was found in animals subcutaneously exposed to FA, Specol, RIBI and TiterMax.

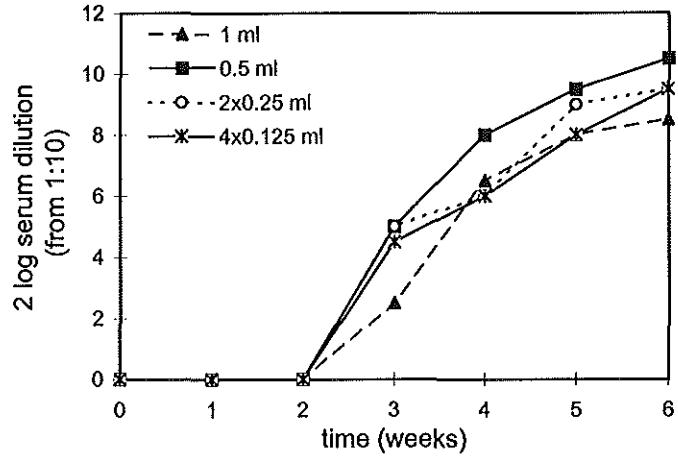

Figure 5 ELISA antibody titres in rabbits s.c. immunized and boosted with FA/SPek15a mixtures (different volumes: 1 ml, 0.5 ml, 2 x 0.25 ml, 4 x 0.125 ml). FCA is used in primary and FIA in secondary immunization. Antibody responses were not detectable in sera of week 0, 1 and 2. The initial serum dilution was 1 : 10 (point 0). Results are shown as the mean value for two rabbits.

Macroscopic examination revealed no abnormalities at the injection sites of control animals except some slight hyperaemia or haemorrhage at the secondary injection site of 4 animals. Inflammatory processes were found at most injection sites of animals injected with FA (70%) and RIBI (90%), while animals injected with Specol and TiterMax developed inflammation at 40% of the injection sites. As reasonable similarity existed between reactions of animals who received the same treatment, these data are taken together in Table 1.

Enlargement of draining lymph nodes was observed in some animals with very severe lesions but otherwise occurred incidentally in all groups. Inspection of internal organs in animals with most severe lesions revealed no gross abnormalities, except for one RIBI-treated animal with multiple small subserous abdominal abcesses, which yielded *Escherichia coli*, *Streptococcus faecalis* and *Staphylococcus aureus*.

Subcutaneous lesions varied in size, character and complexity. Dorso-ventral extension was larger than cranio-caudal, but no fistulous tracts as described by Broderson (1989) were observed.

FCA treated animals mainly showed granulomatous nodules with central necrosis (6 of 8 primary sites), which were often hyperaemic or haemorrhagic. In one *Mycoplasma*-treated animal a flat yellow layer surrounded by severe hyperaemia and haemorrhage was observed. This animal showed the same severe abnormality at the secondary injection site, 2 weeks after FIA injection. Microscopically, the yellow

substance appeared to be a necrotic layer within granulomatous inflamed connective tissue, very rich in capillaries near the necrotic areas. All FIA injections led to hyperaemic or haemorrhagic lesions 2 weeks after injection, that otherwise varied from slight to very extensive flat granulomatous processes, attached to superficial muscle. They showed less necrosis than FCA-induced granulomas.

Specol, in combination with *Mycoplasma pneumoniae,* induced the same yellow caseous plaques as described for FCA, which were very extensive at both sides in both rabbits. After repeat of the latter combination, this time prepared according to the manufacturer's description, the lesions were confined to the secondary injection site and were milder, mainly resembling extensive fibrous proliferation of the subcutis.

Table 1. Pathology findings[a] at injection sites of rabbits immunized (s.c. or i.m.) with various adjuvants mixed with either SPek15a, galactocerebroside or *Mycoplasma pneumoniae* (Experiment 1)

		s.c.		*i.m.*	
Adjuvant	*Antigen*	*P*[b]	*S*[c]	*P*	*S*
FA	SPek15a[d]	+	++	+	++
	Galacto.[e]	++	+	+	-
	M.pneu.[f]	++	++	++	-
Specol	SPek15a	-	+	-	+
	Galacto.	-	-	-	-
	M.pneu.	-	++	+	+
RIBI	SPek15a	++	+	+	+
	Galacto.	++	++	++	++
	M.pneu.	++	++	+	++
TiterMax	SPek15a	-	++	+	+
	Galacto.	-	-	-	+
	M.pneu.	+	++	-	+
-	SPek15a	-	-	-	-
(no adjuvant)	Galacto.	-	-	-	-
	M.pneu.	-	-	-	-

[a] - = no lesion, + = moderate lesion, ++ = severe lesion; Lesions are described in detail in the results section; [b]P=primary immunization; [c]S=secondary immunization; [d]each line represents the average of two s.c. and the average of two i.m. injected animals; [e]galactocerebroside; [f]*Mycoplasma pneumoniae*, only results of repeat experiment of FA and Specol mixed with *Mycoplasma pneumoniae*.

Microscopically the proliferation appeared to be granulomatous inflammation, in part around oil spaces, with minimal necrosis. In combination with other antigens, Specol induced only one lesion in a single animal.

RIBI produced the largest number and the most severe lesions when compared with the other adjuvants. One secondary injection site (*Mycoplasma pneumoniae*) was ulcerated. In addition to one caseous plaque (as described above), in three animals spherical abscess-like processes were found, measuring up to 8 cm diameter. The caseous content was found microscopically to be necrosis of granulomatous inflammatory tissue. At most other injection sites, RIBI induced less voluminous necrotizing granulomas.

Subcutaneous injection of TiterMax induced a few relatively severe changes. At one secondary injection site (SPek15a) ulceration occurred. In the *Mycoplasma*-group, yellow layers were found at one primary and two secondary injection sites (as described above).

Intramuscular lesions were seen as irregular, grey, white, shining processes sometimes containing thick pale yellow pus, tending to spread parallel to muscle fibres. They measured up to 1.5 cm cross diameter and 5 cm in length. Skin lesions and muscle atrophy were not observed in i.m. injected rabbits.

RIBI most consistently produced lesions intramuscularly, 6 as well as 2 weeks post inoculation, and these lesions often showed hyperaemia and pus centrally. FCA and FIA produced changes less frequently and of less severity than RIBI. Specol and TiterMax produced few abnormalities intramuscularly 6 weeks post inoculation, but at 2 weeks post inoculation most injection sites showed granulomas. At 6 weeks post inoculation, FCA induced more lesions than Specol and TiterMax.

Experiment 2

Gross examination of animals s.c. injected with different volumes of FA, revealed remarkable results. The two animals injected with 0.125 ml per injection site showed necrotizing granulomas, measuring up to 3 cm diameter, at 7 out of 8 FCA injection sites. Less severe granulomatous proliferations were found at 7 out of 8 FIA injection sites. Only one of the 1 ml FA injected rabbits showed impressive granulomas at both sides (Fig. 6). FCA lesions in animals injected with 0.5 ml were absent, while both animals injected with 0.25 ml (on two spots) showed two distinctive granulomas; FIA lesions were present at all sites injected with 0.5 and 0.25 ml. Both control animals showed no abnormalities at necropsy.

Experiment 3

On clinical observation, the animals injected with FCA and RIBI showed prominent nodules at both injection sites, measuring approximately 1 cm diameter. One FCA induced nodule was ulcerated centrally. Microscopically, intradermal granulomas around oil spaces were found, with focal necrosis. The RIBI-induced lesions (Fig. 7)

that appeared particularly tender on palpation, were composed microscopically of massive necrosis surrounded by a thin vital rim of granulomatous inflammatory tissue.

Discussion

In this study, Specol is being considered as a possible alternative to FA since Specol produced serum antibody titres comparable to FA but fewer adverse effects.

Primary and secondary antibody response was elicited depending on type of antigen, adjuvant and route of administration. In order to discriminate between poor and potent adjuvants, the antigens used in this study were selected for their low immunogenic power. A dose range of 50-1.000 µg is generally recommended for most protein, carbohydrate and nucleic acid antigens (Johnston *et al.*, 1991). The dose of 200 µg used for the SPek15a and galactocerebroside antigen should be sufficient to elicit an antibody response. The antibody titre of the RIBI/SPek15a-injected animals was very low. This low antibody titre for RIBI adjuvant agrees with the observations by Johnston *et al.* (1991) who used a synthetic peptide as an antigen. However, Cantrell and Finn (1989) and Deeb *et al.* (1992) claim good antibody titres with the RIBI adjuvant. It should be noted that the excellent antibody responses seen with Specol and SPek15a were also observed when the antibodies were tested in an α_1-antitrypsin (native protein) ELISA. This indicates that Specol is efficient not only in SPek15a antibody production but also in the production of anti-native protein crossreactive antibodies (i.e. anti-peptide antibodies that also recognize the native protein).

In the *Mycoplasma pneumoniae* group the antibody titres decreased from week 5 for the animals injected with RIBI or TiterMax. This drop from week 5 after secondary injection was also found for RIBI by Deeb *et al.* (1992) who immunized rabbits i.m. with 0.25 ml RIBI with synthetic polypeptide antigen. In the study of Bennett *et al.* (1992) the antigen was conjugated to HEA or BSA which may have a positive influence on the immune response. In our study no such carriers were used and therefore a low immune responses may be expected. Our aim was not to examine the effect of a potent or less potent carrier protein; we wanted to discriminate between poor and potent adjuvants. The i.m. injections of RIBI seem to elevate the antibody titres more than the s.c. injection of the same adjuvant. It is the other way around for the TiterMax adjuvant. The high antibody titres claimed for TiterMax adjuvant (Hunter and Bennett, 1984; Check *et al.*, 1990) were not observed in this study.

In the galactocerebroside group no antibody titres were detected after s.c. immunization or after i.m. immunization. It remains obscure whether there is no antibody response at all or antibody response cannot be estimated for technical reasons. Fry *et al.* (1976) reported a study in rabbits when an antibody titre was measured by immunoprecipitation in agar and complement fixation. Fry *et al.* injected rabbits with 3 mg of galactocerebroside antigen mixed with 3 mg bovine serum albumine (in 0.3 ml PBS and 0.3 ml FA) by foot pad inoculations (each animal

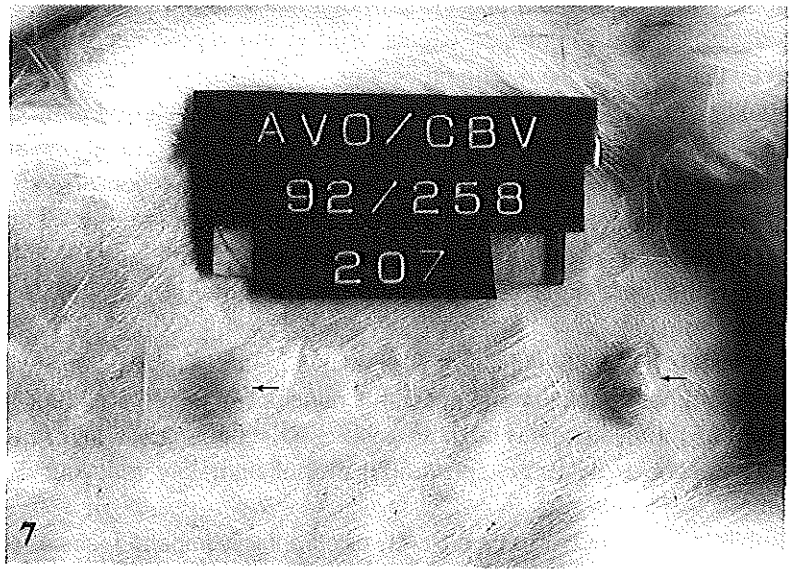

Figure 6 Subcutaneous injection of 1 ml FCA/SPek15a mixture. At necropsy after 42 days, an extensive subcutaneous area of oedema and hyperaemia; centrally irregular grey nodules attached to muscles. Superficial muscle layer removed. Microscopically, granulomatous inflammation with focal necrosis.

Figure 7 Intradermal injection of 0.05 ml RIBI in two sites. At necropsy after 42 days, prominent yellow nodules covered with intact skin, measuring about 1 cm diameter and containing thick pale yellow pus. Microscopically, intradermal granulomatous proliferations with central necrosis.

received 0.15 ml per foot pad). The galactocerebroside antigen used in our study was not coupled to a carrier protein. Further, FA/galactocerebroside used in mice in our laboratory resulted in high titres. This mice serum was used as a positive control in the antibody titrations for galactocerebroside.

On macroscopic observation, lesions were seen after s.c. and. i.m. administration for most of the adjuvants. However, striking differences, both qualitative and quantitative, were seen. The small volume of 0.5 ml, as suggested by Herbert and Kristensen (1986), could not prevent the adverse effects of most adjuvants. The role of the antigen must be considered as having influenced the side effects after administration with an adjuvant. Specol and TiterMax in combination with galactocerebroside antigen hardly gave any side effects, while the RIBI adjuvant in combination with the galactocerebroside antigen showed an inflammatory reaction at all the injection sites. One severe lesion type, the extensive caseous layers with haemorrhagic borders in the subcutaneously injected animals, only occurred after administration of *Mycoplasma pneumoniae* as antigen in combination with four different adjuvants. After administration of the antigens in PBS no inflammatory processes were found at gross examination.

The only difference between the repeat experiment (s.c. injection of FA or Specol with *Mycoplasma pneumoniae*) and the previous test was the procedure of antigen-adjuvant mixing. The different side effects seen in the repeat experiment might be explained by this difference in mixing procedure.

The results of Experiment 2 do not indicate that the injected volume of FA has any influence on lesions or antibody responses. Injection of 0.5 ml at one site seems to be preferable since adverse effects are less than after injection of 0.25 ml in two sites or 0.125 ml in 4 sites and no significant difference was seen in antibody titre. The number of animals used, however, is small (two). Broderson (1989) found that in rabbits, lesions were most severe when larger volumes (0.1-0.3 ml) of adjuvant were injected per site.

After i.d. injection of 0.05 ml FCA or RIBI adjuvant, undesirable effects were found. Thus, the recommended volume (Canadian Council of Animal Care, 1991) of an i.d. injection of 0.05 ml adjuvant still leads to severe effects in animals injected with FCA or RIBI. As described by Broderson (1989) the inflammatory reaction with the i.d. injection route was localized.

The rabbits did not appear to be severely or chronically impaired, since food intake, body weight and locomotor activity were within normal limits for the post-immunization period. The same results were observed in FA-immunized rabbits by Johnston *et al.* (1991) and in FA-immunized mice by Toth *et al.* (1989), while Wanstrup and Christensen (1965) observed decreased activity, dullness of the fur and loss of hair in mice when 0.05-0.2 ml FCA was s.c. injected weekly.

The severe pathological changes observed in this study after immunization with FA confirm the data that it is a disagreeable formula. RIBI and, to a far lesser extent,

TiterMax adjuvants caused lesions and low antibody responses were produced. The adverse effects observed in animals injected with Specol were less severe than with FA. Immune responses produced by Specol were comparable to FA. Specol is therefore considered as being a possible alternative to FA for immunization purposes in rabbits.

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Chapter 4

Comparison of several types of adjuvants for immune potentiating properties and side effects in mice

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Abstract

Four types of adjuvants were evaluated as alternative to the use of Freund's complete adjuvant in mice. The adjuvants evaluated included a water-in-oil emulsion (Specol), a micro-organism (*Lactobacillus*), immune-stimulating complexes with incorporated rabies virus glycoprotein (RV-ISCOMs) and a saponin, Quil A. The adjuvants and saline were combined with three weak immunogens (a synthetic peptide, an autoantigen or a particulate antigen) and given by three different routes (intraperitoneal, subcutaneous or dorsal in the foot). The evaluation was based on clinical observations, behavioural studies, pathological lesions and capacity to support immunological responses to weak immunogens. Lesions were most severe after injection of antigen combined with Freund's adjuvant or Quil A, mild to moderate with Specol and minimal with *Lactobacillus*, RV-ISCOMs or saline. Despite pathological changes, no signs of prolonged pain or distress could be demonstrated based on clinical observations and behavioural studies. Minimal immunological responses were found after injection of antigen in combination with saline or *Lactobacillus*. T cell activation and high antibody responses were found after injection of antigen/RV-ISCOMs or antigen in Freund's adjuvant emulsions. After Specol/antigen immunizations T cell activation was demonstrated and high antibody titres were found except for Specol/autoantigen immunizations. Presented data suggest that Specol is a possible alternative to Freund's complete adjuvant for the induction of an immune response against weak immunogens except possibly autoantigens, for which RV-ISCOMs seem very well suitable.

Introduction

To elicit effective B and T cell responses, Freund's complete adjuvant (FCA) has become the most widely used adjuvant in laboratory animals. However, besides the marked enhancement of the immune response, also a broad spectrum of unwanted side effects can be found (Toth *et al.*, 1989). Pathological changes seen after injection of FCA are known to be very painful in humans (Chapel and August, 1976). Growing concern with respect to the severe side effects caused by FCA is leading to stricter regulations. In Canada (Canadian Council on Animal Care, 1991), the USA (National Institute of Health, 1988) and the Netherlands (Veterinary Public Health Inspectorate, 1993) for example, guidelines have been publised giving recommendations on the use of adjuvant products in laboratory animals. One of the reasons for continuation of the use of FCA is a lack of information on possible alternatives to FCA, with regard to both side effects and immunological properties. Many products with adjuvant activity are available. Dependent on the type of immune response desired, an adjuvant may be applied. In an earlier study (Leenaars *et al.*, 1994) we evaluated adjuvants for the production of polyclonal antibodies and side effects in rabbits.

For the induction of a specific immune response it is important to know the mode of action of the adjuvant. To elicit immune responses efficiently the production of cytokines is required. Two major cytokines produced by activated helper T cells are IL-2 (Kuziel and Greene, 1990) and IFN-γ (Gustafson and Rhodes, 1992). T cell activation can also be studied by revealing the 39 kDa membrane protein (gp39) expressed on activated helper T cells, this molecule is essential for the activation of resting B cells into antibody producing cells (Noelle *et al.*, 1992; Van den Eertwegh *et al.*, 1993).

Since mice are generally used in immunological research we conducted a study to compare side effects and immunomodulating effects of alternative adjuvants to FCA in mice. The adjuvants, selected on their mechanism, low toxiticity and supposed immunological properties, included a water-in-oil emulsion, *Lactobacillus* strains, immune-stimulating complexes and saponin. To mimic normal laboratory problems in evoking immune responses and to ease discrimination between the adjuvants to be evaluated, the antigens used (a synthetic peptide, an autoantigen and a particulate antigen) were weak immunogens.

Animals, materials and methods

Animals

Male and female BALB/c mice were bred specific pathogen free (SPF) at the RIVM breeding facilities, Bilthoven, the Netherlands and were used at 10-14 weeks of age.

At the RIVM, animals were housed in groups of five under SPF conditions in polycarbonate cages, environmental temperature of 20-22°C, relative humidity of 50-70% and with a 12 h day/night cycle. Mice were fed a commercial diet (Hope farms, Woerden, the Netherlands) and provided bottled water *ad libitum*.

Adjuvants

The following adjuvants were used: Freund's adjuvant (water-in-oil emulsion, Freund's incomplete adjuvant (FIA) containing mycobacteria (FCA); Difco Laboratories, Detroit, MI), water-in-oil emulsion (Specol described by Bokhout *et al.* (1981); Institute for Animal Science and Health (ID-DLO), Lelystad, the Netherlands), non-pathogenic micro-organism, *Lactobacillus* (*Lactobacillus plantarum* (ATCC 8014, 10^9 per ml), *Lactobacillus casei* (ATCC 393, 10^9 per ml); Claassen *et al.*, 1994), ISCOM matrix with incorporated rabies virus glycoprotein (RV-ISCOMs) (preparation described below; Claassen and Osterhaus, 1992) and purified saponin Quil A ('Spikoside', Iscotec, Luleå, Sweden).

Rabies virus (RV-Pasteur strain) was propagated in Vero-cell monolayer cultures (van Wezel *et al.*, 1978). Culture supernatant was cleared by filtration and concentrated by Amicon ultrafiltration (cut-off 10^6 Da). Virus was inactivated with ß-propiolactone (BPL) and stored at -70°C at a concentration of 900 µg per ml. This rabies virus antigen was used for the preparation of RV-ISCOMs as described earlier (Feduka *et al.*, 1992).

Antigens

Synthetic peptide (SP215, an analogue of SP29) was synthesized as described by Boersma *et al.* (1989). SP215 is a sequence derived from the hinge region of human IgG2, comprising 21 amino acids. The autoantigen was myelin basic protein from bovine brain (MBP, Van Noort *et al.*, 1993; Sigma, St. Louis, MO), consisting of 173 amino acids (18.5 kDa). The particulate antigen *Mycoplasma pneumoniae* (MAC strain; *M. pneumoniae*) was a kind gift of Dr. A. Angulo (RIVM, Bilthoven, the Netherlands) and was prepared by culture into Chanock broth, concentrated by centrifugation and washed with phosphate buffered saline (PBS). Inactivation was done at 57°C during 30 min. Particles of *Mycoplasma pneumoniae* are 0.1 - 0.8 µm in diameter.

Adjuvant/antigen preparation

For the preparation of adjuvant/antigen mixtures, the antigens (amounts given in Table 1) were diluted in sterile physiological saline and mixed with the adjuvant, except when antigens were injected in combination with RV-ISCOMs. SP215 (2 mg) was coupled covalently to 120 µg RV-ISCOMs using 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDC) as described previously (Deen *et al.*, 1990). MBP (2.1 mg) was coupled to 80 µg RV-ISCOMs using EDC. After coupling for 30 min. at room temperature (RT), RV-ISCOMs were dialysed against PBS overnight to remove

uncoupled antigens and EDC. Freund's adjuvant (FCA in primary and FIA in secondary immunization, abbreviated as FA) and Specol emulsions, containing antigen, were prepared as recommended by the manufacturer of the adjuvant. *Lactobacillus*/antigen mixtures were prepared by adding antigen in sterile physiological saline to *Lactobacillus* (1 : 1) and mixing well. Quil A dissolved in water was added to the antigen and mixed. Intraperitoneally (i.p.) injected animals received 10 µg Quil A per injection while animals injected subcutaneously (s.c.) or at the dorsal site of the hindfoot (d.f.p.) received 20 µg Quil A per injection.

Experimental design

Adjuvants were evaluated in three experiments. In each experiment one type of antigen was combined with each of the adjuvants, with one exception: in combination with *M. pneumoniae*, RV-ISCOMs was substituted by Quil A. The size of this particulate antigen makes it unfeasible to couple to RV-ISCOMs. Per antigen and per injection route, a control group, injected with antigen in sterile physiological saline was included. Injected volume per route of administration and per type of antigen are given in Table 1. Five mice were used per group.

Animals were given a primary injection with the adjuvant/antigen or saline/antigen preparations on day 0. Identical secondary injections were given on day 42, except that FCA was replaced by FIA and the secondary injections of MBP/RV-ISCOMs were given on day 91 due to unavailability of the conjugated antigen.

Blood samples were taken from the tail vein, on day -1 to determine preimmune titres, one week after primary immunization and at 7 day intervals thereafter. Sera were pooled per group except for sera collected at necropsy. These sera were collected and titrated individually. Sera were stored at -20°C. One week after secondary immunization mice were bled after being anaesthetized by intramuscular inoculation of 0.1 ml KRA (mixture of Ketamine (50 mg per ml), Rompun (20 mg per ml) and Atropine (1 mg per ml) in a 7 : 3 : 1 ratio). Animals were examined for gross lesions and relevant tissue samples were collected for histopathology.

Table 1. Injected volumes per type of antigen and per route of administration[a] and dose of antigen applied in this study

		Volume (ml)		
Antigen	*Dose (µg)*	*i.p.*	*s.c.*	*d.f.p.*
SP215	50	0.1	0.1	2x0.04
MBP	100	0.2	0.2	2x0.04
M.pneu[b]	15	0.2	0.1	2x0.04

[a] i.p., intraperitoneal; s.c., subcutaneous; d.f.p., at dorsal site of hind foot;

[b] M.pneu = *Mycoplasma pneumoniae.*

Clinical observation and body weight

Specific changes in common clinical signs to indicate pain, distress or discomfort in experimental animals were evaluated as described by Morton and Griffiths (1985). General condition was evaluated daily and mice were weighed twice a week, starting on day -1. Injection sites were palpated for signs of pain and swelling at least once a week.

Behavioural and physiological state

The behavioural changes and physiological state of the mice were studied in the Primary Observation Test (POT); a systematic quantitative procedure described by Irwin (1968) and modified by Prof. Dr. B. Olivier (Solvay-Duphar, Weesp, the Netherlands).

The POT procedure involved an initial phase of undisturbed observation and a later manipulative phase during which the animal was subjected to different stimuli. Animal behaviour was studied before immunization, three days after primary immunization and weekly thereafter.

The procedure started by placing a group of mice in a viewing jar followed by an adaptation period of 1 h. The observation study began by observing the animal's undisturbed behaviour within the viewing jar, i.e., dispersion in the cage, apathy, startle-reaction, restlessness, watchfulness and respiration. Thereafter, the animals were individually transferred onto the viewing arena for testing: arousal response to transfer, spatial locomotion, gait, slip resistance, righting reflex, exophthalmos and pilo-erection. Throughout individual handling, touch-reflex, provoked-freezing, provoked biting, skin colour, body tone, pupil size, urination-defecation and vocalization were studied. After handling, mice were again placed in the viewing jar and wash activity was observed. Behavioural changes and physiological state of each mouse were scored.

Pathology

Necropsy consisted of complete dissection and examination of injection sites and major organs. Tissue of lesions and organs showing macroscopic abnormalities were preserved in 4% buffered formaldehyde. A sample of the omentum was preserved of all i.p. injected animals. The size of the popliteal lymph node (PLN) of d.f.p. injected animals was scored. The left PLN was fixed in 4% buffered formaldehyde, while the right PLN was immediately frozen in liquid nitrogen for immunohistochemistry for demonstration of cytokine-producing cells and gp39 expression. Frozen tissues were stored at -70°C in air-tight aluminium containers containing a small amount of ice to prevent dehydration on prolonged storage. For histopathological evaluation, a selection of fixed tissue samples was embedded in paraffin, sectioned at 5 µm, and stained with haematoxylin and eosin. In the i.p. injected animals the extent of abdominal lesions was scored based on the relative amount of white plaques in the

visceral and diaphragmatic peritoneum. The severity of peritonitis in these groups was scored based on gross detection of adhesions and omentum retrahens and on microscopic evaluation of (peri)pancreatitis, fat necrosis and micro abscesses. In animals s.c. injected lesions were scored separately for primary and secondary injection site. The score was based on diameter of lesions and on microscopic evaluation of infiltrates and the presence of necrosis. In the d.f.p. injected animals swelling was scored grossly and severity of lesions was scored microscopically by involvement of deep layers and the presence of ulceration. Gross and histopathologic lesions were scored as follows: - = minimal; + = mild; ++ = moderate; +++ = marked.

Immunoassays

Serum antibodies to MBP, SP215 and *M. pneumoniae* were determined using a direct enzyme linked immunosorbent assay (ELISA). For animals immunized with SP215 and MBP, ELISA procedures were performed essentially as described by Zegers *et al.* (1991). Sera were diluted 1:100, 1:300 and 1 : 900.

For *M. pneumoniae* antibodies, ELISA was performed as described in detail by Leenaars *et al.* (1994). The sera were diluted using three-fold dilutions from 1 : 270 to 1 : 65610.

Immunohistochemistry

Detection of cytokine-producing cells and $gp39^+$ cells in PLN were performed essentially as described by Van den Eertwegh *et al.* (1993). Cryostat sections (-20°C, 8 μm) were fixed for 10 min. in fresh acetone containing 0.02% H_2O_2. The murine monoclonal antibody (mAb) DB-1 (specific for rat IFN-γ and cross-reacting with murine IFN-γ) and the rat mAb S4B6 (specific for murine IL-2) both conjugated to alkaline phosphatase (AP) were used as cytokine-specific antibody conjugates. After washing the slides with PBS, AP activity (blue) was demonstrated by naphthol-substrate as described by Claassen *et al.* (1986a). Immunohistochemical demonstration of gp39 was performed with MR-1 (a hamster mAb specific for murine gp39) horizontally overnight under high humidity at 4°C. Slides were washed three times with PBS followed by the murine mAb specific for rat/hamster-Ig*k* (RG-7) chain conjugated to peroxidase (HRP) horizontally for 1 h under high humidity at RT. RG-7 HRP slides were washed three times with PBS and HRP activity (red) was demonstrated by AEC-substrate as described by Claassen *et al.* (1986a).

For both substrate types, the reaction was stopped by transferring slides to PBS. Sections were counterstained with haematoxylin, rinsed with tap water and embedded in glycerin-gelatin. Cytokine-producing cells (IL-2-PC and IFN-γ-PC) and $gp39^+$ cells were counted per cryostat section.

Statistical evaluation

For statistical evaluation change in body weight was calculated as body weight on testday minus body weight on last measured day before. Differences between adjuvant and no-adjuvant groups were evaluated for significance using analysis of variance. Serum antibody responses at necropsy were analyzed by the two-sample Student's *t*-test for comparison of two empirical means in a normally distributed population. A probability of 0.05 or less was considered significant.

Results

Clinical findings

After 7 days, the body weights of immunized animals were equal to or higher than body weights of control mice for the remainder of the post-immunization period. Pilo-erection was observed two days after primary and secondary immunization, mainly in FA groups and when *M. pneumoniae* preparations were injected. Abnormalities (swelling, signs of pain etc.) after immunization are described below per injection route.

i.p. injection

Body weights decreased significantly in groups of mice (n=5) i.p. immunized with FCA/antigen (3 out of 3 groups), Specol/antigen (2/3), *Lactobacillus*/antigen (1/3) or RV-ISCOMs/antigen (2/2).

After primary immunization, i.p. injected animals showed no abnormalities at the injection site and palpation of the abdomen did not lead to any sign of pain such as vocalizing or struggling. Within one hour after secondary immunization (i.p.) with FIA/SP215 emulsion animals died. Within 2-3 days after secondary injection (i.p.) of antigen/RV-ISCOMs, some animals died (combined with SP215, 2/5 and combined with MBP, 5/5).

s.c. injection

When mice were immunized subcutaneously, body weights significantly decreased after primary injection of FCA/antigen (2 out of 3 groups), Specol/antigen (2/3), *Lactobacillus*/antigen (1/3) or RV-ISCOMs/antigen (1/2). At the s.c. injection site of FA/antigen emulsions, palpable nodules, varying in size between 1 and 5 mm, were found. One hour after secondary immunization (s.c.) with FIA/SP215 emulsion, one mouse died. In some animals s.c. injected with *M. pneumoniae* combined with Specol or *Lactobacillus* a yellow spot was found at the site of injection. Secondary injection of Quil A/*M. pneumoniae* preparations (s.c.) resulted in nodules of 2-5 mm.

d.f.p. injection

In animals injected with FCA, body weights were significantly decreased 2 days after immunization. An ulcerative lesion was found at the injection site in two animals injected with Quil A/*M. pneumoniae*

Behavioural changes and physiological state

Pilo-erection was observed in the first days after primary immunization (i.p.) with the FCA/SP215 or FCA/MBP emulsions. No significant changes in behaviour or physiological state of the mice were observed in other groups after primary immunization. After secondary injections, pilo-erection was observed in all groups i.p. or s.c. injected with *M. pneumoniae* preparations. Secondary injections resulted in mortality in some groups (specified above).

Pathological findings at necropsy

i.p. injection

Table 2 shows gross and histopathologic lesions at necropsy. The most prevalent abdominal abnormality was white smooth shiny thickening of the peritoneum covering liver, diaphragm and spleen. Microscopically, these 'plaques' are granulomatous peritonitis. This was present in all FA and Specol treated animals. In animals that died immediately after FIA/SP215 injection (i.p.) the same plaques were found. Moreover they showed cyanosis of extremities and congestion of intestinal serosa. Animals that died after secondary injection (i.p.) of RV-ISCOMs/antigen showed a red nose and congestion of intestinal serosa and liver while no abdominal granulomatous lesions were found. Histology of the kidneys of three animals showed acute degeneration of convoluted tubules. Omentum retrahens was present in all groups treated with *M. pneumoniae*/adjuvant preparations. Intestinal adhesions were only found in FA/MBP (3/5 animals) and in RV-ISCOMs/antigen (1/3) injected animals. This last animal showed arteritis in the pancreas.

s.c. injection

FA injection sites most consistently showed lesions both 1 week after FIA as 7 weeks after FCA application. FCA injection resulted in white subcutaneous nodules measuring 1-9 mm, microscopically designated as fibrosing granulomas. FIA induced lesions grossly showed white fluid diffusely in the s.c. fat. Microscopy revealed oil spaces and fat necrosis surrounded by active fibrous tissue rich in granulocytes. At the primary injection site of Specol/*M. pneumoniae* in 3/5 mice thin-walled cysts (2-3 mm) with yellow fluid were present, microscopically diffuse pyogranulomatous panniculitis. Secondary injection of Specol/*M. pneumoniae* induced subcutaneous white lesions which microscopically are granulomas around large oil spaces. A soft yellow mass was found after secondary injection of *Lactobacillus/M. pneumoniae* and

Quil A/*M. pneumoniae*, which microscopically appeared to be exudative panniculitis and diffuse necrotizing panniculitis respectively.

Table 2. Score of gross and histopathologic lesions at necropsy (7 weeks after primary and 1 week after secondary injection) of mice injected with various adjuvant/antigen or saline/antigen mixtures via different routes[a]

		Pathological findings[b]					
		i.p.		*s.c.*		*d.f.p.*	
Adjuvant	*Antigen*	*extent*[c]	*severity*[c]	*primary*[c]	*secondary*[c]	*gross*[c]	*histo*[c]
FA	SP215	[d]		++	[e]	++	+
	MBP	++	+++	+++	+	++	+
	M.pneu.	+++	+++	+	++	++	++
Specol	SP215	+	-	-	+	+	-
	MBP	+	-	-	-	++	-
	M.pneu.	++	+	++	+	++	+
Lactob.[f]	SP215	-	-	-	-	-	-
	MBP	-	-	-	-	-	-
	M.pneu.	+	+++	-	++	+	-
RV-ISCOMs	SP215	-[g]	-	-	-	-	-
	MBP[h]	[i]		-	+	-	-
Quil A	M.pneu.	+	+++	-	++	+++	+++
Saline	SP215	-	-	-	-	-	-
	MBP	-	-	-	-	-	-
	M.pneu.	-	-	-	-	-	-

[a] Intraperitoneal (i.p.), subcutaneous (s.c.) or at the dorsal site of the hind feet (d.f.p.);
[b] comparison of data is possible intraroute and not interroute because of anatomical differences;
[c] - = minimal; + = mild; ++ = moderate; +++ = marked;
[d] no data comparable since animals died within 1 h after secondary injection (see: Results section);
[e] primary and secondary injection on the same site;
[f] *Lactoabacillus* spp;
[g] in 1/3 animals lesions were found (see: Pathological findings at necropsy, i.p. injection section);
[h] 14 weeks after primary injection;
[i] no data comparable since animals died within 48 h after secondary injection (see: Results section).

d.f.p. injection

Injection of FA/antigen or Specol/antigen preparations resulted in diffuse swelling of the metatarsal region and local swellings at the dorsal side just proximal to the metatarso-phalangeal joints and of the tarsal region. Histologically granulomatous inflammation in the subcutis was present. In the FA treated animals lesions also involved muscles, tendon sheaths and periost. Lesions were most severe after d.f.p. injection of Quil A/*M. pneumoniae*. Feet were swollen and hyperaemic and two animals showed an ulcerative lesion. Microscopically these lesions were extremely exudative and also involved the deep layers. The relative enlargement of the PLN after d.f.p. injection is shown in Table 3.

Table 3. T cell activation, enlargement of popliteal lymph nodes (PLN), and antibody response of animals d.f.p. injected with various adjuvant/antigen preparations

Antigen	*Adjuvant*	*IL-2*[a]	*IFN-γ*	*gp39*	*PLN*[b]	*antibody response*[c]
SP215	FA	+	+	+	++	1.68±0.4
	Specol	+	+	+	++	1.78±0.2
	Lactob.[d]	-	-	+	-	0.19±0.2
	RV-ISCOMs	+	+	+	-	1.97±0.05
	Saline	-	-	-	-	0.04±0.5
MBP	FA	+	+	+	++	0.15±0.2
	Specol	+	+	+	++	0.07±0.03
	Lactob.	-	-	-	-	-
	RV-ISCOMs	+	+	+	++	1.83±0.5
	Saline	-	-	-	-	-
M.pneu.	FA	+	+	+	++	6
	Specol	+	+	+	++	6
	Lactob.	+	+	+	+	5
	Quil A	+	+	+	+	6
	Saline	+	+	+	-	4

[a] IL-2-PC, IFN-γ-PC, gp39[+] cells; - = no staining observed, + = staining observed; [b] PLN; - = not enlarged, + = twice enlarged, ++ = three times or more enlarged;[c] serum antibody responses one week after secondary injection; SP215 and MBP results are expressed as absorbance at 405 nm for diluted (1:300) sera. Values represent the mean ± SD of antibody responses of five mice. *M. pneumoniae* results are shown as ^{3}log serial dilutions with a serum dilution from 1:270 (1) to 1:65610 (6);[d] *Lacotabacillus* spp.

Antibody response

High serum anti-SP215 responses were found after injection of SP215 in combination with FA, Specol or RV-ISCOMs (Fig. 1). The d.f.p. injection resulted in antibody responses comparable to s.c. injection. Primary injection of SP215 emulsified in FCA or Specol resulted in high antibody responses which reached a plateau at week 5 while secondary injection had limited effect on antibody responses. The secondary injection of RV-ISCOMs/SP215 had enhancing effect on the immune response. When RV-ISCOMs/SP215 were injected d.f.p., antibody responses were low until secondary immunization. A secondary injection (d.f.p.) of RV-ISCOMs/SP215 enhanced the antibody response to a level comparable to FA/SP215 or Specol/SP215 emulsions.

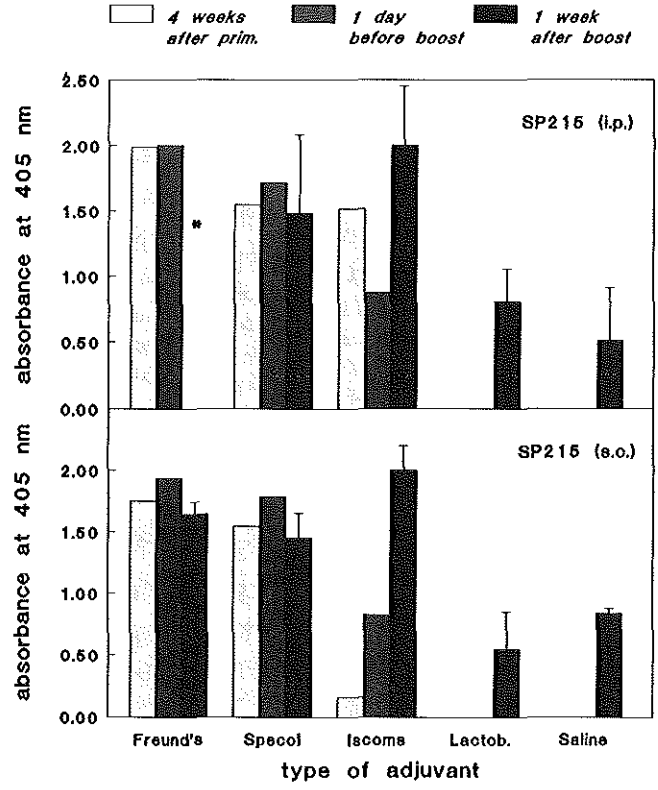

Figure 1 Serum anti-SP215 antibody responses in a direct ELISA. Mice were primary immunized and boosted (i.p. or s.c.) with different adjuvant/SP215 preparations. The results are expressed as absorbance at 405 nm for diluted (1:300) sera, collected 4 weeks after primary immunization, one day before secondary injection and one week after secondary injection. *, no data available. Data from one week after secondary injection represent mean ± SD of antibody responses of five mice.

The i.p. injection of FA/MBP emulsions resulted in high antibody titres (Fig. 2). After RV-ISCOMs/MBP injection (i.p.) serum antibody responses were still rising at the moment of secondary immunization. When MBP was s.c. injected (Fig. 2) antibody titres were similar to those produced after d.f.p. injection. Low anti-MBP antibody responses were found after injection of FA/MBP emulsions via s.c. or d.f.p. route also after a secondary injection. After primary injection of RV-ISCOMs/MBP (s.c. or d.f.p.) anti-MBP antibodies increased till secondary immunization (week 13). Secondary injection (s.c. or d.f.p.) of RV-ISCOMs/MBP enhanced the immune response significantly.

After primary injection of adjuvant/*M. pneumoniae* preparations minimal differences in antibody responses were observed between the adjuvants (data not shown). A secondary injection resulted in enhanced antibody responses in all groups. The effect of secondary injection of adjuvant/*M. pneumoniae* preparations on antibody responses was higher than in the saline/*M. pneumoniae* injected animals.

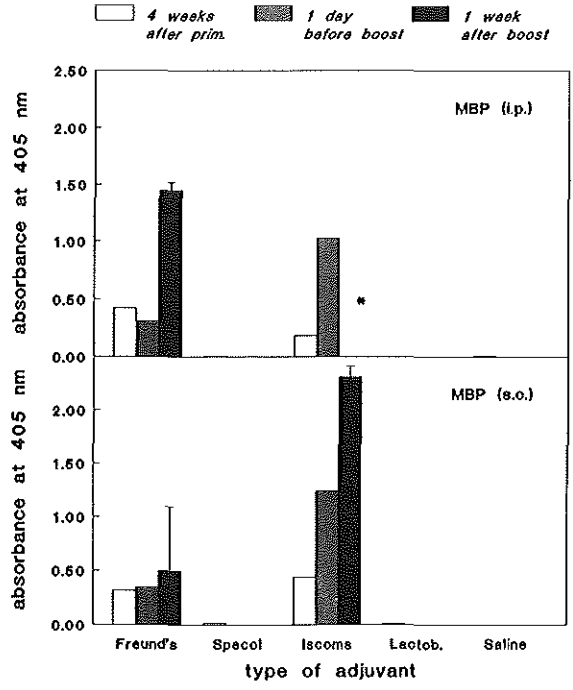

Figure 2 Serum anti-MBP antibody responses in a direct ELISA. Mice were immunized and boosted (i.p. or s.c.) with different adjuvant/MBP preparations. The results are expressed as absorbance at 405 nm for diluted (1:300) sera, collected 4 weeks after primary immunization, one day before secondary injection and one week after secondary injection. *, no data available. Data from one week after secondary injection represent mean ± SD of antibody responses of five mice.

T cell activation

Cytokine production (IL-2, IFN-γ) and gp39 expression were demonstrated by immunohistochemistry (Table 3). IL-2-producing cells (IL-2-PC) and IFN-γ-PC cells were observed in PLN of animals injected with FA/SP215 or Specol/SP215 emulsions or with RV-ISCOMs/SP215 (Fig. 3(a)). $Gp39^+$ cells were demonstrated in PLN of all animals injected with different adjuvant/SP215 preparations. No gp39 expression or cytokine production were demonstrated in PLN of saline/SP215 injected animals.
In PLN of animals injected with FA/MBP, Specol/MBP, or RV-ISCOMs/MBP, IL-2-PC, IFN-γ-PC and $gp39^+$ cells were demonstrated. No cytokine-producing cells or $gp39^+$ cells could be demonstrated in animals injected with *Lactobacillus*/MBP or saline/MBP mixtures. IL-2-PC, IFN-γ-PC and $gp39^+$ cells (Fig. 3(b) - 3(d)) were observed in PLN of all animals injected with preparations containing *M. pneumoniae* as antigen.

Discussion

In a comparative study in mice we here show that RV-ISCOMs can be used as an effective adjuvant for the production of specific antibodies to autoantigen and small peptides while inducing minimal side effects when injected subcutaneously. In a previous study in rabbits (Leenaars *et al.*, 1994) we concluded that Specol might be an acceptable alternative to FA. Based on data of this study with 3 antigens, we suggest that this conclusion can be extended to the mouse. An exception was autoantigen MBP, where only a very small amount of anti-MBP antibodies could be detected in serum of Specol/MBP injected animals. Cytokine-producing cells and $gp39^+$ cells were demonstrated in PLN of animals d.f.p. injected with all three antigen/Specol emulsions while PLN were enlarged and many plasma cells were present (data not shown). The enlargement of the PLN can be explained by the aspecific immune-stimulating effects of Specol as described by Boersma *et al.* (1992). The low level of antibodies might be explained by the minimal presence of specific B cells for autoantigen (MBP). Furthermore, we were unable to induce Experimental Allergic Encephalomyelitis (EAE) in SJL/J mice with Specol/MBP as opposed to FCA/MBP (data not shown), indicating yet again that T cells were not antigen specifically activated by Specol/MBP. High antibody responses found after immunization with Specol/SP215 confirm the data of Boersma *et al.* (1989) who found 25 µg of SP29 emulsified in Specol to be sufficient in mice to elicit an anti-peptide response. Specol can be a possible alternative to FCA; however, more research is needed to confirm that it is an alternative to FCA for a wide range of antigens. Pathological changes after i.p. injection of Specol emulsions were similar to those observed after injection of Freund's adjuvant emulsions, but the area affected was less extensive.

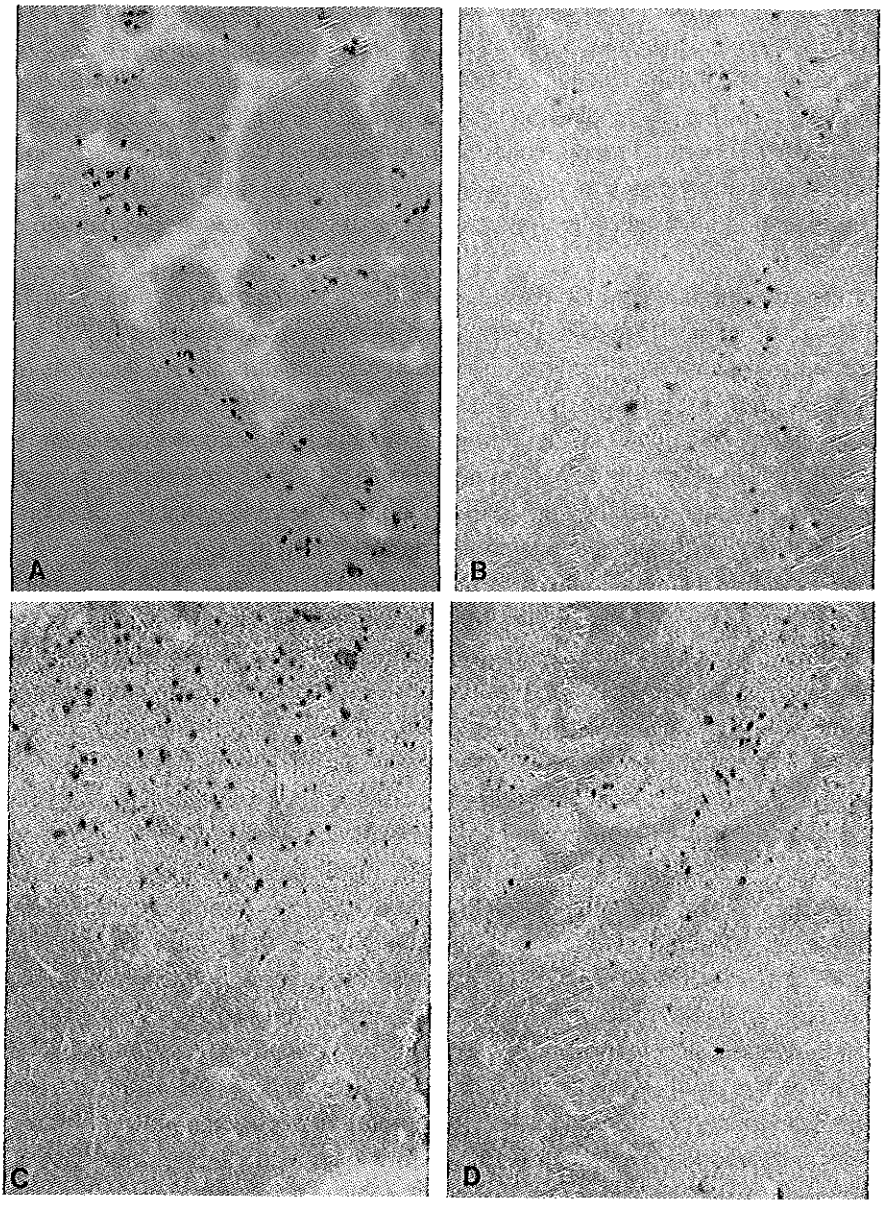

Figure 3 Immunohistochemical visualization of IL-2-producing cells (IL-2-PC) and gp39$^+$ cells in the PLN after secondary immunization with antigen/adjuvant mixtures. Cryostat sections of murine lymphoid tissue were incubated with specific immuno-conjugates, followed by the appropriate substrates, as described in Animals, materials and methods section. IL-2-PC in a cryostat section of the PLN of a mouse, one week after secondary immunization with FIA combined with SP215 (a). Gp39$^+$ cells in a cryostat sections of the PLN of a mouse, one week after secondary immunization with Specol/*M. pneumoniae* (b), *Lactobacillus*/*M. pneumoniae* (c) or Quil A/*M. pneumoniae* (d).

Only the injection of RV-ISCOMs/MBP induced high antibody responses to MBP after s.c. and d.f.p. injection. The responses exceeded those induced by Freund's adjuvant. The secondary injection of RV-ISCOMs/MBP was given in week 13 owing to the availability of the conjugated antigen. At week 7 anti-MBP antibody levels in the RV-ISCOMs/MBP were already higher than those found in the FA/MBP group which received a secondary injection at week 6. Moreover, levels still increased in the period until the secondary immunization (at week 13) and had not reached a plateau at that time.

For the coupling of antigen to RV-ISCOMs, the amounts of antigen given in Table 1 were used per animal. However, possibly not all antigen was coupled to RV-ISCOMs and therefore the amount of antigen injected in combination with RV-ISCOMs may be lower than given in Table 1. It has been shown that when antigen is coupled to ISCOMs, immunization can be performed at reduced antigen concentra-tions (Morein *et al.*, 1987). Coupling of haptens and peptides to ISCOMs is a laborious process and not all types of antigen can be coupled. These are disadvantages if ISCOMs are to be used as a ready-to-use product that serves as alternative to FCA.

Besides the adjuvant, also the type of antigen and the route of injection influenced the severity of the lesions found after immunization with the adjuvant/antigen preparations. When different antigens were compared most severe lesions were found after injection of *M. pneumoniae* preparations. The nature of the antigen injected may be involved in the severity of induced lesions. When different injection routes were compared, i.p. injection resulted in relatively severe lesions (including intestinal adhesions, (peri)pancreatitis, abscesses) compared to s.c. injection and in some groups animals died after secondary i.p. immunization. Within 1 h after secondary i.p. injection of FIA/SP215 emulsions, animals most probably died as a result of anaphylactic shock as suggested by time course and observed circulatory disturbances (peripheral cyanosis and abdominal congestion). After the secondary i.p. injection of RV-ISCOMs/SP215, two animals died after 2-3 days (not suitable for microscopy). In one survivor arteritis was observed which suggests the possibility of a type III hypersensitivity reaction. Secondary i.p. injection of RV-ISCOMs/MBP resulted in death of the whole group within 48 h. No evidence of acute immune complex disease was found (i.c. no endothelial proliferation or polymorphonuclear infiltration in vessels or glomeruli) in these five mice. The presence of free Quil A in ISCOM preparations is not considered the cause of death. Quil A is known to have intrinsic haemolytic activity *in vitro*. When Quil A is incorporated in ISCOM, the haemolytic activity is ten times lower than observed with free Quil A (Kersten, 1990). Macroscopically, no signs of haemolysis were found and microscopically no haemoglobin was present in renal tubular epithelium. Morein *et al.* (1987) and Speijers *et al.* (1988) observed minimal pathological effects after injection of ISCOMs. Speijers *et al.* (1988) injected 0.25 ml measles virus ISCOMs containing 90 µg Quil A intramuscularly in rats and observed minimal local and no haematological effects.

Other immunopathologic events may have caused the death of these animals since these animals died within 48 h after secondary injection while circulatory disturbances were found as may be observed in shock state. Besides these immunopathologic events after i.p. injection, minimal pathological lesions were observed after injection of RV-ISCOMs.

At necropsy severe pathological changes were found after injection of FA. Despite the severe lesions we could not demonstrate signs of prolonged pain or distress based on clinical observations, weight gain or several behavioural studies. This is in correspondence with results of Toth *et al.* (1989) who did not find prolonged signs of pain in mice after i.p. injection of 0.5 ml FA/saline (1:1). Van den Broek *et al.* (1994) however, suggest that i.p. injection of 0.1 ml FCA may lead to severe distress. The reasons for the fact that we could not demonstrate prolonged pain or distress while severe lesions were found could be that these lesions do not induce pain and distress in mice or the methods we used were not sufficient to detect prolonged animal distress. In order to control possible pain and distress, the induction of severe pathological changes need to be kept to a minimum. Amyx (1987) suggested that most of the undesirable side effects of the use of Freund's complete adjuvant can be eliminated by careful control of injection quantity and site selection.

In conclusion, when alternative adjuvants are compared, the effect of the nature of antigen on immune responses and side effects, should be taken into account. We suggest that Specol is a possible alternative to FCA for the production of specific antibodies to small peptide and particulate antigen. We demonstrated that s.c. injection of RV-ISCOMs can be used for the production of specific antibodies to autoantigen or small peptide and cause minimal side effects.

Acknowledgements

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Chapter 5

Assessment of side effects induced by injection of different adjuvant/antigen combinations in rabbits and mice

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Abstract

In this paper, an evaluation is made of the side effects induced by injection of Freund's adjuvant (FA) and alternative adjuvants combined with different antigens. Freund's complete adjuvant was used in primary and Freund's incomplete adjuvant in secondary immunizations. This is abbreviated by FA. Rabbits and mice were injected subcutaneously (s.c.), intramuscularly (i.m.; rabbits) or intraperitoneally (i.p.; mice) with different adjuvants (FA, Specol, RIBI, TiterMax, Montanide ISA50, DDA, Gerbu, ISCOMs, Quil A, Lactobacilli) in combination with several types of antigens (synthetic peptides, autoantigen, glycolipid, protein, Mycoplasma or viruses). Parameters used to evaluate the effect of treatment on animals' well-being included: clinical and behavioural changes (POT and LABORAS procedures) and gross and histopathological changes. In rabbits, no indications were found that injection of adjuvant/antigen mixtures induce acute or prolonged pain and distress. In mice, behavioural changes were observed immediately after (predominantly secondary) immunization. Injection of several adjuvant/antigen mixtures resulted in severe pathological changes, depending on adjuvant, type of antigen, animal species used and route of injection. In rabbits and mice, pathological changes were marked to severe after injection of FA while changes were variable, ranging from minimal to marked, after Specol and Montanide ISA50 injections. Pathological changes after RIBI injections were severe in rabbits while slight in mice. After TiterMax injections pathological changes were moderate in rabbits and severe in mice. ISCOMs, DDA, Lactobacillus or Gerbu induced minimal pathological changes as found at necropsy. Quil A/*Mycoplasma pneumoniae* induced severe pathological changes. In conclusion, injection of FA in accordance with existing guidelines resulted in most cases in severe pathological changes but in minimal clinical and behavioural indications of prolonged severe pain. From our data it appeared that Montanide ISA50 and Specol induce acceptable antibody titres and less pathological changes than FA. We therefore conclude that Montanide ISA50 and Specol can be an alternative to FA.

Introduction

Immunization procedures are performed on a large scale in laboratory animals, often to produce specific polyclonal antibodies to different kinds of weakly immunogenic antigens. To enhance the immune response, an adjuvant is added to the antigen. In laboratory animals, Freund's complete adjuvant (FCA) is commonly used for this purpose since it evokes high-level and long-lasting humoral and cellular immunity to a wide range of antigens. However, the severity of the side effects induced by FCA has resulted in guidelines discouraging its use in laboratory animals (e.g. Canadian Council on Animal Care, 1991; Veterinary Public Health Inspectorate, 1993). The use of alternative adjuvants is recommended to reduce pain in immunized animals. Many products with adjuvant activity are available (reviewed in Claassen and Boersma, 1992; Vogel and Powell, 1995; Cox and Coulter, 1997). An alternative to FCA should be an adjuvant that induces minimal side effects and acceptable antibody responses and is easy to prepare, commercially available and inexpensive. Most evaluation studies on adjuvants emphasize the immunological properties of the product. Since the aim of replacing FCA is to reduce pain and distress, it is necessary to also evaluate the side effects upon administration.

The severity of side effects can be evaluated based on clinical, behavioural, biochemical, and pathological parameters. Morton and Griffiths (1985) and Wallace *et al.* (1990) issued guidelines on the recognition of pain, distress and discomfort in experimental animals. The assessment includes general appearance, body weight, clinical signs, unprovoked behaviour, and responses to appropriate stimuli. Elevated levels of hormones or metobolites may be used as indicators of pain, however, problems may occur when interpreting these levels in pain and distress (Broom and Johnson, 1993). Irwin (1968) developed the Primary Observation Test to assess pain after injection of pharmacological agents. Jansen van 't Land and Hendriksen (1995) described a system to evaluate discomfort based on locomotion activity. Recently, a behaviour registration system LABORAS (Bulthuis *et al.*, 1997; Van de Weerd, 1996) was developed which automatically records six different categories of behaviour (immobility, locomotion, climbing, grooming, eating, drinking) during prolonged periods of time. This system seems promising for extensive behavioural studies. Side effects of adjuvants can also be assessed based on gross- and histopathological changes. Many studies on side effects after administration of FCA have been published, while only limited data (Deeb *et al.*, 1992; Johnston *et al.*, 1991) are available on side effects induced by alternative adjuvants as compared to FCA.

To obtain more information on side effects induced by Freund's adjuvant and alternative adjuvants, we performed comparative studies in which rabbits and mice were given different combinations of adjuvant and antigen via different routes. Besides antibody responses, clinical, behavioural, and pathological changes were

studied. Emphasis is put on five adjuvants (Freund's adjuvant, Specol, RIBI, TiterMax and Montanide ISA50) which are commercially available and easy to prepare; other adjuvants (DDA, Gerbu, ISCOMs, Quil A and *Lactobacilli*) were studied in less detail.

Animals, materials and methods

Animals

New Zealand White rabbits (*Oryctolagus cuniculus*) of both sexes were obtained from the specific pathogen free (SPF) breeding centre of the RIVM. The rabbits were about 6 months old and weighed between 3.5 and 4.0 kg initially. They were individually housed in stainless steel or plastic cages, environmental temperature of 20-21°C and under a 12 h day-night light cycle. The relative humidity was between 45 and 60%. The rabbits were fed rabbit chow (80 g/day, Hope Farms BV, Woerden, the Netherlands) and tap water was available *ad libitum*.

BALB/c/Rivm mice of both sexes were bred SPF at the RIVM breeding facilities and were used at 10-14 weeks of age. At the RIVM, animals were housed in groups of five mice (one sex) under SPF conditions in Macrolon type II cages, environmental temperature of 20-22°C, relative humidity of 50-70% and with a 12 h day/night cycle. Mice were fed a commercial diet (Hope Farms BV, Woerden, the Netherlands) and provided with tap water *ad libitum*. One exception is the behavioural experiment (LABORAS) at Utrecht University. For this experiment, female BALB/cAnCrRyCpbRivU mice were maintained at the Central Laboratory Animal Institute (GDL) of Utrecht University, the Netherlands, and were used at 10-12 weeks of age. At the GDL (Utrecht), mice were housed individually in Macrolon type II cages, environmental temperature of 20-22°C, relative humidity of 50-70% and with a reversed 12 h day/night cycle.

Adjuvants

Freund's complete adjuvant (FCA; Difco Laboratories, Detroit, MI) consists of 85% paraffin oil (mineral oil; Bayol F) and 15% emulsifier (mannide monooleate) with added heat-killed *Mycobacterium butyricum* (0.5 mg/ml). Freund's incomplete adjuvant (FIA; Difco Laboratories, Detroit, MI) is the same product as FCA except that no Mycobacteria were added. The term Freund's adjuvant (FA) is used in this paper to indicate that FCA was used in primary and FIA in secondary immunization. Specol (ID-DLO, Lelystad, the Netherlands; Bokhout *et al.*, 1981; Boersma *et al.*, 1992) is 90% mineral oil (Marcol 52) and 10% emulsifier (Span 85 and Tween 85). RIBI (Sanbio BV, Uden, the Netherlands; Rudbach *et al.*, 1995) consists of metabolizable oil (squalene; 2% in final volume of adjuvant/antigen), emulsifier (Tween 80) and microbial components: 0.5 mg/ml monophosphoryl lipid A (MPL), 0.5 mg/ml synthetic trehalose dicorynomycolate (TDM) and (when used in rabbits)

0.5 mg/ml cell wall skeleton (CWS). TiterMax (CytRx, Norcross, GA; Hunter *et al.*, 1995) consists of a metabolizable oil (squalene), emulsifier (sorbitan monooleate 80), a patented block copolymer CRL-8941 and microparticulate silica coated with CRL-8941). Montanide ISA50 (Seppic, Paris, France; Ganne *et al.*, 1994) consists of 85% mineral oil and 15% emulsifier (mannide oleate). When mixed with aqueous antigen solution, FCA, FIA, Specol, Montanide and TiterMax form water-in-oil emulsions while RIBI forms an oil-in-water emulsion. ISCOM matrix with incorporated rabies virus glycoprotein (RV-ISCOMs) were kindly provided by Dr. I. Claassen (RIVM, Bilthoven, the Netherlands). Quil A ('Spikoside', Iscotec, Luleå, Sweden) is a purified saponin (Campbell and Peerbaye, 1992). Lactobacilli (*Lactobacillus plantarum* [ATCC 8014, 10^9 per ml], *Lactobacillus casei* [ATCC 393, 10^9 per ml]) are non-pathogenic micro-organisms (Claassen *et al.*, 1995) and were kindly provided by Dr. W. Boersma (TNO-PG; Leiden, the Netherlands). Gerbu adjuvant (Gerbu BIOTECHNIK GmbH, Gaiberg, Germany) contains GMDP (N-acetylglucosaminyl-N-acetylmuramyl-L-alanyl-D-isoglutamine) in combination with natural synergists, supplied as a lyophilisate. dimethyldioctadecylammonium bromide (DDA; Eastman Kodak Company, NY; kind gift from Dr. H. Snippe, Utrecht, the Netherlands) is a synthetic adjuvant which is surface active (Hilgers and Snippe, 1992).

Antigens

Synthetic peptide (SPek15a, 27 amino acids, containing a B and T cell epitope) was synthesized as described by Zegers *et al.* (1993). Synthetic peptide (SP215, 21 amino acids, a homologue to the hinge region of the human IgG2 molecule) was synthesized as described by Boersma *et al.* (1989). Myelin basic protein (MBP; Sigma, St. Louis, MO) is an autoantigen which is derived from bovine brain and consists of 173 amino acids, 18.5 kDa (Van Noort *et al.*, 1993). Galactocerebroside (MBL-TNO, Rijswijk, the Netherlands; Gerritse *et al.*, 1993) is a glycolipid. Bovine Serum Albumin (BSA) was obtained from Sigma, St. Louis, MO (A-9647). Measles virus (Edmonston strain; 10^7 $CCID_{50}$/ml; van Binnendijk *et al.*, 1994), rubella virus (HPV-77 strain; 10^7 $CCID_{50}$/ml; Parkman *et al.*, 1966) and mumps virus (Enderson strain; 10^7 $CCID_{50}$/ml) was kindly supplied by Dr. N. Elzinga (RIVM, Bilthoven, the Netherlands). HIV peptides conjugated to tetanus toxoid were prepared by Dr. P. de Vries (RIVM, Bilthoven, the Netherlands). *Mycoplasma pneumoniae* (MAC strain) is a particulate antigen (0.1-0.8 µm) and was a kind gift from Dr. A. Angulo (RIVM, Bilthoven, the Netherlands). The antigens (amounts per rabbit in Table 1 and per mouse in Table 2) were diluted in sterile phosphate buffered saline (PBS) except for galactocerebroside which was diluted in dimethyl sulphoxide (DMSO).

Adjuvant/antigen preparation

Adjuvant/antigen and adjuvant/PBS mixtures, were prepared according to the manufacturers instructions. Freund's adjuvant emulsions (FCA in primary and FIA in secondary immunization, abbreviated by FA) were prepared by mixing FA and aqueous antigen solution (1 : 1), using two glass syringes with Luer Lock connector. The antigen was added to FA via the connector and mixed for 1 min. Specol emulsions were prepared by adding the aqueous antigen solution (Specol:antigen=5 : 4) dropwise to Specol while vortexing. RIBI emulsions were prepared by warming the RIBI vial to 40-45°C for 5 min., reconstituting a final volume of 2 ml with the aqueous antigen solution in the RIBI vial and vortexing for 3 min. TiterMax was emulsified with aqueous antigen solution (1 : 1) the same way as FA, except that the aqueous antigen solution was added to the oil phase in two steps. Montanide ISA50 emulsions were prepared by pouring Montanide ISA50 in a glass tube, adding the aqueous antigen solution with a syringe and making 10 up and down strokes. Gerbu/antigen mixtures were prepared by adding antigen in PBS to the Gerbu powder and shaking carefully (per rabbit: 10 µg Gerbu per injection). DDA was dis-solved in PBS and mixed with antigen (per rabbit: 400 µg DDA per injection). When RV-ISCOMs were used as an adjuvant, the antigens (SP215 and MBP) were coupled to the RV-ISCOMs as described in Leenaars *et al.* (1995). Per injection mice received 3-4 µg RV-ISCOMs. Quil A was dissolved in water, added to the antigen in PBS and mixed (per mouse: 10 µg Quil A per intraperitoneal (i.p.) injection; 20 µg Quil A per subcutaneous (s.c.) injection). *Lactobacillus*/antigen mixtures were prepared by adding antigen in PBS to Lactobacilli (10^9 per ml) in 1 : 1 proportion and mixing well.

Experimental design

Comparative studies were performed in rabbits and mice. Adjuvant-antigen combinations inducing obvious problems were not unnecessarily repeated. Experimental designs are described in Table 1 for the studies in rabbits and Table 2 for studies in mice. In rabbits, four experiments were performed, injecting animals s.c. on the flank or intramuscularly (i.m.) in the the posterior thigh. To discriminate between primary and secondary inoculations, single adjuvant/antigen injections were given on separate sites. This was not possible in rabbit exp nr. III and IV which were combined with routinely performed polyclonal antibody production experiments. Here, 4 injections were given (s.c.) due to concentration of antigen. In mice, three experiments were performed, injecting animals s.c. in the neck on primary injection and s.c. in the groin on secondary injection or i.p. Control rabbits and mice received antigen only. At selected time points blood samples were taken to determine antibody production, except for mice exp nr. III (see Table 2), which was a behavioural study only. Clinical and behavioural abnormalities occurring during the

experiment, and pathological changes at the end of each experiment were studied as described below.

Table 1. Experimental design for immunization studies in rabbits

Exp nr.	*Adjuvant*	*Antigen[a] (μg per rabbit)*	*Route/ Volume (ml)*	*#1[b]*	*#2[c]*	*Boost (day)[d]*	*End (day)[e]*	*Additional[f]*
I[g]	-FA -Specol -RIBI -TiterMax -none(PBS)	-SPek15a (200) -Galacto (200) -M.pneu. (87)	-s.c./0.5[h] -i.m./0.5[h]	30	2	28	42	W, T, L
II	-FA -Specol -Montanide ISA50 -Gerbu -DDA -none(PBS)	-Rubella[i] -BSA (1000)	-s.c./0.1 -i.m./0.1	24	2	28	42	W
III	-FA -Specol	-Measles[i] -Mumps[i]	-s.c./4x0.25	4	5	35	49	-
V	-FA -Specol -Montanide ISA50 -none(PBS)	-HIV-pept/tt I -HIV-pept/tt II	-s.c./4x0.1	8	1	28,84[j]	92	-

[a] SPek15a = synthetic peptide; Galacto = Galactocerebroside; M.pneu. = *Mycoplasma pneumoniae*; BSA = bovine serum albumin; HIV-pept/tt = HIV peptides conjugated to tetanus toxoid; [b] number of combinations studied in experiment, combining one adjuvant, antigen, and route; [c] number of animals injected per combination given in column #1; [d] day of secondary immunization; [e] day of necropsy; [f] additional parameters studied to assess side effects; W= body weight, T = body temperature, L = locomotion activity; [g] also described in Leenaars *et al.* (1994); [h] RIBI emulsions: 2 x 0.5 ml; TiterMax emulsions: 0.08 ml (as recommended by the manufacturers); [i] amount of antigen described in *Antigen* section of 'Animals, materials and methods'; [j] third immunization on day 84.

Clinical and behavioural parameters

General condition (appearance and clinical abnormalities) of the rabbits and mice was observed daily as described by Morton and Griffiths (1985). Abdomen of i.p. injected mice and s.c./i.m. injection sites were palpated to monitor signs of pain. Injection sites were examined for swelling the day after injection and weekly thereafter. Additional clinical or behavioural parameters were studied to evaluate side effects (see Table 1 and 2). These parameters are described below.

Table 2. Experimental design for immunization studies in mice

Exp nr.	*Adjuvant*	*Antigen[a] (µg per mouse)*	*Route/ Volume(ml)*	*#1[b]*	*#2[c]*	*Boost (day)[d]*	*End (day)[e]*	*Addi-tional[f]*
I[g]	-FA -Specol -RV-ISCOMs -Lactobac -none (PBS)	-MBP (100)	-s.c./0.2 -i.p./0.2	10	5	42	49	W, P
I[g]	-FA -Specol -Quil A -Lactobac -none (PBS)	-M.pneu.(15)	-s.c./0.1 -i.p./0.2	10	5	42	47	W, P
I[g]	-FA -Specol -RV-ISCOMs -Lactobac -none (PBS)	-SP215 (50)	-s.c./0.1 -i.p./0.1	10	5	42	47	W, P
II	-FA -Specol -RIBI -TiterMax -Montanide ISA50 -none (PBS)	-SP215 (50) -none (PBS)	-s.c./0.1[h] -i.p./0.2	24	5	42	47	-
III	-FCA -none (PBS)	-none (PBS)	-i.p./0.2	2	4	-	18	L

[a] MBP = myelin basic protein (autoantigen); M.pneu. = *Mycoplasma pneumoniae*; SP215 = synthetic peptide; [b] number of combinations studied in experiment, combining one adjuvant, antigen, and route; [c] number of animals injected per combination given in column #1; [d] day of secondary immunization; [e] day of necropsy; [f] additional parameters studied to assess side effects; W= body weight, P = Primary Observation Test, L = LABORAS, automated behaviour registration system; [g] also described in Leenaars *et al.* (1995); [h] TiterMax emulsions 0.05 ml (as recommended by the manufacturer).

Body weight, body temperature, locomotion activity

Rabbits were weighed (exp nr. I and II) weekly and mice (exp nr. I) were weighed before injection, 3 days after injection and twice weekly thereafter. Body temperature of rabbits (exp nr. I) was measured twice a week in a restraining box; after an initial acclimatisation of 30 min., the rectal temperature was recorded four times in 30 min. The average of these four temperatures was taken as the body temperature. Locomotion activity of rabbits was studied during the first week after primary and secondary injection by studying the animals moving freely in the animal room.

Primary Observation Test

Behavioural changes and physiological state of the mice were studied (mice exp nr. I) in the Primary Observation Test (POT). POT is a systematic quantitative procedure described by Irwin (1968) and modified by Prof. Dr. B. Olivier (Solvay Duphar, Weesp, the Netherlands). The POT procedure was performed before injection, 3 days thereafter and then weekly. The POT procedure involved an initial phase of undisturbed observation and a later manipulative phase during which the animal was subjected to different stimuli. The procedure started by placing a group of five mice in an observation chamber followed by an adaptation period of 1 h. The observation study began by observing the animal's undisturbed behaviour within the observation chamber, i.e. dispersion in the cage, apathy, startle-reaction, restlessness, watchfulness and respiration. Thereafter the animals were individually transferred onto the viewing arena to observe: arousal response to transfer, spatial locomotion, gait, slip resistance, righting reflex, exophthalmos and piloerection. Then, throughout individual handling, touch-reflex, provoked-freezing, provoked biting, skin colour, body tone, pupil size, urination-defecation and vocalisation were studied. After handling, mice were placed in the observation chamber and grooming was observed. Behavioural changes and physiological state were scored per mouse.

Behaviour registration system LABORAS

A newly developed behaviour registration system LABORAS (Bulthuis *et al.*, 1997; Van de Weerd, 1996) was used to register behavioural patterns of individually housed mice (mice exp nr. III). The LABORAS system is a fully automated device which can deduce the behavioural categories: locomotion, immobility, climbing, grooming, eating, drinking. The animal is placed in a Macrolon type II cage which is located on a sensing platform. The mechanical vibrations caused by movements of the animal are transduced into electrical signals and recorded. The signals are translated into the six separate behavioural categories by a computer. On day 0, fifteen min. before the start of the dark period (= 15 min. before start of recording period), mice were injected with FCA or PBS (0.2 ml i.p.) and placed in the LABORAS system (i.e. in cage on sensing platform). Movements of the mouse were recorded during the first 4 hours of the dark period every other day during 16 days. Per day (i.e. first 4 hours of dark period) the relative time spent on each behavioural category was calculated and analysed. After a square-root transformation, data were statistically analysed using a *t*-test to detect significant differences in recorded behaviour between FCA and PBS injected mice. The level of statistical significance was pre-set at $p < 0.05$.

Pathology

At the end of each experiment, rabbits were anaesthetized by intravenous injection of 2.5 ml sodiumpentobarbital (60 mg/ml) before bleeding via cardiac puncture. Mice were anaesthetized before orbital bleeding by i.m. injection of 0.1 ml of a mixture of ketamine (50 mg/ml), xylazine (20 mg/ml) and atropine (1 mg/ml) in a 7 : 3 : 1 volume ratio. Necropsy in rabbits and mice included dissection and examination of injection sites and at least abdominal organs. For histopathological evaluation, tissue of injection sites, a sample of the omentum of all i.p. injected mice, and organs showing macroscopic abnormalities, were fixed in 4% neutral buffered formal-dehyde, embedded in paraffin, sectioned at 5 µm, and stained with haematoxylin and eosin. Gross lesions were documented during necropsy and microscopy was performed in selected representative samples. In rabbits and in s.c. injected mice primary and secondary injection sites were scored separately, based on the area of involvement and character of the lesions found. In individual rabbits the severity of lesions was scored according to Table 3. Microscopy was used to verify macroscopical observations (Fig. 1A-D). In individual mice, the severity of s.c. lesions was scored (macroscopically and microscopically) according to Table 4. In an attempt to grade pathological lesions, taking into account the supposed discomfort, categories were defined as shown in Table 3 and 4, which served as a general guideline for classification. In i.p. injected mice, the severity of abdominal lesions was mainly assessed macroscopically, based on the presence of abdominal effusions, adhesions, intestinal dilatation, presence of 'plaques' and omentum retrahens. Microscopical severity of peritonitis was determined to verify macroscopical observations. Examples of microscopic severity grade of lesions in mice are given in Fig. 1E-H (s.c.) and Fig. 1I-J (i.p.).

Antibody responses

During the experiments (except mice exp nr. III), blood samples were taken to determine antibody production. Since this paper emphasizes the side effects induced by adjuvants, only summarising results on antibody production will be given. More detailed information on antibody titres of rabbit exp nr. I and mice exp nr. I, can be obtained from Leenaars *et al.* (1994) and Leenaars *et al.* (1995), respectively. Antibody production after adjuvant/antigen injection was related to antibody production after FA/antigen injection. Antibody titre after FA/antigen injection was set on 100% and antibody titres after other adjuvant/antigen injections were expressed relative to the titre after FA/antigen.

Table 3. Pathological findings at injection sites of rabbits s.c. or i.m. injected with different adjuvant/antigen combinations; applied classification and grading[a]

Subcutaneous lesions[b]

grade	
8	• extensive exudative lesions (generally accompanying granulomatous lesions) with severe haemorrhage, > 40 cm^2 • abscess-like lesions, diameter > 2 cm • ulcus > 1 cm
6	• exudative lesions (generally accompanying granulomatous lesions) with haemorrhage < 20 cm^2 • extensive nodular granulomatous lesions 20-100 cm^2; marked central necrosis in granulomas; minor signs of acute inflammation; • ulcus < 1 cm
4	• nodular granulomatous lesions 1 - 10 cm^2, without marked central necrosis and haemorrhage
2	• nodular granulomatous lesions < 1 cm^2 • minimal diffuse opacity (flat granulomatous proliferation) and/or minimal or local hyperaemia or petecchial bleeding 5 - 25 cm^2
0	• negative, or minimal local opacity or hyperaemia/petecchiae <5 cm^2

Intramuscular lesions[b]

grade	
6	• granuloma(s) > 1 cm Ø[c], with marked central necrosis • granuloma(s) > 2 cm Ø[c], without marked central necrosis
4	• granuloma(s) 0.5 - 2 cm Ø[c], without marked central necrosis
2	• minimal white or red streaks in muscle, or slight hyperaemia or haemorrhage < 0.5 cm Ø[c]
0	• negative

[a] In some cases lesions were given a score between the mentioned values;

[b] when different lesions were present, highest scoring one was taken to classify the injection site;

[c] transverse diameter, perpendicular to muscle fibres.

Table 4. Pathological findings at injection sites of mice s.c. injected with different adjuvant/antigen combinations; applied classification and grading[a]

	Primary		*Secondary*	
grade[b]	*macroscopy*	*microscopy*	*macroscopy*	*microscopy*
3	• s.c. nodule > 4 mm	• marked to severe fibrosis, diffuse inflammation and cellularity; (epi)dermal abnormalities[c]	• nodule externally visible; s.c. nodule > 5 mm or large amount of white tissue around thigh muscles	• severe diffuse inflammation with marked cellularity (and generally necrotising granuloma)
2	• s.c. nodule 2 - 4 mm	• marked fibrosis and slight to moderate diffuse inflammation, cellularity and granuloma formation	• bulging white mass externally visible; s.c. marked amount of white tissue	• marked diffuse inflammation with moderate cellularity (and generally necrotising granuloma)
1	• s.c. nodule 1 - 2 mm	• slight to moderate fibrosis, diffuse inflammation, cellularity and granuloma formation	• white mass externally visible, no swelling; s.c. slight to moderate amount of white tissue	• moderate diffuse inflammation
0.5	• s.c. nodule < 1 mm • s.c. negative; hairless area <2 mm	• minimal fibrosis, diffuse inflammation, cellularity and granuloma formation	• negative externally; s.c. minimal amount of white tissue	• minimal to slight diffuse inflammation

[a] In some cases scores were given in between mentioned values;

[b] the macroscopic and microscopic severity grade of a lesion is not necessarily the same;

[c] exudative nature usually dominates granuloma formation.

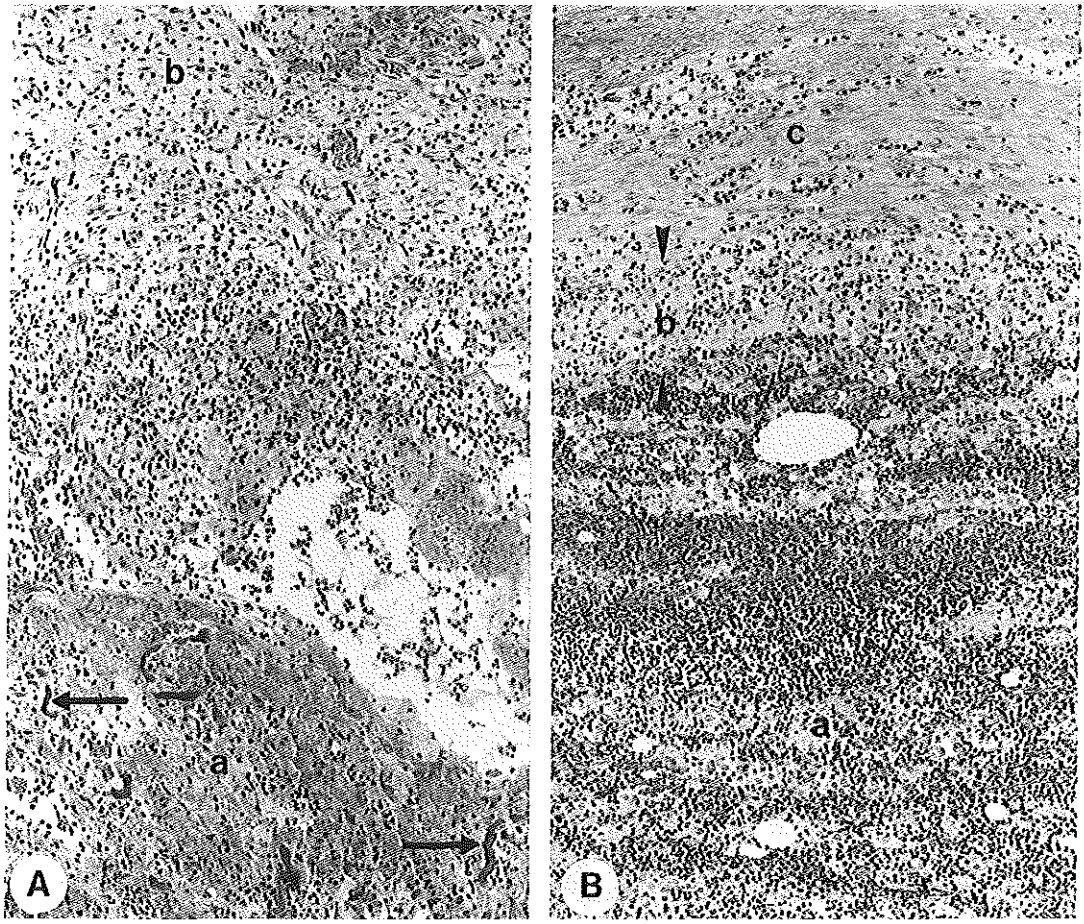

Figure 1

A

Rabbit exp nr. III, s.c. FIA/measles virus, 2 weeks after secondary injection, score 8. Microscopical detail of palpable, extensive haemorrhagic lesions, total surface 300 cm^2. a: Necrosis of pre-existent tissue, as shown by pre-existent collagen fibres (arrows), between cellular debris and fibrin. b: Fibroangioblastic proliferation with hyperaemia and haemorrhage. Considered primarily exudative, no granulomatous component present. HE, x110.

B

Rabbit exp nr. IV, s.c. Specol/HIV-pept/It II, 9 weeks after secondary injection, score 6. Microscopical detail of nodular complex lesions, total surface 60 cm^2. a: Marked central necrosis of granuloma, in which some oil spaces and no collagen fibres are present. b: Thin rim of vital granuloma. c: Fibroblast capsule. HE. x110.

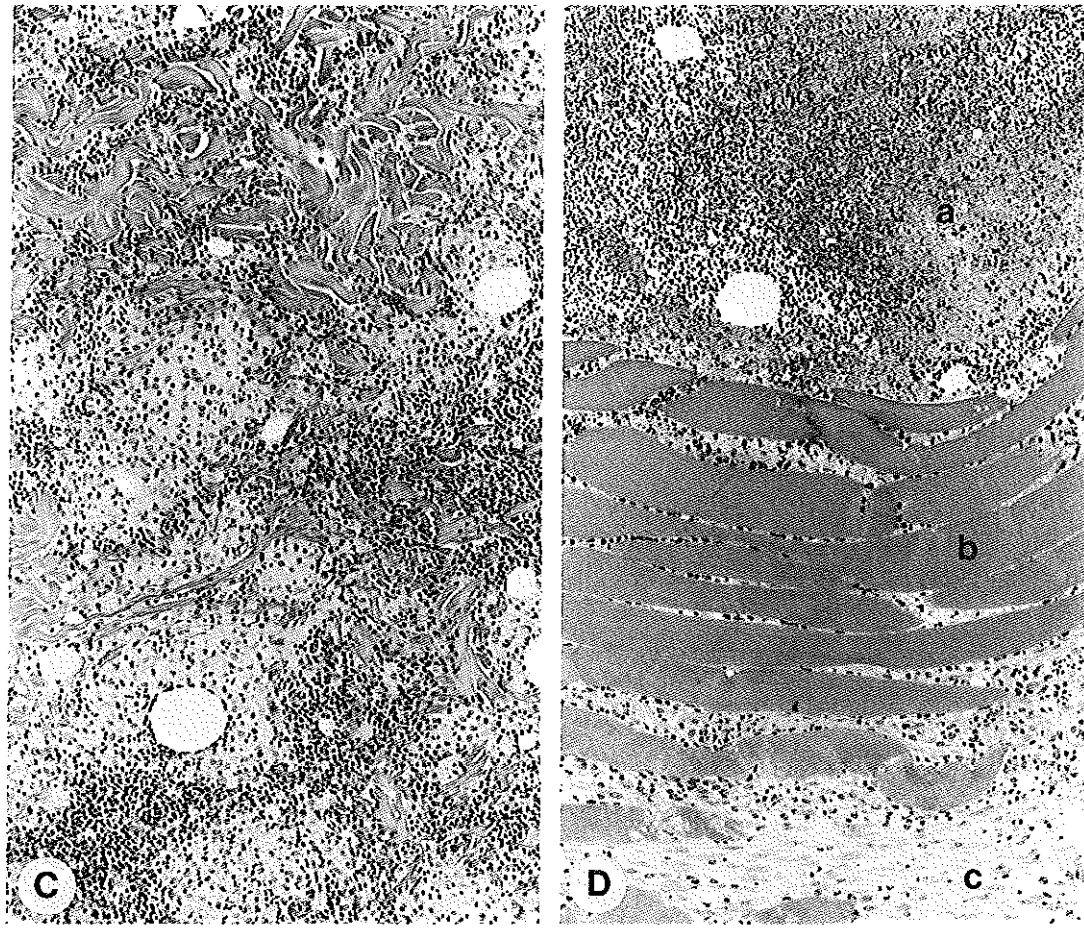

Figure 1

C

Rabbit exp nr. II, s.c. Montanide ISA50/BSA, 2 weeks after secondary injection, score 4. Microscopical detail of a palpable lesion, grossly described as faint pink tissue with central hyperaemia and slight haemorrhage, measuring 12 cm^2 and maximum width 0.5 cm. Epithelioid granulomas, partly around oil spaces, and lympho-plasmacellular infiltrate between subcutaneous collagen fibres. Necrosis absent. HE, x110.

D

Rabbit exp nr. I, i.m. RIBI/galactocerebroside, 6 weeks after primary injection, score 6. Microscopical detail of a lesion, described grossly as 1 cm ∅ grey firm elongated mass, centrally containing thick yellow pus-like material surrounded by a red zone. a: Centrally in the lesion cellular debris and oil spaces. b: Necrotic muscle fibres. c: Reactive fibroblast capsule. Considered primarily exudative, no evidence for granulomatous component. HE, x110.

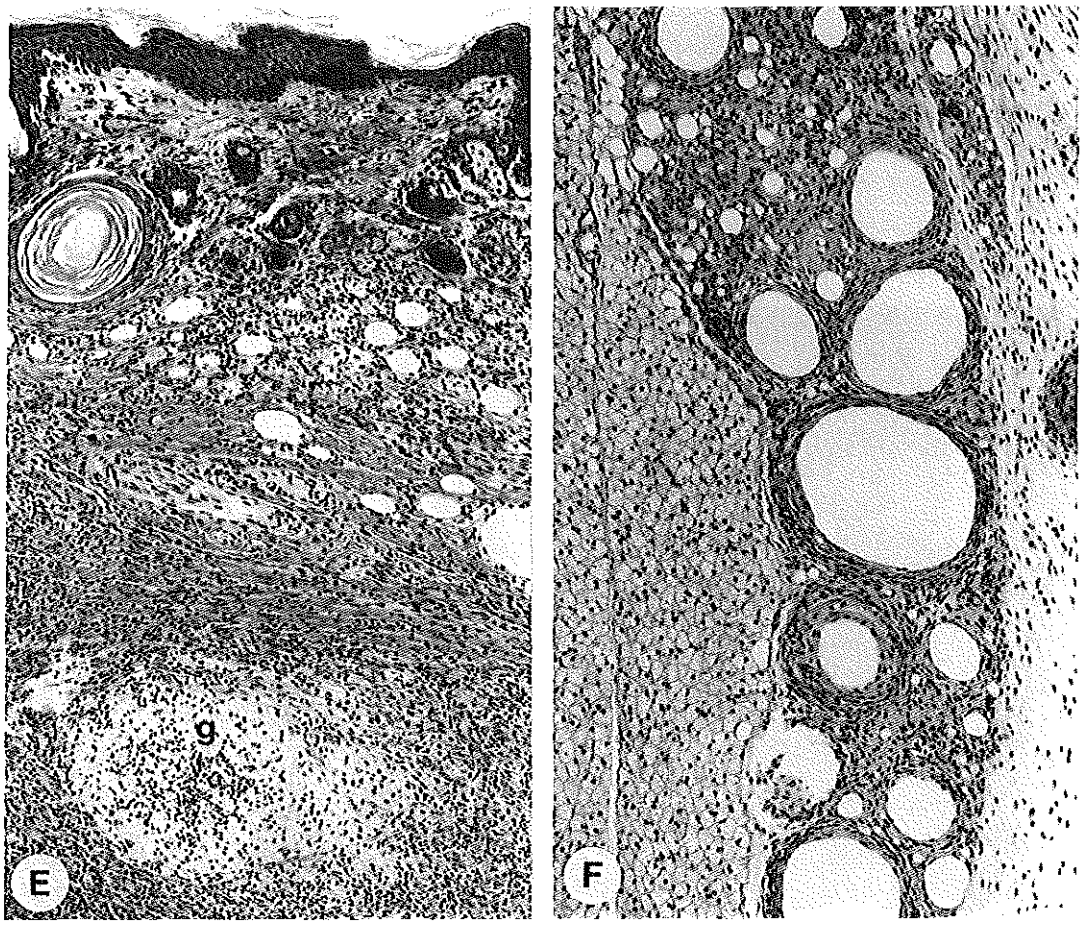

Figure 1

E

Mouse exp nr. II, s.c. TiterMax/SP215, 7 weeks after primary injection. Grossly hairless area, s.c. pale-yellow nodule 5 mm with slight haemorrhage, score 3. Microscopical detail shows acanthosis, severe fibrosis and diffuse inflammation, with marked cellularity and an occasional small granuloma (g) with central necrosis, score 3. HE, x110.

F

Mouse exp nr. II, s.c. Specol/SP215, 7 weeks after primary injection. Grossly white nodules < 2 mm and slight petechial bleeding in interscapular brown fat, score 1. Microscopically slight granuloma formation around oil spaces, moderate diffuse inflammation (not observable in this detail) and moderate fibrosis, score 1. HE, x110.

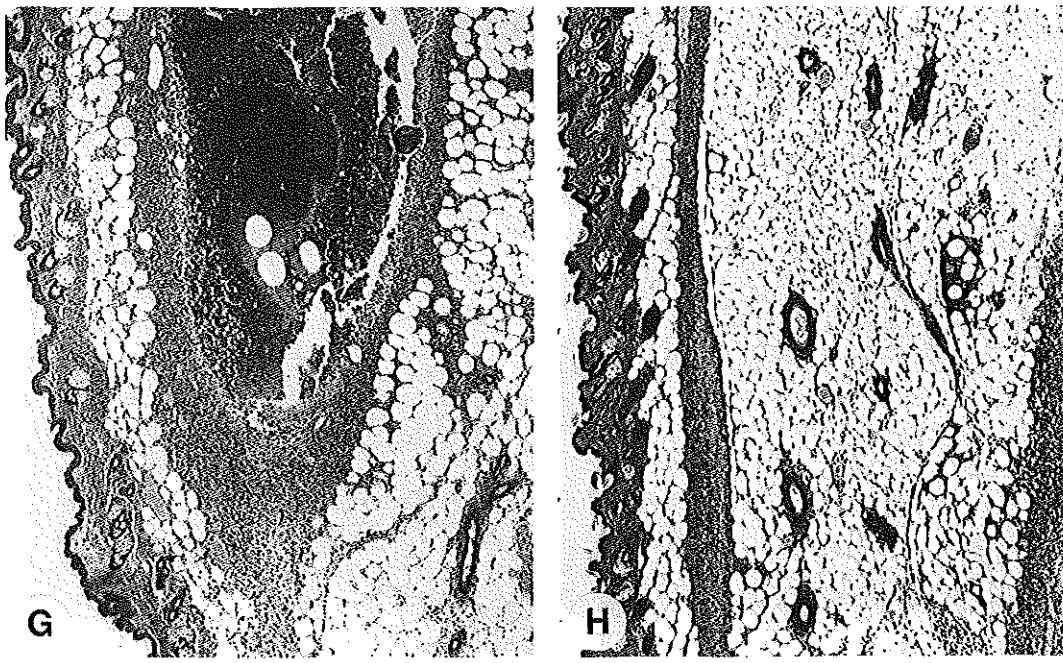

Figure 1

G

Mouse exp nr. II, s.c. TiterMax/SP215, 5 days after secondary injection. Grossly prominent nodule, white viscous material inside thin capsule in s.c. fat, score 3. Microscopically fairly circumscribed lesion, essentially composed of large amount of cellular debris within reactive capsule, score 2.5. HE, x44.

H

Mouse exp nr. II, s.c. RIBI/no antigen (PBS), 5 days after secondary injection. Grossly minimal amount of white tissue s.c. in the groin, not externally visible, score 0.5. Microscopically moderate diffuse inflammation, score 1. HE, x44.

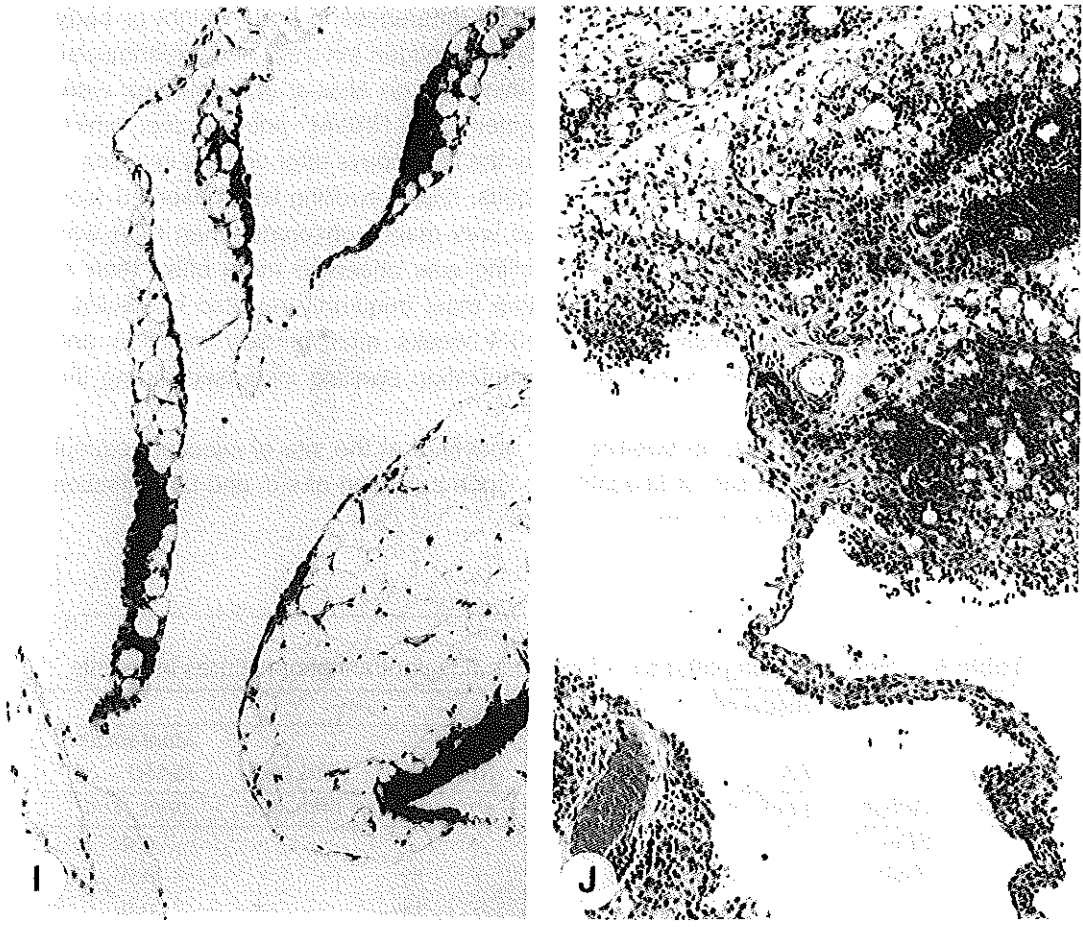

Figure 1

I

Mouse exp nr. II, i.p., omentum control: no adjuvant (PBS)/SP215, 5 days after secondary injection. Grossly negative, microscopically milky spots (physiological aggregates of lymphocytes and macrophages) and adipose tissue covered with inconspicuous mesothelium. HE, x110.

J

Mouse exp nr. II, i.p., omentum Montanide ISA50/SP215, 5 days after secondary injection. Grossly slight consolidation of omental edge, score 0.5 (see Table 7). Microscopically in contrast granulomatous inflammation, mesothelium prominent or absent, score 3. Also unaffected omentum present (not shown). HE, x110.

Results

Clinical and behavioural signs

Rabbits

Both after s.c. and i.m. injection, no significant differences in body weight or body temperature were observed between the control (antigen injected) and experimental (adjuvant/antigen injected) groups. After injection, no clinical abnormalities were observed, except for one rabbit i.m. injected with RIBI/*M. pneumoniae*, which showed discomfort during locomotion activity the first 4 days after primary injection.

Exp nr. I + II At s.c. injection sites in rabbits, tissue swelling was observed (see Table 5). Swelling at primary injection sites was mainly observed when FCA was injected. At secondary injection sites, swelling was observed more often than at primary injection sites. FA and RIBI injection most frequently resulted in swelling. Swelling, if present, increased the first 3-4 weeks after primary injection and decreased thereafter. After secondary immunization swelling increased during the first week post injection.

In i.m. injected rabbits, no swelling was observed at the site of injection, except secondary i.m injection site of Specol/BSA. Upon palpation of s.c. and i.m. injection sites, no signs indicating pain were observed.

Table 5. Tissue swelling after s.c. of injection adjuvant/antigen mixtures in rabbits

		Adjuvant[b]									
		FA		*Specol*		*RIBI*		*TiterMax*		*Montanide ISA50*	
Exp nr.	*Antigen*[a]	*prim*[c]	*sec*[c]	*prim*	*sec*	*prim*	*sec*	*prim*	*sec*	*prim*	*sec*
I	SPek15a	-[d]	-	-	-	-	-	-	-	n.d.	
I	Galacto	-	++	-	-	-	++	-	-	n.d.	
I	M.pneu.	-	++	-	+	-	++	-	+	n.d.	
II	Rubella	++	+	-	-	n.d.		n.d.		-	-
II	BSA	++	+	-	++	n.d.		n.d.		+	++

[a] Antigens described under Table 1;

[b] antigen controle (PBS + antigen injection): no abnormalities;

[c] maximum swelling at primary (prim) or secondary (sec) injection site;

[d] - = no abnormalities; + = swelling < 2 cm; ++ swelling >> 2 cm; n.d. = not done.

Exp nr. III + IV Swelling was observed at most injection sites of rabbits given measles- or mumps antigen combined with FA or Specol, especially after secondary injection (5-20 cm^2). Swelling after injection of mumps antigen combined with Specol was less extensive than combined with FA. Injection of HIV-peptide conjugates combined with FA resulted in severe swelling, persistent till the end of the experiment while injection of HIV-peptide conjugates combined with Specol resulted in swelling immediately after injection and minimal palpable swelling 4 weeks later. In rabbits injected with Montanide/HIV-peptide conjugates minimal swelling was observed.

Mice

Exp nr. I Body weight decreased in mice the first days after primary injection of FCA/antigen (5 out of 6 groups; 5/6), Specol (4/6), RV-ISCOMs (3/4) and Lactobacillus (2/6). Within one week, body weights were back to normal. The first days after adjuvant/antigen injection, some clinical abnormalities were observed in mice. Piloerection was observed in mice the first two days after i.p. primary and secondary immunization with FA/SP215 or FA/MBP emulsions. After secondary immunization, piloerection was observed in all groups i.p. or s.c. given *M. pneumoniae* combined with an adjuvant. At s.c. injection sites of FCA/SP215 and FCA/MBP a nodule (1-4 mm) was palpated. Nodules were also observed when mice were given s.c. *M. pneumoniae* with FA, Specol or Quil A. The FA induced nodules were present from one week after injection till the end of the experiment. Quil A and Specol nodules had disappeared 4 weeks after injection. In exp nr. I, secondary i.p. injection resulted in mortality of mice when injecting FIA/SP215 (5 out of 5), RV-iscom with coupled SP215 (2/5) and RV-ISCOMs with coupled MBP (5/5). It should be noted that these deaths were antigen but not adjuvant related, probably related to the persistent high antibody titres observed in these animals. Palpation of injection site did not result in signs of pain.

Exp nr. II Mice showed piloerection and hunched posture the first day after priming i.p. with FCA or TiterMax emulsions. The same clinical changes were found after secondary immunization with FA, TiterMax, Specol or Montanide emulsions. At necropsy (5 days after secondary injection) FA, TiterMax and Specol injected mice still showed piloerection. After primary s.c. injection nodules were palpable in mice injected with FA/SP215 (4-8 mm) or TiterMax (5 mm). Two mice died after secondary i.p. immunization with FA/SP215 (2/5). Palpation of the abdomen of i.p. injected mice or nodules at the s.c. injection sites did not lead to any sign of pain such as vocalising or struggling.

Using the POT procedure, we did not observe changes in behaviour and physiological state of the mice (besides piloerection as described above). Assuming that lesions induced by i.p. FCA/antigen injection are painful, we compared activity of mice at three weeks after i.p. injection of FCA/SP215 or PBS/SP215. No differences

between these two groups were observed (data not shown), indicating that, at that time, mice were minimally disturbed in their locomotion activity by FCA injection.

Behaviour registration system LABORAS

In mice exp nr. III, no clinical abnormalities were observed. Using the LABORAS system, significant differences in behavioural patterns between FCA and PBS injected animals were observed. On the day of injection, locomotion, climbing, grooming, eating and drinking were significantly ($p < 0.01$) decreased in FCA injected mice while immobility was significantly ($p < 0.01$) increased in FCA injected mice as compared to PBS injected mice (Fig. 2). Two days later immobility was still significantly ($p < 0.01$) increased in FCA injected mice. Four days post immunization, no significant differences in recorded behaviour were observed.

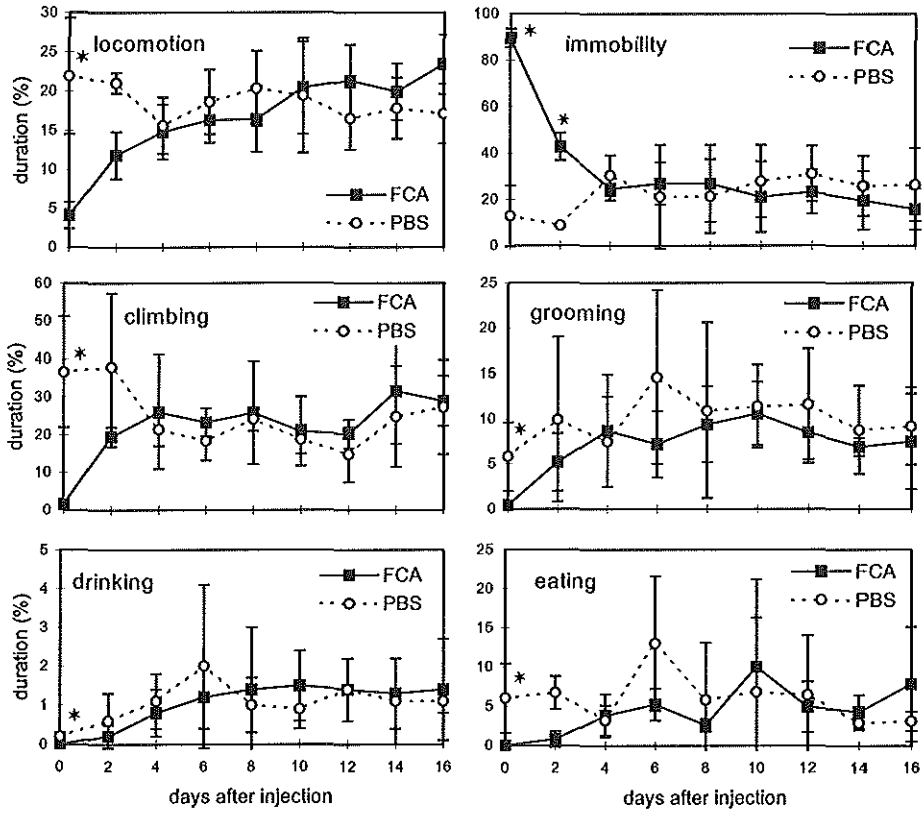

Figure 2 Results of LABORAS behaviour registration system (mice exp nr. III). Behavioural patterns of FCA and PBS injected (0.2 ml i.p.) mice (n = 4) are shown. Relative mean time (± SD) spent on behaviour per day during 4 hours measuring period. * $p < 0.01$.

Pathological changes

Rabbits

Despite the absence of changes in clinical and behavioural parameters in rabbits after injection of adjuvant/antigen mixtures, severe pathological changes did occur in some of these animals. Pathological changes in rabbits of exp nr. I and II at primary and secondary injection sites of different adjuvant/antigen mixtures are summarized in Table 6.

Gerbu injections induced no and DDA injections moderate lesions; as Gerbu and DDA gave negative immunological results, these adjuvants are not considered relevant and therefore are not included in Table 6.

S.c. route rabbits RIBI and FA induced most and more severe lesions compared to other adjuvants. Lesions were often extensive (up to 100 cm^2) and voluminous, combining proliferative and exudative features. Abscess-like lesions were only observed in RIBI treated rabbits. Specol, TiterMax and Montanide resulted in lesions at secondary injection sites mainly (Fig. 1C). Their presence and severity depended on the antigen added. For the overall severity score of s.c. lesions per adjuvant/antigen combination (Fig. 3), the sum of the mean primary and secondary lesion score was taken, after applying a multiplication factor of 1.5 for the former score, to take into account possible reduction over time and longer duration of primary lesions.

Pathological changes of rabbit exp nr. III and IV are not summarized in Table 6, since in these studies four injections, instead of one at a time, were given. When Specol was introduced to replace FA in routine polyclonal antibody production, for measles- and mumps antigen unexpected severe lesions were found. In the next series Specol and FA were compared, and with both antigens extensive (with measles antigen: more than 200 and more than 300 cm^2 respectively and with mumps antigen almost 100 and more than 200 cm^2 respectively) haemorrhagic lesions were induced (exp nr. III) (Fig. 1A). Measles antigen without adjuvant (n = 7) did not induce gross lesions, while mumps antigen (n = 1) induced an abscess-like lesion (60 cm^2 totally). Injection of 4 x 0.25 ml FA without antigen (n = 1) induced extensive necrotising granulomas. The same amount of Specol without antigen (n = 1) resulted in (extensive: 100 cm^2) primarily exudative lesions, while after a single dose of 0.1 ml (n = 1) minimal reaction was found (data not shown). In exp nr. IV, Specol and Montanide combined with antigen at relatively low doses (4 x 0.1 ml) were shown to induce exudative lesions (Fig. 1B), which at the most recently injected sites were more severe than those induced by FIA.

Table 6. Pathological findings at injection sites of rabbits s.c. or i.m. injected with different adjuvant/antigen combinations

		s.c. injection[c]		*i.m. injection*	
Adjuvant[a]	*Antigen*[b]	*primary*	*secondary*	*primary*	*secondary*
FA	SPek15a	4[d]	6	2	5
	Galacto	6	4	2	0.5
	M.pneu.	6	6	5	0
	Rubella	4	5	6	3.5
	BSA	5	6	6	5
Specol	SPek15a	0	3	0	6
	Galacto	0	0	0.5	0.5
	M.pneu.	2	6	2	4.5
	Rubella	0	0	0	0
	BSA	4	6	4	6
RIBI	SPek15a	7	4	5	3
	Galacto	7	7	6	6
	M.pneu.	5	8	5	6
TiterMax	SPek15a	1	5	2	5
	Galacto	1	2	0	2
	M.pneu.	3	6	0	3
Montanide	Rubella	0	0	0	1
ISA50	BSA	2	4	2.5	4.5

[a] Antigen controls (PBS + antigen): all primary injection sites negative; secondary injection sites: Galacto (i.m.), M.pneu. (i.m.) = severity score 1; Galacto (s.c.) = severity score 2; [b] SPek15a = synthetic peptide; M.pneu. = *Mycoplasma pneumoniae*; Galacto = Galactocerebroside; BSA = bovine serum albumin; [c] comparison is not possible between routes; [d] mean of severity score at injection site of two rabbits; score as described in Table 3 in 'Animals, materials and methods' section.

I.m. route rabbits Intramuscular lesions generally consisted of granulomas spreading parallel to muscle fibres and showed less variation in size and character than s.c. ones. Maximum cross diameter was 4 cm and in general length was 2 to 4 times the cross diameter. RIBI and FA induced more and more severe lesions than the other adjuvants, resulted in lesions mainly at secondary injection sites. The presence and severity of lesions depended on the antigen used. Necrosis in i.m. granulomas was most common after RIBI injections. Necrosis of pre-existent tissue was restricted to 4 RIBI cases (Fig. 1D) and 1 exceptionally severe Specol (BSA) case. The overall severity score of i.m. lesions is given in Fig. 3; the same calculation was applied as described for s.c. lesions.

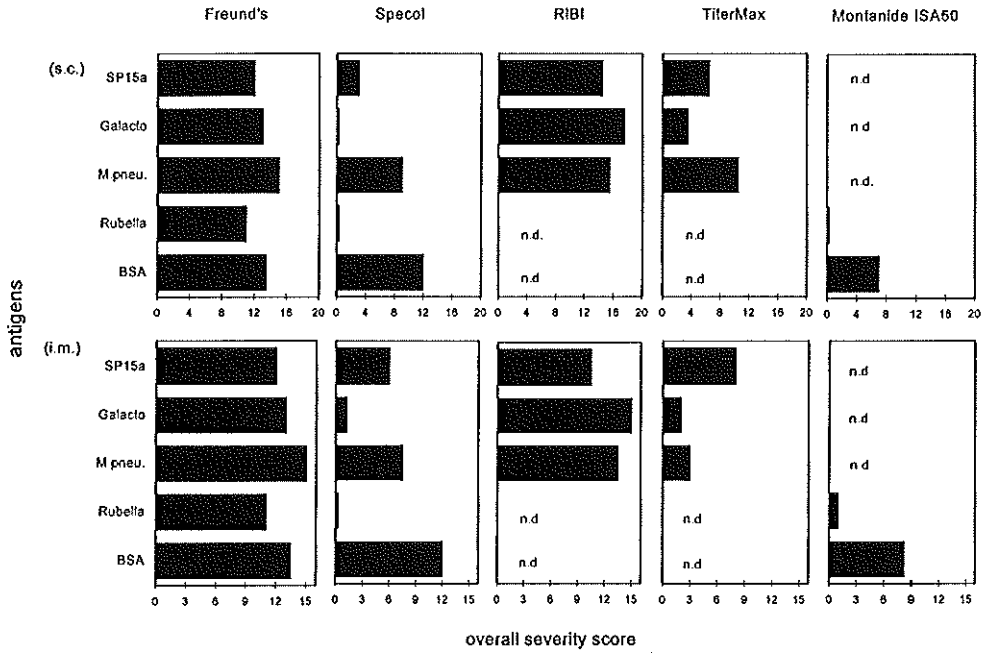

Figure 3 Overall pathological severity score of lesions after s.c. or i.m. injection of different adjuvant/antigen mixtures in rabbits (n = 2). For the overall severity score of lesions per adjuvant/antigen combination per route of injection, primary and secondary lesion scores were summed after applying a multiplication factor of 1.5 for the former score (see Pathological changes of 'results' section. Antigen controls (PBS + antigen) are described under Table 6.

Mice

Pathological changes in mice after injection of FA, Specol, RIBI, TiterMax and Montanide ISA50 are described later in this paragraph per route of injection. Data of RV-ISCOMs, Lactobacillus and Quil A are not included in Fig. 4 or Table 7, because of limited availability (RV-ISCOMs), negative immunological results (Lactobacillus) and limited immunological and pathological data (Quil A). When RV-ISCOMs were used, lesions (adhesion between omentum and mesorchium after i.p. inoculation) were found only in one mouse; however, 7 out of 20 mice died after secondary i.p. inoculation of RV-ISCOMs with coupled antigen. Lactobacillus induced pathological abnormalities only when combined with *M. pneumoniae*. (s.c.: exudative panniculitis; i.p.: omentum retrahens and fat necrosis). Quil A with *M. pneumoniae* resulted in comparable lesions (s.c. necrotising panniculitis; i.p.: marked omentum retrahens and peritoneal microabscesses).

S.c. route mice Gross lesions found at the primary injection site varied slightly within groups, while lesions at secondary injection sites were almost identical within groups. At most primary injection sites of FA, TiterMax and Montanide, white or yellow nodules (2-10 mm) were observed. Microscopically, granuloma formation, exudative diffuse inflammation and fibrosis were present at varying degrees (Fig. 1E-F). Specol resulted only in marked lesions at the primary injection site when it was combined with *M. pneumoniae*. After secondary injection of Specol/*M. pneumoniae* and in some animals after secondary injection of TiterMax, circumscribed and/or hyperaemic nodules (up to 10 mm) were found. At most secondary injection sites of other adjuvant/antigen combinations, grossly white shiny tissue attached to muscles was found. Depending on the amount of this apparently innocuous reaction, it was visible externally as a white s.c. area after moistening the coat and caused bulging of the skin in the groin. Microscopically, diffuse exudative inflammation usually dominated (Fig. 1G-H), the severity of which was mostly uniform within groups and did not always parallel the gross findings. To come to a mean severity score of lesions per injection site, the sum of macroscopic and microscopic score was taken. To obtain an overall severity score per injected adjuvant/antigen combination, the sum of the mean primary lesion score (multiplied by 1.5) and the mean secondary lesion score was taken (as described for s.c. lesions in rabbits). The overall severity scores obtained in mice exp nr. II are visualized in Fig. 4. The score of FA in this figure (± 12) is representative for other antigens when combined with FA. The score of Specol when combined with *M. pneumoniae* was higher (10), and when combined with SP215 (exp nr. I) or MBP lower (± 2) than the depicted overall severity score of ± 5 for Specol/SP215. Injection of FA and RIBI in combination with antigen resulted in lesions comparable to those observed after injection of FA and RIBI without antigen. Specol and Montanide ISA50 without antigen induced lesions that were slightly milder than

those found after s.c. inoculation of the same adjuvant combined with antigen. TiterMax lesions were milder when s.c. injected without antigen compared with TiterMax/antigen injection. All antigen controls (antigen+PBS) were grossly negative. Microscopically, *M. pneumoniae*/PBS and SP215/PBS (mice exp nr. II) injection resulted in minimal or slight diffuse inflammation at the secondary injection site.

I.p. route mice Injection of FA and TiterMax resulted most consistently in severe pathological abnormalities. Severity of lesions in the case of Specol depended on the antigen used. Pathological changes in i.p. treated mice are summarized in Table 7. Findings were very homogenous within groups. Adhesions, often extensive, were seen between small and large intestine, spleen and abdominal wall or in the hypogastrium, with shortening of the ligamentum latum. The pattern of adhesions was strikingly comparable within groups. Other signs of peritoneal irritation were milky effusions and dilatation of the small intestine. In less severe cases, white smooth, shiny thickening of the peritoneum covering liver, diaphragm and spleen (designated plaques), and/or omentum retrahens were observed. Microscopically, severity of peritonitis was determined evaluating mesothelial alteration, volume and character of peritoneal infiltrate, hyperaemia and fibrosis. When no omentum could be recognized due to severe omentum retrahens, peritoneum covering the pancreas was analysed instead. Marked gross signs of active peritonitis were judged severe, regardless of microscopical score. In some groups gross abnormalities were minimal or slight, while microscopy revealed peritonitis of unexpected severity. When this was the case, the total score was increased when compared to the macroscopic overall score. Mice in exp nr. III (LABORAS experiment) showed no pathological changes after injection with PBS (0.2 ml i.p.) while FCA/PBS injection (0.2 ml i.p.) resulted in marked adhesions and plaques (Table 7).

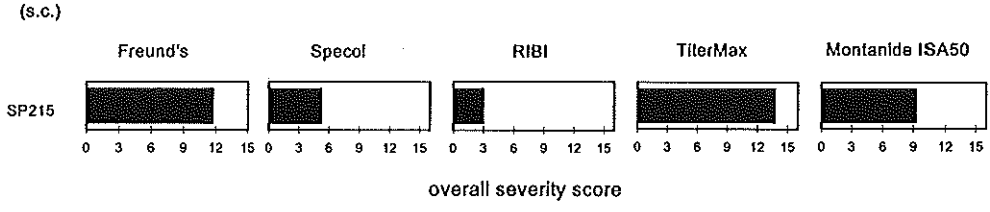

Figure 4 Overall pathological severity score of lesions after s.c. injection of adjuvant/SP215 mixtures in mice (n = 2-4). The overall severity score was calculated as described under Figure 3.

Table 7. Pathological findings in mice i.p. injected with different adjuvant/antigen combinations

		Macroscopy[c]					*Macro-scopy*[d]	*Micro-scopy*[e]	
Adjuvant[a]	*Antigen*[b]	*effu-sions*	*adhe-sions*	*intestinal dilata-tion*	*pla-que s*	*omentu m retra-hens*	*overall score*	*severity perito-nitis*	*total score*[d]
FA	MBP	0	2	0	2	2	4	4.3	4
	M.pneu.	0	0	0	2	1.5	2	4	2
	SPII[f]	2	2	0	0	2	4	3	4
	- (PBS)	1	0	0.5	0.5	1	3	3	3
Specol	SPI	0	0	0	1	0	1	2	1
	MBP	0.5	0	0	0.5	0	1	3	1
	M.pneu.	0	0	0	1	1.5	2	3	2
	SPII	0	2	2	0	2	4	4.5	4
	- (PBS)	0	1	2	0	1	4	n.d.	4
RIBI	SPII	0	0	0	0.5	0	0	1.7	1
	- (PBS)	0	0	0	0	0	0	1.8	1
TiterMax	SPII	2	1	1	0	2	4	2.8	4
	- (PBS)	2	1	0.5	1	1	4	n.d.	4
Montanide	SPII	0	0	0	1	0.5	1	3.3	2
ISA50	- (PBS)	0	0	0	0.5	0.5	1	1.7	1
FCA	- (PBS)	0	2	0	1	0	4	n.d.	4

[a] Antigen controls (PBS + antigen): grossly and microscopically negative; [b] SPI = synthetic peptide (mice exp nr. I); MBP = myelin basic protein (autoantigen); M.pneu. = *Mycoplasma pneumoniae;* SPII = synthetic peptide (mice exp nr. II); - = PBS, no antigen; [c]mean macroscopic severity score (n = 4-5): 0 = absent; 1 = moderate; 2 = marked; [d] 0 = negative-minimal; 1 = slight; 2 = moderate; 3 = marked; 4 = severe; [e] mean microscopic severity score (n = 3-5): 0 = negative; 1 = minimal; 2 = slight; 3 = moderate; 4 = marked; 5 = severe; [f] two out of five mice died after secondary immunization.

Antibody responses

This paper emphasizes the side effects induced by adjuvants. Consequently, only relevant results on antibody production are given. In Figure 5, mean relative antibody titres (FA = 100%) of five commercially available adjuvants (Freund's adjuvant, Specol, RIBI, TiterMax and Montanide ISA50), injected in rabbits (s.c. or i.m.) and mice (s.c. or i.p.) combined with different antigens, are shown. Injection of FA/antigen resulted in high titred specific antibody responses independent of the antigen combined with it. RIBI did not induce acceptable antibody responses in our studies in rabbits and mice. TiterMax was not effective in inducing antibody responses in rabbits and mice, except when i.p. injected in mice. Specol and Montanide ISA50 induced antibody responses which were comparable to those induced by FA in rabbits and mice. Except for Specol/autoantigen injection in mice which did not result in detectable antibody responses; Montanide ISA50 and also RIBI and TiterMax were not injected in combination with autoantigen. RV-ISCOMs with coupled antigen (autoantigen or synthetic peptide) induced very high specific antibody titres but this very potent and promising adjuvant is not (yet) commercially available and therefore can not readily replace FCA. Antibody titres were low when DDA, Gerbu or Lactobacillus were used as adjuvant when compared with FA.

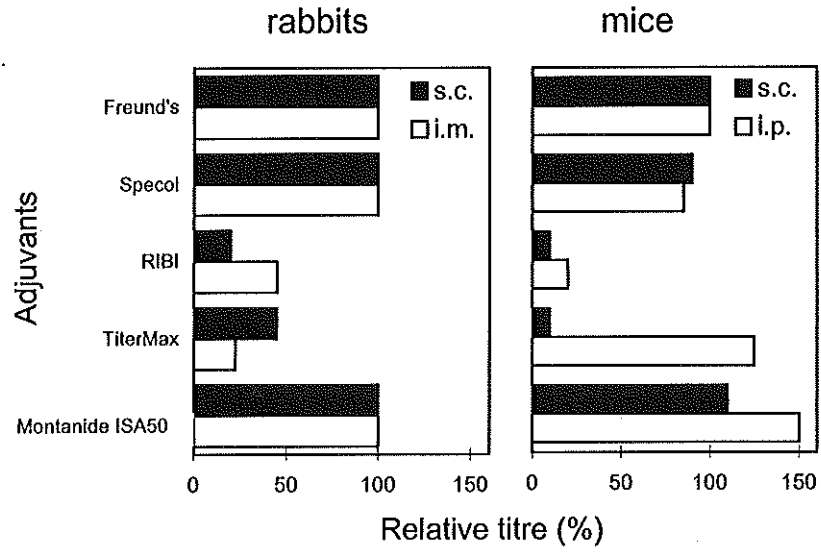

Figure 5 Relative antibody titre in serum of rabbits and mice after injection of different adjuvant/antigen combinations. Antibody titre in serum of FA/antigen injected animals was set on 100%.

Discussion

In this paper, FA and alternative adjuvants were evaluated on their side effects to select an alternative which combines minor side effects with high antibody titres for a broad range of antigen types. Injection of FA resulted in moderate to severe pathological changes (Fig. 3 and 4; Table 6 and 7), confirming data of Broderson *et al.* (1989), Toth *et al.* (1989) and Wiedemann *et al.* (1991). Literature data on side effects induced by alternative adjuvants compared with FA are very limited. Information of side effects is often only obtained from clinical observations. We consider necropsy necessary to determine the side effects induced by adjuvant injection (e.g. intramuscular lesions cannot properly be monitored in the living animal). Sometimes, we observed extensive lesions at necropsy while no swelling or nodules were found upon palpation. Lesions in rabbits after RIBI injection were more severe than those induced by FA (Fig. 3). Johnston *et al.* (1991) compared FA, RIBI and Montanide ISA50 and observed no differences in macroscopical abnormalities induced by these adjuvants. In contrast, Deeb *et al.* (1992) observed mild lesions after RIBI injection in rabbits. In our studies in mice, RIBI induced minimal pathological changes (Fig. 4 and Table 7). After i.p. injection of RIBI in mice Lipman *et al.* (1992) observed lesions (adhesions and white plaques on organs) which were less extensive than FA induced lesions. After injection of TiterMax in rabbits (Fig. 3) we observed slight lesions while injection of TiterMax in mice resulted in severe lesions (Fig. 4 and Table 7). Bennett *et al.* (1992) studied histopathological changes after i.m. injection of 2 x 40 µl TiterMax/LHRH-BSA and found mild lesions. Montanide ISA50 injections resulted generally in less severe lesions when compared to FA injections, both in rabbits and mice (Fig. 3 and 4; Table 6 and 7). As described above Johnston *et al.* (1991) injected rabbits with Montanide ISA50 and observed similar lesions as those induced by FCA. This may be explained by the fact that different antigens were used in both studies.

Pathological investigation was performed at the end of each experiment. Consequently, continuous monitoring of the lesions by macroscopy and microscopy was not possible. The monitoring time point generally was 2 weeks or 5 days after secondary injection in rabbits and mice, respectively, lesions at primary injection sites being at least 6 weeks old at that time. To discriminate between primary and secondary injection, these injections were given on separate sites. Primary injection sites were negative more often than secondary ones. Besides healing of the inflammation, a role for immunopathological phenomena (as a result of repeated introduction of antigen) in secondary lesions is likely. Due to the one point of monitoring, rapidly resolving inflammatory reactions may have been missed, especially in rabbits (lesions at least 2 weeks old). A certain lesion, found at the primary injection site (at least 6 weeks old), is supposed to have impaired the animal more than an identical lesion found at the secondary injection site. To compensate for this, scores of primary injection sites were augmented by a factor 1.5 before

summing up primary and secondary scores to reach an overall severity score per adjuvant/antigen combination (Fig. 3 and 4).

Microscopy was performed since this adds useful observations to gross data. Lesions at s.c. injection sites in mice showed some remarkable differences in macroscopic and microscopic findings. At the s.c. primary injection site in mice, grossly observed impressive nodules occasionally appeared to exist only of one or several large 'oil spaces' surrounded by slight or moderate (fibro)granulomatous proliferation. On the other hand macroscopically identically scored lesions, histologically could display marked diffuse and granulomatous inflammation and fibrosis around variably sized 'oil spaces'. Microscopical examination of s.c. secondary injection sites in mice revealed grossly unexpected severe inflammation in some groups. Why these microscopically marked exudative lesions are seen macroscopically as not remarkable, remains obscure. Haemorrhage was absent in lesions of mice but was present in lesions of rabbits, that showed marked hyperaemia in exudative lesions despite exsanguination. When upon macroscopical examination of s.c. and i.m. injection sites no pathological changes are found, additional information from microscopy depends on successful sampling. The i.p. route can always be monitored microscopically and mild or resolving peritonitis (missed grossly) can be assessed in a more sensitive way. Since in rabbits macroscopical severity correlated well with microscopical severity, microscopy was only used to verify gross findings. The same holds true for the i.p. route in mice. In s.c. treated mice macro- and microscopical findings were taken together, because the latter were unpredictable from the former.

Besides the adjuvant, other factors determine the severity of lesions, e.g. antigen type and injection route. The importance of the antigen in induction of pathological changes was observed when *M. pneumoniae* was used as antigen. Lesions were relatively severe after injection of this antigen combined with all tested adjuvants in rabbits and mice while negligible lesions were observed when this antigen was injected without an adjuvant. In combination with measles or mumps antigen, Specol induced extensive lesions (less extensive than FA) in rabbits while in combination with other antigens (e.g. synthetic peptide, glycolipid) lesions were minimal to mild. This may be explained by the volume injected (4 x 0.25 ml) or the particulate character of the antigens. It is tricky to compare severity of lesions between injection routes. In our opinion the i.p. injection route should be rejected, due to the extensive surface of the peritoneum, its high susceptibility to physicochemical damage and its intimate contact with various vital organs. Especially the acute phase of peritonitis is supposed to be painful. In abdominal tissue of mice, acute exudative inflammation was observed 2 to 96 h after i.p. FCA/TNP-KLH injection (0.2 ml) (data not shown). Jansen van 't Land and Hendriksen (1995) showed decreased locomotion activity the first day after i.p. injection of 0.2 ml FCA. In an experiment in the automated behavioural system LABORAS, locomotion, climbing, grooming, eating and drinking

were significantly reduced after i.p. injection of FCA/PBS (Fig. 2). The i.p. route also yielded some deaths after booster immunization, probably due to systemic reactions. From the pathology results in rabbits, a preferential route is difficult to select. The i.m. route is more prone to injection error (i.e. wrong localization of adjuvant/antigen mixture) and more painful than the s.c. one and i.m. lesions are more difficult to monitor clinically. The s.c. loose areolar tissue will permit spread of inoculum and inflammation more easily than the rigid muscle compartment, indeed resulting in larger s.c. lesions. I.m. lesions probably exert more pressure on the surrounding tissue. So, when considering the s.c. and i.m. route, pain experienced by the animal is not only a function of lesion size and character. Although s.c. lesions sometimes were very extensive, we prefer the s.c. route to the i.m. route.

An effective adjuvant (Fig. 5) that induces minimal pain in the animals is preferred for immunization. In rabbits, no clinical or behavioural changes indicating pain and distress were observed. In mice, indications of acute pain and distress (e.g. weight loss, decrease in activity and piloerection) were found the first days after injection. Although no signs of prolonged severe pain and distress were observed, marked to severe pathological changes did occur after injection of several of the adjuvant/antigen mixtures (Fig. 3 and 4; Table 6 and 7). It is not possible to give an estimation of pain associated with the observed pathological changes, because we did not observe indications of prolonged pain and distress, in mice, only acute distress was found. The available methods may be inadequate to monitor prolonged pain and distress, or there was no significant pain and distress despite the severe pathological changes. To monitor pain and distress in rabbits, the methods may not be sufficient to detect all possible signs of pain, because rabbits do not readily exhibit pain signs (Wallace *et al.*, 1990; Griffiths, 1991). In mice, signs of acute pain were found while later in time pain is not detectable by clinical and behavioural changes, indicating that the animals can cope with the induced pathological changes or that these changes are not or less painful than excepted. Devices like the LABORAS system (Bulthuis *et al.*, 1997; Van de Weerd, 1996) enable the registration of behavioural changes in mice very accurately, suggesting adequate possibilities to monitor severe pain and distress. We injected animals, in accordance with the Dutch guidelines for immunization of laboratory animals, e.g. small volumes of FCA and FCA in primary and FIA in secondary immunization. This may declare that minimal pain and distress were found. Johnston *et al.* (1991) did not observe indications for pain in rabbits while marked pathological changes were found. Amyx (1987) suggested that most undesirable effects of FCA can be eliminated by careful control of injection quantity and site selection. The pathological changes induced by FCA are thought, based on the analogy principle with humans, to be (potentially) very painful. Reports on accidental and intentional human injections provide another source of information on pain induced by FCA. Chapel and August (1976) reported severe pain after accidental injection in humans. However, all persons who suffered

severe pain were positive to the tuberculin test prior to the accidental injection, indicating that the reaction was a result of repeated exposure to Mycobacteria (Stills and Bailey, 1991). Wanstrup and Christensen (1965) injected mice weekly with FCA and observed decreased activity, dullness of fur and loss of hair.

In conclusion, to minimize pain and distress in immunization experiments, it is essential to use the adjuvant properly, i.e. in accordance with existing guidelines. When doing so, in our studies no clinical and behavioural changes indicative for severe prolonged pain and distress (acute distress was observed) were found, despite severe pathological changes observed with some adjuvants. Pathological changes are considered an essential parameter to study side effects of adjuvants. The s.c. route should be preferred above i.m. (rabbits) or i.p. (mice) route. When an adjuvant is sought that induces less pathological changes than FA and acceptable antibody responses, we suggest, based on our data, to use Montanide ISA50 and Specol as alternatives to FA.

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Chapter 6

Immune responses and side effects of five different oil based adjuvants in mice

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submitted

Abstract

In this study, five different oil based adjuvants were compared to assess efficacy and side effects. Mice were injected subcutaneously (s.c.) or intraperitoneally (i.p.) with a weak immunogen (synthetic peptide; SP215) emulsified in Freund's adjuvant (FA; Freund's complete adjuvant on primary and Freund's incomplete adjuvant on secondary injection), Specol, RIBI, TiterMax or Montanide ISA50. Control groups received antigen in phosphate buffered saline (PBS) or PBS emulsified in adjuvant. Side effects were evaluated based on clinical and behavioural abnormalities and (histo)pathological changes. Efficacy of adjuvants was evaluated based on their properties to induce peptide specific IgG1, IgG2a and total IgG antibodies, native protein cross reactive antibodies and cytokine production. Pilo-erection and hunched posture developed in mice after primary i.p. injection of TiterMax and after secondary injection of FIA, TiterMax, Specol or Montanide ISA50. Based on histopathological data, severity of lesions induced by adjuvant injection can be ranked as: (s.c. injection) FA = TiterMax > Montanide ISA50 > Specol = RIBI and (i.p. injection) FA = TiterMax = Specol > Montanide ISA50 > RIBI. Although marked differences in isotype profile and height of titre are observed among the different adjuvants used, we found that FA, Montanide ISA50 and Specol worked equally well in the s.c. and i.p. route, TiterMax functioned only when given i.p. and RIBI did not perform up to par. When comparing the number of cytokine producing cells (interferon-gamma, interleukin-4) in spleen sections of mice, RIBI yielded significantly higher numbers of positive cells compared with the other adjuvants used in this study, five days after secondary injection (i.p.). Combined with the low antibody responses after RIBI injection, these data suggests that cytokine profiles after secondary injection of adjuvant/antigen, not necessarily correlate with desired antibody titres and isotype distribution of antibodies.
In conclusion, from our data it appeared that high peptide specific antibody levels with minimal side effects can be obtained by s.c. injection of peptide combined with Montanide ISA50 or Specol as alternatives to FA.

Introduction

Adjuvants are substances which stimulate the immune system. In experimental immunology, adjuvants are used to induce specific antibody responses to an antigen. In laboratory animals, Freund's complete adjuvant (FCA) is, because of its overall applicability, routinely used for this purpose. FCA consists of a non-metabolizable mineral oil with added heat-killed Mycobacteria, which makes it a very effective but also a harmful adjuvant. Severe side effects in laboratory animals were observed when FCA was injected (Broderson, 1989; Toth *et al.*, 1989). The severity of the side effects induced by FCA, resulted in concern about its use in laboratory animals. Several adjuvants are developed as an alterrnative to FCA (Claassen and Boersma, 1992; Vogel and Powell, 1995). These alternatives should ideally be as effective as FCA but induce minimal side effects. The components and constitution of an adjuvant determine to a large extent the efficacy and side effects. To minimize side effects, adjuvants have been developed in which harmful components of FCA have been replaced (e.g. by synthetic analogues) or deleted. Since the quality of the mineral oil and Mycobacteria in FCA are an important inducer of side effects, selected mineral oil adjuvants were introduced which do not include Mycobacteria or any other microbial components. Examples of such mineral oil adjuvants, Specol (Bokhout *et al.*, 1981) and some products of the Montanide series (Ganne *et al.*, 1994). Mineral oils can also be replaced by metabolizable oils to reduce the side effects (Scalzo *et al.*, 1995). Metabolizable oils, however, are less potent. Alternative adjuvants were developed, consisting of metabolizable oil with an added immunomodulator. Examples of such adjuvants are: TiterMax (Hunter *et al.*, 1995) and RIBI (Rudbach *et al.*, 1995), which contain, a nonionic block polymer (CRL-8941) and synthetic microbial products, respectively.

The number of studies in which the side effects and efficacy of these alternatives to FCA have been compared is very limited. Side effects of adjuvants can be assessed by clinical, behavioural (Morton and Griffiths, 1985) and pathological changes. Evaluation of adjuvant efficacy can be based on antibody production by B cells. In the development of antibody responses, T-B cell interactions play a central role (Laman and Claassen, 1996). Upon activation, T helper (Th) cells express the ligand for CD40 (CD40L) and produce cytokines which are both essential for T cell-dependent B cell activation. After the initial B cell activation, Th cell-derived cytokines are thought to regulate the differentiation of B cells into antibody forming cells. The pattern of cytokine secretion of Th cells is thought to be crucial in the selection of isotype produced by antibody-forming B cells. It is generally accepted that interferon-gamma (IFN-γ; produced by Th1 cells) promotes antibodies of the IgG2a isotype, whereas interleukin (IL-4; produced by Th2 cells) promotes an immune response dominated by antibodies of the IgG1 isotype (reviewed by Finkelman *et al.*, 1990; Laman and Claassen, 1996). Consequently, cytokine

production may be another indicator to study adjuvant efficacy. To evaluate the balance between efficacy and side effects of commercially available adjuvants, five different oil based adjuvants were injected (s.c. or i.p.) in mice combined with a weakly immunogenic antigen (synthetic peptide) and compared for their properties to induce antibody and cytokine production and side effects.

Animals, materials and methods

Animals

Female BALB/c mice were bred specific pathogen free (SPF) at the RIVM breeding facilities, Bilthoven, the Netherlands and used at 10-14 weeks of age. Animals were housed under SPF conditions and had free access to pelleted food (Hope Farms, Woerden, the Netherlands) and tap water. The experiments were approved by the Animal Ethics Committee of the RIVM.

Antigen

The antigen used is a synthetic peptide of 21 amino acids (SP215), a homologue to the hinge region of the human IgG2 molecule, which was synthesized as described by Boersma *et al.* (1989). SP215 is weakly immunogenic, it does not induce a detectable antibody response when injected without an adjuvant or carrier. SP215 was diluted in phosphate-buffered saline (PBS) and administered at a concentration of 50 µg per mice for both the primary and secondary inoculation.

Adjuvants

Freund's incomplete adjuvant (FIA) and Freund's complete adjuvant (FCA) were obtained from Difco Laboratories (Detroit, MI). FIA is a non-metabolizable mineral oil (Bayol F) and an emulsifier. FCA is FIA with added heat-killed *Mycobacterium butyricum* (0.5 mg/ml). Specol (ID-DLO, Lelystad, the Netherlands) is a non-metabolizable mineral oil (Marcol 52) and emulsifiers (described by Bokhout *et al.*, 1981). TiterMax (CytRx, Norcross, GA) contains a metabolizable oil (squalene), emulsifier and a patented block copolymer CRL-8941. When these oil adjuvants are mixed with aqueous antigen solution, water-in-oil emulsions are formed. Montanide ISA50 (Seppic, Paris, France) is a non-metabolizable mineral oil and emulsifier. RIBI (Sigma Immunochemicals, St. Louis, MO) consists of a metabolizable oil (squalene), emulsifier and microbial components: 0.5 mg/ml monophosphoryl lipid A (MPL) and 0.5 mg/ml synthetic trehalose dimycolate (TDM). Mixing of RIBI with aqueous antigen solution results in an oil-in-water emulsion.

Preparation of oil emulsions

The oil emulsions were prepared by mixing oil and aqueous phase as recommended by the manufacturer. The adjuvants were mixed with either PBS/SP215 or PBS. Water-in-oil emulsions of FA (Freund's adjuvant; FCA in primary, FIA in secondary immunization) were prepared using two glass syringes. The aqueous phase was added to the oil phase (oil : aqueous phase = 1 : 1) and emulsified by mixing back and forth for 1 min. Specol emulsions were prepared by mixing the oil phase thoroughly on a vortex and adding the aqueous phase dropwise (oil : aqueous phase = 5 : 4). RIBI was prepared by warming the RIBI vial in a 40°C water bath for 5 min., aqueous phase was added to the RIBI vial and vortexed for 3 min. TiterMax was prepared the same way as FA using two glass syringes, except that now the aqueous phase was added in two steps. Montanide ISA50 emulsions were made by transferring the oil phase into a plastic tube, adding aqueous phase, and emulsify by making 10 up and down strokes using a syringe (oil : aqueous phase = 1 : 1).

Experimental design

To evaluate the properties of oil based adjuvants to induce side effects, specific antibodies and cytokine production, mice were injected with SP215 emulsified in adjuvant (FA, Specol, RIBI, TiterMax or Montanide ISA50). Control groups received antigen in PBS or PBS emulsified in adjuvant. Per combination, five mice were injected subcutaneously (s.c.) and five intraperitoneally (i.p.). Mice were injected s.c. at the base of the neck in primary and in the groin in secondary immunization to distinguish between injection sites for pathological studies. The s.c. injected volume was 0.1 ml per injection except for TiterMax injections when 0.05 ml was injected (as recommended by the manufacturer). The i.p. injected volume was 0.2 ml per injection. Animals were given a primary injection on day 0 and an identical secondary injection on day 42, except that FCA was replaced by FIA. Blood samples were taken on day -3, 7, 14, 28 and 41 via orbital punction under ether anaesthesia. Five days after secondary immunization, mice were bled after being anaesthetized by intramuscular injection of 0.1 ml of a mixture of ketamine (50 mg/ml), xylazine (20 mg/ml) and atropine (1 mg/ml) in a 7:3:1 volume ratio. Animals were examined for gross lesions and relevant tissue samples were collected for histopathological study. Spleens were removed and immediately frozen in liquid nitrogen and stored at -70°C for immunohistochemistry.

Clinical observation and pathology

General condition of the mice was evaluated daily, based on general appearance and clinical signs. Injection sites were palpated for signs of pain and examined for swelling once a week. Diameter of swelling was measured in s.c. injected animals. Five days

after secondary immunization, animals were bled and necropsy consisted of examination of injection sites and major organs.

For histopathological evaluation, tissue of s.c. injection sites, a sample of the omentum of all i.p. injected animals and organs showing macroscopic abnormalities were preserved in 4% buffered formaldehyde, embedded in paraffin, sectioned at 5 µm, and stained with haematoxylin and eosin. Lesions were scored macroscopically and microscopically. In s.c. injected animals, lesions were scored separately at primary and secondary injection site. The score was based on diameter of lesions and on microscopic evaluation of infiltrates and the presence of necrosis and fibrosis. In the i.p. injected animals the severity of lesions was mainly assessed macroscopically, based on abdominal effusions, adhesions, intestinal dilatation, presence of 'plaques' and omentum retrahens. Microscopic severity of peritonitis (volume and character of peritoneal infiltrates) was added to correct for macroscopic underscoring. Gross and histopathologic lesions were scored and finally classified as follows: 0 = negative/minimal; 1 = slight; 2 = moderate; 3 = marked; 4 = severe.

Immunoassays

Peptide specific IgG1 and IgG2a antibody levels

In serum samples, SP215 specific IgG1 and IgG2a antibody levels were determined by enzyme-linked immunosorbent assay (ELISA). Plates were coated with SP215 (10 µg/ml) and blocked with PBT. Diluted serum samples were incubated and then plates were incubated with peroxidase-conjugated goat antibodies specific for mouse IgG1 (0.4 µg/ml) or IgG2a (0.4 µg/ml) antibodies (Nordic, Tilburg, the Netherlands). TMB (3,3',5,5'-tetramethylbenzidine), 100 mg/ml in 0.11 M CH_3COONa (pH 5.5), was used as a substrate. After 10 min. the reaction was stopped by adding H_2SO_4 (2M). The absorbance was read at 450 nm against the signal from preimmune serum as a blank. A pool of sera from mice (of this experiment) producing high levels of specific antibodies served as a standard and was used for calibration of both isotypes. The level of SP215 specific IgG1 and IgG2a antibodies are related to this standard and expressed as relative titre (% of standard).

Peptide specific and native protein cross-reactive IgG antibody levels

In serum samples, peptide (SP215) specific and native protein (huIgG2) cross-reactive IgG antibody levels were determined by ELISA. Plates were coated with SP215 (10 µg/ml) or huIgG2 (10 µg/ml) and blocked with PBT (PBS containing 0.5% BSA and 0.05% Tween 20). Diluted serum samples were incubated and then plates were incubated with alkaline phosphatase (AP)-conjugated goat antibodies specific for mouse IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD)(4 µg/ml). PNPP (*p*-nitrophenyl phosphate) in 10 mmol diethanolamine and 1 mmol of $MgCl_2$ per litre,

was used as substrate. The absorbance was read after 30 min. at 405 nm against the signal from preimmune serum as a blank. A pool of sera from mice producing high levels of specific antibodies served as a standard and was used for calibration. The level of IgG antibodies is related to this standard and expressed as relative titre (% of standard).

Immunohistochemistry

Cryostat sections of spleen (-20°C, 8 µm), three of every mouse for each staining, were picked up on glass slides and kept overnight under high humidity. Slides were fixed in fresh acetone, containing 0.02% H_2O_2 and then air dried. Detection of activated T cells and cytokine producing cells was performed as described previously (Van den Eertwegh *et al.*, 1993). Slides were incubated overnight with monoclonal antibodies (mAb) to detect activated T cells and cytokine producing cells. The mAb used were: biotinylated hamster mAb MR-1 (specific for murine CD40L; expressed on activated T cells), the biotinylated rat mAb 11B11 (specific for murine IL-4; Ohara and Paul, 1985), the biotinylated murine mAb DB-1 (specific for rat IFN-γ and cross-reacting with murine IFN-γ; Van der Meide *et al.*, 1989). Incubation with biotinylated mAb was followed by incubation with avidin conjugated to horseradish peroxidase (HRP). Histochemical revelation of HRP (red) activity was demonstrated as previously described (Claassen and Adler, 1988). Sections were counterstained with haematoxylin, and embedded. Activated T cells and cytokine producing cells were counted per cryostat section and calculated per mm^2.

Results

Clinical findings

To evaluate induced pain or distress, condition of the animals was studied based on general appearance and clinical signs.

s.c. injection

After s.c. primary immunization no clinical changes were observed while, after s.c. secondary immunization, mice showed piloerection and a hunched back when given SP215 in FA, Montanide ISA50 or TiterMax. One day after s.c. injection, clinical changes were no longer seen. When examining s.c. injection sites during the experiment, we observed swelling measuring 4-8 mm at primary injection sites in mice given FCA or TiterMax emulsions. Palpation of nodules or swelling at the s.c. injection sites did not lead to signs of pain such as vocalizing or struggling. One mouse died after secondary injection of TiterMax/SP215.

i.p. injection

The first hours after primary immunization (i.p.), mice given FA- or TiterMax-emulsion showed piloerection and hunched back. These changes were also found after secondary immunization with FA-, TiterMax-, Specol- or Montanide ISA50-emulsion. During five days after i.p. secondary immunization with SP215 combined with FA, TiterMax, or Specol, piloerection was observed. Within a few hours after secondary immunization with FA/SP215 two mice died. These phenomena are probably related to the persistent high antibody titres in these animals. Palpation of the abdomen of i.p. injected mice did not lead to signs of pain.

Pathological findings at necropsy

To obtain more data on side effects of the evaluated adjuvants, pathological changes were studied at the end of the experiment (summarized in Fig. 1).

s.c. injection

Primary s.c. injection sites showed some variation within groups while secondary injection sites were almost identical within groups. At most primary s.c. injection sites of FA/SP215 and TiterMax/SP215, white or yellow nodules from 2-10 mm were observed. Microscopically, marked to severe granuloma formation, diffuse exudative inflammation and fibrosis were present at these injection sites, as well as (epi)dermal signs of inflammation. Some primary s.c. injection sites of Montanide ISA50/SP215 showed nodules (around 3 mm in diameter), which microscopically were less severe (mainly fibrosis) than forementioned groups. Secondary s.c. injection sites grossly showed white shiny tissue attached to muscles, the amount of which was most abundant in FA/SP215 and TiterMax/SP215 injected groups. Microscopically, impressive diffuse inflammation dominated in all groups, whereas cellularity was highest in TiterMax groups. When the adjuvants were combined with PBS (instead of antigen), lesions found were comparable (FA, RIBI), slightly milder (Specol, Montanide ISA50), or milder (TiterMax).

i.p. injection

In i.p. injected mice findings were very homogenous within groups. Milky abdominal effusions were present in FA and TiterMax treated animals. These two adjuvants and Specol, resulted in intestinal adhesions, while Specol and TiterMax caused dilatation of the small intestine. These signs of active peritonitis were judged severe. Marked omentum retrahens was present in the three forementioned groups. Intraperitoneal inoculation of Montanide ISA50 and RIBI resulted in slight and minimal white shiny plaques in the peritoneum respectively, accompanied by minimal omentum retrahens in the case of Montanide ISA50. Microscopical examination confirmed the severity of peritonitis and absence of unaffected omentum in FA, TiterMax and Specol injected groups. In Montanide ISA50 and RIBI injected animals normal parts of omentum were present but affected parts

microscopically showed obvious peritonitis (Montanide ISA50 moderate, RIBI slight). When the adjuvants were combined with PBS (instead of antigen), the peritoneal lesions were somewhat milder in Specol, TiterMax and RIBI groups and markedly milder in FA and Montanide ISA50 groups.

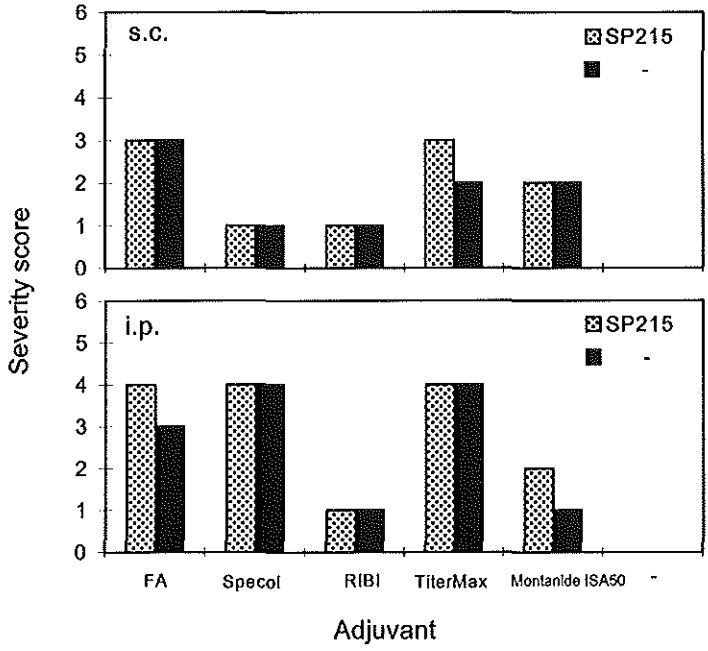

Figure 1 Severity score of pathological changes at necropsy 5 days after secondary injection (day 47) of different adjuvants combined with SP215 or PBS (-) via s.c. or i.p. route; n=5. Severity score: 0 = negative/minimal; 1 = slight; 2 = moderate; 3 = marked; 4 = severe.

Peptide specific IgG1 and IgG2a antibody production

To study antibody responses and isotype distribution of peptide specific antibodies after adjuvant/SP215 immunization, SP215 specific IgG1 and IgG2a antibody levels were determined in serum of day -3, 7, 14, 28, 41, 47. On day -3 peptide specific antibodies were not detectable while on day 7 and 14 antibody levels were low but detectable after i.p. or s.c. immunization with SP215 combined with FCA, Specol, TiterMax or Montanide ISA50 (data not shown).

After s.c. injection, SP215 specific IgG1 antibody levels were of comparable height when mice were given SP215 combined with FA, Montanide ISA50 or Specol and minimal when given with TiterMax or RIBI. SP215 specific IgG2a antibody levels were highest after FA/SP215 injection and slightly lower in serum of mice s.c. given SP215 combined with any of the other adjuvants used (data not shown).

After i.p. injection, SP215 specific IgG1 antibody levels were very high in serum of mice injected with Montanide ISA50/SP215, high when SP215 was i.p. injected combined with FA, TiterMax, somewhat lower with Specol, and minimal when injected with RIBI (Fig. 2). A clear booster effect in IgG1 response was observed when Montanide ISA50 was used as an adjuvant. SP215 specific IgG2a antibody levels were high after i.p. immunization with TiterMax/SP215. These SP215 specific IgG2a responses were at least six times higher than after immunization with any of the other adjuvants. When SP215 was injected without an adjuvant or adjuvant without SP215, no SP215 specific IgG1 and IgG2a antibodies were detectable.

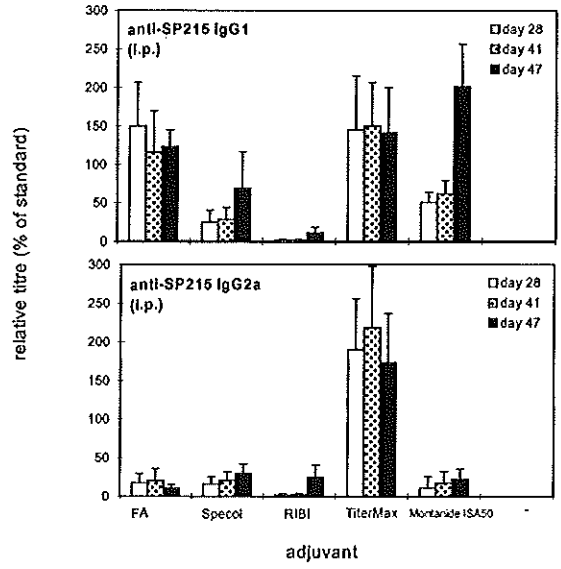

Figure 2 SP215 specific IgG1 and IgG2a antibody responses (day 28, 41 and 47) after i.p. injection of different adjuvants or PBS (-) combined with SP215. On day 42, mice were given a secondary immunization. Values are expressed as relative titre (% of standard) as detected in ELISA (arithmetic mean ± s.d.; n=5).

Peptide specific and native protein cross-reactive IgG antibody production

To investigate cross-reactivity of peptide specific antibodies with the native protein (huIgG2), SP215 and huIgG2 specific IgG responses were determined in serum from mice given SP215 combined with different adjuvants. After s.c. injection, SP215 specific IgG responses were comparable when mice were given SP215 combined with FA, Montanide or Specol, low when given with TiterMax and minimal with RIBI on day 47 (Fig. 3). HuIgG2 specific IgG responses were low after s.c. injection of SP215 combined with each adjuvant. After i.p. injection, SP215 specific IgG responses were high when mice were given SP215 combined with Montanide or TiterMax (Fig. 3).

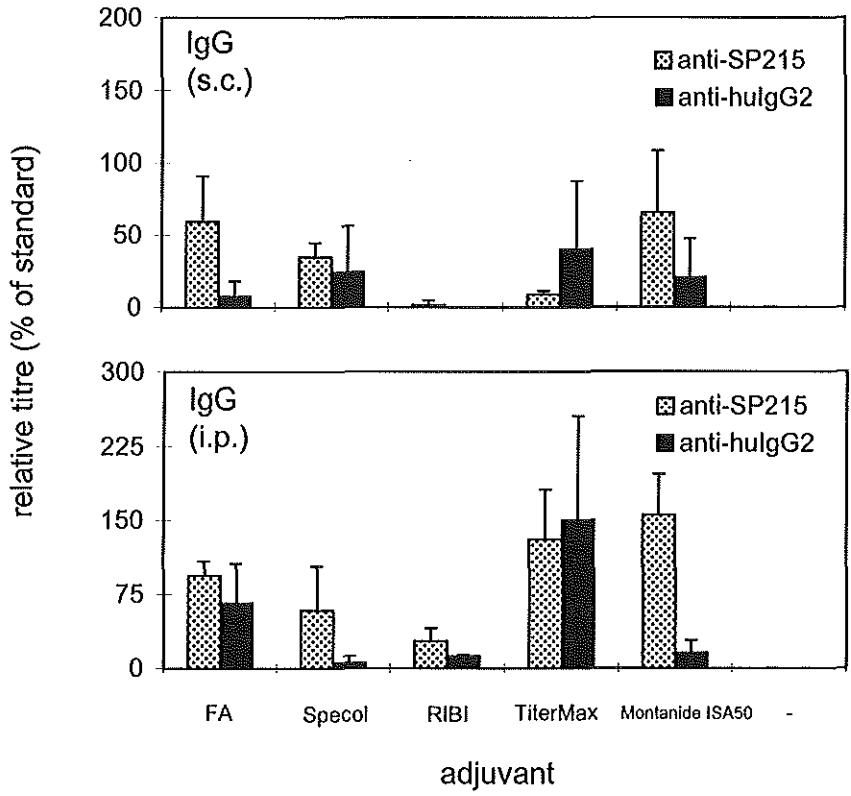

Figure 3 SP215 specific IgG and native protein (huIgG2) cross-reactive IgG antibody responses on day 47 after s.c. and i.p. injection of different adjuvants or PBS (-) combined with SP215. On day 42, mice were given a secondary injection. Values are expressed as relative titre (% of standard) as detected in ELISA (arithmetic mean ± SD; n=5).

These responses were twice as high as those observed after i.p. injection of SP215 combined with FA or Specol. SP215 specific IgG responses were low when mice were i.p. given SP215 combined with RIBI. HuIgG2 specific IgG responses were high (twice as high as after FA) after i.p. injection of SP215 combined with TiterMax, while low levels of huIgG2 specific IgG antibodies were observed after i.p. injection of SP215 combined with Specol, Montanide or RIBI. Injection of SP215 without an adjuvant resulted in undetectable SP215 or huIgG2 specific antibody production.

Activated T cells and cytokine producing cells in spleen sections of i.p. injected mice

To investigate whether isotypes distribution (IgG1 and IgG2a) could be correlated with cytokine production (IL-4 and IFN-gamma respectively) the number of IL-4 and IFN-gamma producing cells in spleen sections was assessed 5 days after secondary i.p. injection. T cell activation was studied by CD40L-expression (Th cells expressing the ligand for CD40). As shown in Fig. 4, relatively high frequencies of positive cells were observed in spleen sections of mice given RIBI combined with SP215 or with PBS. This was also observed when IL-2 and IL-5 producing cells were studied (data not shown). Immunization with FA (with or without antigen) resulted in low numbers of positive cells. These low numbers were comparable to those observed in spleen sections of mice given SP215 without an adjuvant. The number of positive cells was higher after Specol/SP215 injection than after Specol/PBS injection.

Discussion

In this study, efficacy and side effects of five commercially available oil adjuvants were compared in mice. We showed that Montanide ISA50 and Specol are suitable alternatives to FA preferably when given by the subcutaneous route.

FCA (mineral oil and Mycobacteria) was used as a 'gold' standard since this adjuvant is very effective in inducing a specific immune response to antigens. The effective immune-stimulating properties of FCA (Lipman *et al.*, 1992) as well as the induction of severe pathological changes by FCA (Toth *et al.*, 1989) were confirmed in our study. When Specol (mineral oil without Mycobacteria) was injected in combination with SP215, antibody levels were comparable to those observed after FA/SP215 injection. This confirms earlier findings (Leenaars *et al.*, 1995) in which Specol/SP215 was injected i.p. or s.c. in mice. Specol induced mainly antibodies of the IgG1 isotype. This confirms earlier findings (Boersma *et al.*, 1992). Anti-native protein antibodies were low after Specol/SP215 injection. This may be explained by the dose of antigen used. Boersma *et al.* (1989) described that the required dose of peptide is higher when an anti-native response is needed (200 µg) than is strictly required for an anti-peptide response only (50 µg).

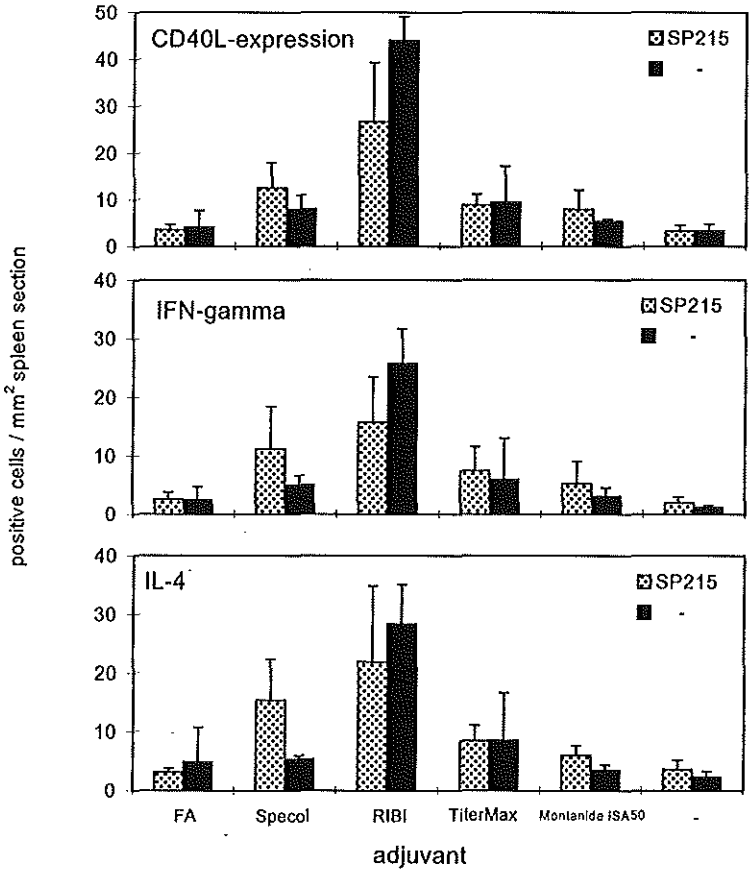

Figure 4 Number of activated T cell (CD40L expression) and cytokine producing cells (IFN-gamma and IL-4) in spleen sections, five days after secondary i.p. injection (day 47) of different adjuvants or PBS (-) combined with SP215 or PBS (-). Values represent mean ± SD of number of positive cells per mm^2; n=5.

After s.c. injection of Specol, slight pathological changes were observed while i.p. injection resulted in comparable pathological changes as those observed after FA injection. The data after i.p. injection are in contrast with our earlier findings (Leenaars *et al.*, 1995), when we observed minimal pathological changes also after i.p. injection of Specol/SP215. This may be due to the difference in the injected volume (0.1 ml earlier experiment; 0.2 ml in this study). Antibody responses after injection of Montanide ISA50 (mineral oil without Mycobacteria) combined with SP215 were comparable or even higher than when FA/SP215 was injected. These data confirm findings of Jones *et al.* (1990), who observed higher antibody

responses when Montanide ISA50 was i.p. injected in mice compared with FA injection. Montanide ISA50/SP215 injection resulted in antibodies of IgG1 isotype, predominantly. Less severe pathological changes were found after Montanide ISA50 injection as compared with FA injection. Our findings indicate that the balance between efficacy and safety of these Montanide ISA50 injections is better than of FA injections. After s.c. injection of TiterMax, antibody responses were much lower than after FA, while i.p. injection of TiterMax/SP215 was observed to be effective (higher antibody levels than with FA/SP215) in inducing, peptide specific IgG, IgG1, IgG2a antibodies and native protein cross-reactive antibodies. Comparable antibody responses after TiterMax and FA injection were also observed by Bennett *et al.* (1992) and Daly and Long (1996). We observed IgG2a antibody responses that were much higher after i.p. injection of TiterMax than after any of the other adjuvants. These high IgG2a levels are in accordance with findings that TiterMax induces cell-mediated immunity (Kast *et al.*, 1993). Zhou and Afshar (1995) observed comparable anti-isotype antibody levels when mice were s.c. given pseudo rabies virus in FA or in TiterMax, but did not observe anti-idiotype antibodies when TiterMax was used as compared with FA. Claims, that TiterMax is safe for use in laboratory animals, could not be confirmed in our study. Both after i.p. and s.c. injection, pathological changes were comparable after TiterMax and FA injection. Therefore, despite high antibody responses after i.p. injection, TiterMax is not considered the ultimate alternative to replace FA. After injection of mice with RIBI/SP215, antibody levels were very low and minimal pathological changes were observed. Intermediate and low antibody responses after RIBI injection were observed by Tiong *et al.* (1993) and Kenney *et al.* (1989), respectively. These and our data conflict with findings of Geerligs *et al.* (1989), Lipman *et al.* (1992) and Daly and Long (1996), who observed comparable antibody responses after injection of FCA and RIBI. This may be explained by the antigen to which an immune response is required. A relatively high level of IgG2a antibodies was observed after RIBI injection. This confirms earlier findings that MPL and TDM stimulate production of antibodies of IgG2a isotype (Rudbach *et al.*, 1995).

Despite low antibody responses after RIBI/SP215 injection, significantly higher numbers of activated T cells (= CD40L expressing cells) and cytokine producing cells were observed in spleen sections of mice 5 days after secondary i.p. injection of RIBI compared with the other adjuvants. Based on data of Van den Eertwegh *et al.* (1993), we studied spleen sections 5 days after secondary injection. Van den Eertwegh *et al.* (1993) showed a peak in CD40L expressing cells and KLH specific antibody forming cells 4-5 days after secondary i.v. injection of KLH, suggesting that CD40L expression plays a role in the specific antibody production. Grun and Maurer (1989) observed preferential stimulation of phenotypically different T helper cell subsets and found corresponding IgG1 and IgG2a antibodies in serum after injection of FCA and Alum in mice. Our findings, however, suggest, that antibody levels in

serum and the numbers of activated T cells and cytokine producing cells in spleen sections do not necessarily correlate 5 days after secondary immunization. Fox (1992) detected differences in antibody titres and isotypes following injection with different adjuvants but did not find differential affects of adjuvants on Th1 and Th2 cells. Valensi *et al.* (1994) observed that it was difficult to identify a consistent relationship between systemic cytokine responses measured and the antigen specific circulating antibody subclasses induced with different adjuvants. In our study, injection of adjuvant combined with or without antigen, resulted in comparable numbers of activated T cells and IFN-γ and IL-4 producing cells, indicating non-specific immune stimulation by the adjuvant. The induction of cytokines by the adjuvant may overrule the specific response to the antigen. We studied the immune-stimulating effects 5 days after secondary immunization. Maybe the antigen specific antibodies are generated at later time points after secondary immunization.

Antibody responses were comparable after injection via s.c. or i.p. route, except for Montanide ISA50 and TiterMax where higher antibody responses were found when these adjuvants were injected i.p. compared to s.c. Contradictory results were observed by Bennett *et al.* (1992) when injecting TiterMax in mice. Considering the severe pathological changes after i.p. injection, s.c. route should be preferred above i.p. route.

From the data of our study, it can be concluded that s.c. injection of Montanide ISA50 or Specol are alternatives to induce acceptable antibody titres to synthetic peptide with less pathological changes than FCA.

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Chapter 7

Model to test efficacy of immunization procedures by early cytokine production in mice

P.P.A.M. Leenaars, L. Nagelkerken, C.F.M. Hendriksen and E. Claassen.
In: *Animal Alternatives Welfare and Ethics*, L.F.M. van Zutphen and M. Balls (Eds.), Elsevier Science, Amsterdam, *in press*

Abstract

To investigate the possibilities for efficacy prediction of immunization procedures in laboratory animals, we studied cytokine production shortly after adjuvant/antigen injection in mice using immunohistochemistry and *in vitro* cell cultures. A weak immunogen, SP215 (peptide of human IgG2 molecule) was studied. Cytokine producing cells (IL-4, IL-5, IL-2 and IFN-γ) were counted in spleen sections 0, 2, 4, 6, 96 hours after intraperitoneal (i.p.) injection of Freund's complete adjuvant (FCA) combined with SP215. Numbers of cytokine producing cells were low and comparable to unimmunized mice at these time points. At 5 and 14 days after i.p. injection of FCA/SP215, spleen cells were stimulated *in vitro* with SP215. Minimal antigen specific proliferation and cytokine production (IL-4 and IFN-γ) were observed. At 14 days after subcutaneous (s.c.) injection of FCA/SP215, draining lymph node cells were stimulated *in vitro* with SP215. Significant levels of antigen specific IFN-γ production were observed. To study adjuvant effect on antigen specific cytokine production (IL-4 and IFN-γ) and isotype induction, mice were s.c. injected with FCA/SP215, FIA/SP215, Specol/SP215 or PBS/SP215. Two weeks later mice were bled and draining lymph node cells were cultured. Levels of antigen specific IFN-γ production were comparable for all three adjuvants while IL-4 production was below the detection limit of 5 pg/ml. SP215 specific IgG1 antibody levels were significantly higher in Specol/SP215 compared to FCA/SP215 injected animals. IgG2a antibody levels were comparable in all adjuvant injected animals. Based on our data we conclude that early cytokine production measured by immunohistochemistry or in lymphoid cell cultures is not a useful model to predict the outcome of immunization procedures.

Introduction

In immunization procedures, adjuvants are used to induce effective immune responses. In laboratory animals Freund's complete adjuvant (FCA) is often used for this purpose. The severity of the side effects induced by FCA, have resulted in guidelines discouraging its use in laboratory animals (Canadian Council on Animal Care, 1991; National Institute of Health, 1988; Veterinary Public Health Inspectorate, 1993). Alternative adjuvants are recommended. A useful tool to choose an adjuvant is a model to predict the outcome of immunization procedures in which a weak immunogen is combined with an adjuvant of unknown efficacy for the particular combination under study. Cytokines are regulating glycoproteins which play an important role in the initial phase of an immune response and thereby can give information on the efficacy of an immunization. Several studies (reviewed by Finkelman *et al.*, 1990) have shown that cytokines like interleukin-4 (IL-4) and interferon-gamma (IFN-γ) direct the quality of immune responses after infection or vaccination. IL-4 promotes an immune response dominated by antibodies of the IgG1 and IgE isotypes while IFN-γ promotes antibodies of the IgG2a isotype. The concentration of these two cytokines, IL-4 and IFN-γ, shortly after immunization can give information on what type of immune response will be induced. Cytokine production can be measured on different levels, e.g. cytokine producing cells in sections of lymphoid organs, protein level (e.g. in serum), mRNA level. To make IL-4 and IFN-γ production detectable, lymphoid cells may be stimulated *in vitro* and cytokine production measured in supernatants of cell cultures. We studied the possibilities to predict the immune response based on early production of cytokines (IL-4 and IFN-γ) using a weak immunogen (SP215, synthetic peptide).

Animals, materials and methods

Animals

Female BALB/c mice were bred specific pathogen free (SPF) at the RIVM breeding facilities, Bilthoven, the Netherlands and used at 10-14 weeks of age. Animals were housed under SPF conditions and had free access to pelleted food (Hope Farms, Woerden, the Netherlands) and tap water. The experiments were approved by the Animal Ethics Committee of the RIVM.

Antigen

Synthetic peptide (SP215, homologous to hinge region of human IgG2 molecule) was synthesized as described by Boersma *et al.* (1989). Per animal 50 µg SP215 was injected.

Adjuvants

FIA and FCA were obtained from Difco Laboratories (Detroit, MI). FIA is a non-metabolizable mineral oil. FCA is FIA with added heat-killed *Mycobacterium butyricum* (0.5 mg/ml). Specol (obtained from ID-DLO, Lelystad, the Netherlands) is a non-metabolizable mineral oil of which the constituents are approved for animal use by the USA Food and Drug Administration (described by Bokhout *et al.* 1981). When these oil adjuvants are mixed with dissolved antigen, water-in-oil emulsions are formed.

Experimental design

To study the number of cytokine (IL-4, IL-5, IL-2, IFN-γ) producing cells in spleen, twelve mice were injected intraperitoneally (i.p.) with 0.2 ml FCA/SP215. After 2, 4, 6 and 96 hours three mice were bled and spleens were removed and snap-frozen in liquid nitrogen for immunohistochemistry. From three unimmunized, mice spleens were also snap-frozen as control spleens. To analyse whether immunization of this weak immunogen (SP215) in FCA resulted in proper cytokine production, lymphoid cells were stimulated *in vitro* with SP215. Spleen cells were obtained from six mice 5 and 14 days after i.p. injection of 0.2 ml FCA/SP215. Alternatively to i.p. injection, subcutaneous (s.c.) injections were performed. Fourteen days after s.c. injection of 4x50 µl FCA/SP215, draining lymph node and spleen cells were cultured *in vitro* with SP215. To study the effect of adjuvant on cytokine production and IgG isotype of antibodies, mice, each group consisted of 6 mice, were injected s.c. (4x50 µl) with FCA/SP215, FIA/SP215, Specol/SP215 or PBS/SP215. After 14 days, three mice of each group were sacrificed to study cytokine production of lymph node- and spleen cells *in vitro*. The remaining three mice were used to take blood samples to follow the immune response in time.

Immunohistochemistry

Cryostat sections of spleen (-20°C, 8 µm) were fixed in fresh acetone, containing 0.02% H_2O_2 and then air dried. Slides were incubated overnight with monoclonal antibodies (mAb) to detect cytokine producing cells. These mAb were: DB-1 conjugated to alkaline phosphatase (AP) for interferon-gamma producing cells (IFN-γ-PC), S_4B_6 conjugated to AP for interleukin-2-PC (IL-2-PC), TRFK-5 conjugated to AP for IL-5-PC and biotinylated 11B11 followed by streptavidin conjugated to horseradish peroxidase (HRP) for IL-4-PC. Histochemical revelation of the AP and HRP activity was demonstrated as previously described (Claassen and Adler, 1988). Sections were counterstained with haematoxylin, and embedded. Cytokine producing cells were counted per cryostat section and calculated per mm^2.

Cell cultures

Single cell suspensions of spleens and lymph nodes were prepared in RPMI1640 (Gibco, Paisley, Scotland) supplemented with penicillin (100 IU/ml), streptomycin (100 mg/ml), L-glutamine (2 g/l), ß-mercapthoethanol ($5x10^{-5}$ M), and 5% Fetal Calf Serum (FCS; Seralab Ltd., Sussex, Crawley Down, UK). In spleen cell suspensions, erythrocytes were lysed by incubation with ice cold lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.4) for 1 min. Spleen cells and draining lymph node cells (collected 5 or 14 days after immunization) were restimulated *in vitro* with SP215 (25 µg/ml), SP215+IL-2, IL-2 or immobilized anti-CD3 mAb (145-2c11) coated in 96-well plates overnight at 4°C (10 µg/ml). After 3, 4 and 5 days culturing, supernatants were collected and stored at -20°C until cytokine assays (IL-4 and IFN-γ) were performed. To study proliferation, the remaining cultures were pulsed for 6 hours with 0.25 mCi [^{3}H]TdR per well (RCC, Amersham, UK) and harvested onto glassfibre filter paper using a Micromate 196 multiple sample harvester. Radio activity was determined by using a Matrix 96 ß-counter.

Cytokine assays

IL-4 and IFN-γ were assayed by ELISA as described previously (Abrams *et al.*, 1992). For IL-4, 11B11 mAb was used as capture and biotinylated BVD4 as detecting antibody. For IFN-γ, R46A2 mAb was used as a capture and biotinylated AN18 as detecting antibody. The detection limits for IL-4 and IFN-γ were 5 and 10 pg/ml respectively. Standard curves were constructed with recombinant cytokines calibrated against DNAX standards.

Determination of IgG1 and IgG2a

In serum samples two isotypes (IgG1 and IgG2a) were determined by ELISA. Plates were coated with SP215, blocked with PBS containing 0.05% Tween 20 and 0.5% BSA. After washing, diluted serum samples were incubated, then plates were washed and incubated with peroxidase-conjugated goat antibodies specific for mouse IgG1 (0.4 µg/ml) or IgG2a (0.4 µg/ml) (Nordic, Tilburg, the Netherlands). After washing, 100 mg/ml TMB (3,3',5,5'-tetramethylbenzidine) in 0.11 M CH_3COONa (pH 5.5) was added to the plates. The reaction was stopped after 10 min. by adding H_2SO_4 (2M). A pool of sera from mice producing high SP215 specific antibodies levels served as a standard and was used for calibration of both isotypes. The level of IgG1 and IgG2a antibodies are related to this standard and expressed as relative titre (% of standard). The relative titre should only be used for comparison within that particular isotype.

Statistical analysis

Results were subjected to statistical analysis by using the Mann-Whitney U-test.

Results

Cytokine producing cells in spleen sections

At 2, 4, 6 and 96 hours after i.p. injection of FCA/SP215, we observed low numbers of cytokine producing cells (IFN-γ, IL-2, IL-4 and IL-5) in spleen sections using immunohistochemical techniques. These low numbers were not higher than in unimmunized mice.

Proliferation and cytokine production by spleen cells in vitro, after i.p. priming with FCA/SP215

To analyse whether immunization of this weak immunogen (SP215) in FCA results in detectable cytokine production, we stimulated spleen cells *in vitro* with SP215 at 5 and 14 days after i.p. injection of FCA/SP215, and studied whether proper antigen specific proliferation or cytokine production could be found. Plate-bound anti-CD3 was used as a positive control. Whereas this mitogenic stimulation resulted in a proliferation of about 3000 counts per min. and in about 100 pg/ml IL-4 and 6000 pg/ml IFN-γ, no antigen specific proliferative response or cytokine production was detectable on day 5 after immunization. Two weeks after immunization, low levels of antigen specific IL-4 were detectable, provided that IL-2 was added to the cultures; IL-2 alone gave 10 pg/ml IL-4 whereas SP215 + IL-2 resulted in 20 pg/ml IL-4. Also for IFN-γ, exogenous IL-2 was required to show a modest antigen specific production. These data thus indicate that early antigen specific cytokine production is not easily detectable after i.p. immunization employing a weak immunogen.

In a separate experiment, we found that two weeks after s.c. immunization of this weak antigen in FCA resulted in significant levels of antigen specific IFN-γ production by draining lymph node cells provide that exogenous IL-2 was present. Therefore, additional experiments were performed to address the possibility that s.c. priming with adjuvant/SP215 is useful to discriminate different adjuvants with respect to cytokine induction.

Effect of adjuvants on cytokine production in vitro and IgG isotype of antibodies in serum after subcutaneous injection

To investigate whether FCA, FIA, Specol and PBS differ in their ability to induce antigen specific cytokine production in draining lymph node cells, these cells were cultured two weeks after s.c. immunization of SP215 combined with these adjuvants. Moreover, we determined the presence of SP215 specific IgG1 and IgG2a antibodies as a read-out of the activity of IL-4 producing Th2 cells or IFN-γ producing Th1 cells *in vivo*. In lymph node cell cultures, IFN-γ production was enhanced in an antigen specific fashion in the presence of exogenous IL-2 (Table 1). However, IFN-γ levels were comparable in all three adjuvant groups and low in the control group.

IL-4 production was below the detection limit of 5 pg/ml in lymph node cell cultures. In spleen cell cultures, no antigen specific stimulation of either IL-4 or IFN-γ production was found. None of the adjuvants enhanced proliferation of lymph node or spleen cell cultures in an antigen specific fashion.

Table 1. SP215 specific IFN-γ production of lymph node cell cultures and corresponding SP215 specific antibody production (IgG1 and IgG2a) in serum, 14 days after s.c. injection of SP215 combined with FCA, FIA, Specol or no adjuvant (-)

Adjuvant	*Animal ID*	*IFN-γ (pg/ml)*	*IgG2a (% of standard)*	*IgG1 (% of standard)*
FCA	1	40	1.2	2.0
FCA	2	2970	2.2	2.2
FCA	3	160	0.7	2.4
FIA	4	30	0.7	2.0
FIA	5	200	0.9	5.1
FIA	6	160	1.5	4.7
Specol	7	50	2.7	3.2
Specol	8	190	0.6	7.0
Specol	9	-	1.4	4.5
-	10	-	-	-
-	11	20	-	-
-	12	-	-	-

- = under detection limit

In serum of all adjuvant/SP215 injected mice, SP215 specific IgG1 and IgG2a antibodies were observed on day 14 (Fig. 1). When comparing different adjuvants in their ability to induce SP215 specific IgG1 or IgG2a antibodies, we observed significantly ($p<0.05$) higher SP215 specific IgG1 antibody levels in Specol/SP215 injected animals compared to FCA/SP215 injected animals (Fig. 1). IgG2a antibody levels were comparable for all three adjuvants. These data indicate that antigen specific antibody levels can give more information on the efficacy of an immunization procedure than antigen specific cytokine production (Table 1).

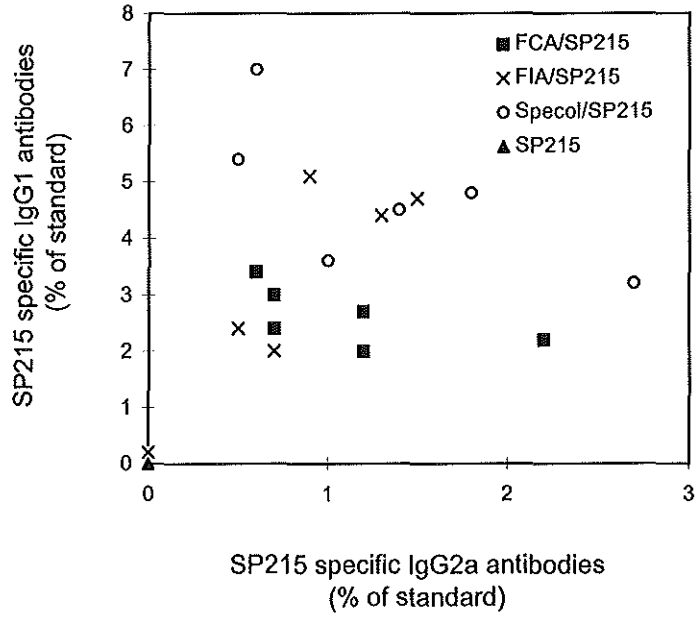

Figure 1 SP215 specific IgG1 and IgG2a antibodies levels in serum, 14 days after s.c. injection of SP215 combined with FCA, FIA, Specol or no adjuvant (PBS).

Discussion

In this report we investigate the possibilities to predict the efficacy of adjuvant/antigen injections in mice. Using immunohistochemistry and *in vitro* cell cultures we could not predict the outcome of an immunization procedure shortly after immunization when testing a weak immunogen (SP215, synthetic peptide). When a strong immunogen (TNP-KLH) was used, we observed increased numbers of IFN-γ producing cells and IL-5 producing cells in spleen sections four hours after i.p. injection of FCA/TNP-KLH (data not shown). This may be explained by the fact that TNP-KLH is a strong immunogen and has an adjuvant effect by itself (Van Ommen *et al.*, 1994) while the antigen under study is a weak immunogen. Using a strong immunomodulator (50 μg LPS), Jotwani *et al.* (1994) found endogenous cytokine levels in the bloodstream (e.g. 2500 pg/ml IFN-γ) of mice, 6 hours after i.p.

immunization. In our experiments, IL-4 and IFN-γ levels in serum were below the detection limits (5 and 10 pg/ml respectively) 4 hours, 5 days and 14 days after FCA/SP215 injection (data not shown).

Fourteen days after s.c. immunization of adjuvant/SP215, we did not observe IL-4 production in lymph node cell cultures while high IgG1 levels in serum were found. Indicating that in our test system, IL-4 production *in vitro* does not necessarily correlate with IgG1 production *in vivo* fourteen days after immunization. IL-4 induces the isotype switching from IgM to IgG1. A very small amount of IL-4 may be enough to induce this isotype switch.

In conclusion, since we wanted a model for the very early prediction (hours) of immune responses, getting data 14 days after immunization is late, while in our testsystem using a weak immunogen in BALB/c mice, IL-4 and IFN-γ production *in vitro* did not correlate with IgG1 and IgG2a production *in vivo* respectively. From the results it appeared that isotype distribution in serum on day 14 can give information on the outcome of the immune response and therefore we suggest to use this as an read-out instead of cytokine production *in vitro* 14 days after immunization.

Acknowledgements

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Chapter 8

Increased adjuvant efficacy in stimulation of antibody responses after macrophage elimination *in vivo*

P.P.A.M. Leenaars, H.F.J. Savelkoul, C.F.M. Hendriksen, N. van Rooijen and E. Claassen, *Immunology*, 1997, 90, 337-343.

Summary

To investigate whether macrophages influence the efficacy of adjuvants, we locally eliminated lymph node macrophages with dichloromethylene diphosphonate containing-liposomes before primary immunization. After macrophage elimination, animals were immunized with a soluble antigen (TNP-KLH; 2,4,6-trinitrophenyl-keyhole limpet haemocyanin) either in phosphate buffered saline (PBS), in Freund's complete adjuvant (FCA), or in Specol. Specol is a water-in-oil emulsion, considered to be less aggressive than FCA, but equally effective. A secondary immunization followed with TNP-KLH. *In vivo* macrophage elimination before Specol/TNP-KLH immunization resulted in increased adjuvant efficacy as measured by (primary) antibody responses. This suggests that the activity of Specol is not primarily mediated through macrophages but rather through other antigen-presenting cell types (e.g. dendritic cells, B cells, fibroblasts). The overall quality of produced antibodies, in terms of isotype distribution and affinity maturation, remained the same. After primary injection, FCA/TNP-KLH immunization resulted in significantly higher antibody responses in macrophage-depleted animals and antibody responses did not increase significantly after secondary immunization. However, a booster effect was found when macrophages were not eliminated before FCA/TNP-KLH immunization, suggesting that the presence of macrophages during the first weeks of the primary immune response is essential for the induction of an effective secondary response in FCA immunizations. In conclusion, macrophage depletion before immunization with a soluble T cell-dependent antigen combined with Specol may enhance specific antibody responses without changing isotype or affinity of the antibodies.

Introduction

Adjuvants are used to enhance immune responses to antigen resulting in higher titred specific antibody formation of desired isotypes (Karagouni and Hadjipetrou-Kourounakis, 1990; Kenney *et al.*, 1989). Besides effective immune responses Freund's complete adjuvant (FCA) induces severe side effects (Broderson, 1989; Toth *et al.*, 1989). In earlier studies we evaluated several adjuvants as possible alternatives to FCA. We found Specol (a well-defined and reproducible water-in-oil emulsion; Bokhout *et al.*, 1981) to be an animal-friendly efficient replacement for FCA (Leenaars *et al.*, 1994; Leenaars *et al.*, 1995). The mechanism of action of Specol is largely unknown. One way in which water-in-oil adjuvants are thought to enhance immunogenicity is through the activation of macrophages (MΦ) (Allison and Gregoriadis, 1990). The induction of effective immune responses involves different types of cells, antigen-presenting cells (APC), T and B cells. The type of antigen influences the way in which B cells are stimulated. When the antigen is T cell-independent, e.g. polysaccharides, B cells may be stimulated directly by interaction of the antigen with the B cells, in those cases an adjuvant is not always effective. However, when the antigen is T cell-dependent (TD), e.g. proteins, glycoproteins, APC are required for sufficient antigen processing and presentation to T cells for the induction of antibody responses (Van den Eertwegh *et al.*, 1992). Several cell types can function as APC. Particulate antigens are taken up almost exclusively by MΦ (Van Rooijen, 1992) while soluble antigen are processed and presented to the immune system by B cells and dendritic cells mainly (Ibrahim *et al.*, 1995). The antigen-presenting function of MΦ in generating an immune response has been studied *in vitro* (Lanzavecchia, 1990), however, *in vitro* studies do not necessarily reflect actual events *in vivo* (Van den Eertwegh *et al.*, 1992).

The role of MΦ *in vivo* can be studied by selective depletion of MΦ from tissues in the live animal using dichloromethylene-diphosphonate (Cl_2MDP) containing-liposomes (Van Rooijen and Sanders, 1994). These liposomes are phagocytosed by MΦ after injection and once digested release of Cl_2MDP kills the MΦ. Which MΦ subpopulations are depleted depends on the route by which Cl_2MDP-liposomes are injected. Injection of Cl_2MDP-liposomes in the footpad results in local depletion of MΦ in the popliteal lymph node while leaving other cell types like dendritic cells, B and T cells intact (Delemarre *et al.*, 1990).

In this report, we studied the role of macrophages in adjuvant efficacy by the induction of humoral immune responses to 2,4,6-trinitrophenyl-keyhole limpet haemocyanin (TNP-KLH), either given with phosphate buffered saline (PBS) or emulsified in adjuvant after local elimination of macrophage by Cl_2MDP-liposomes. The adjuvant properties of FCA and Specol were compared. In macrophage-depleted and control mice the efficacy of the adjuvants was judged by the level of antigen

specific antibodies elicited after primary and secondary immunization and the quality of the antibodies in terms of isotype-distribution and affinity maturation.

Materials and methods

Animals

Female BALB/c mice were bred specific pathogen free (SPF) at the RIVM breeding facilities, Bilthoven, the Netherlands and used at 10-14 weeks of age. Animals were housed in groups of five under SPF conditions and had free access to pelleted food and tap water. The experiments were approved by the Animal Experiments Committee of the RIVM and were performed as described in the Law on Animal Experiments.

Antigen

TNP-KLH was prepared as previously described (Claassen *et al.*, 1986b). The dose of antigen was 50 µg TNP-KLH per injection and was diluted in PBS.

Adjuvants

Freund's complete adjuvant (FCA; Difco Laboratories, Detroit, MI) is an oil emulsion consisting of 85% paraffin oil (= mineral oil), 15% mannide monooleate, and heat-killed *Mycobacterium butyricum* (0.5 mg/ml). Water-in-oil (w/o) emulsion of FCA/TNP-KLH, was prepared by emulsifying FCA with an equal volume of TNP-KLH in PBS using two syringes and a double hub connector. TNP-KLH was added to FCA via the connector and mixed for 1 min.

Specol (ID-DLO, Lelystad, the Netherlands) is an oil emulsion consisting of mineral oil (Marcol 52, paraffin's and cycloparaffin's) and emulsifiers: Sorbitan trioleate (Span 85^{R}) and Polyoxyethylene-20 Sorbitan trioleate (Tween 85^{R}) mixed in ratio of 9/1 (v/v) (Bokhout *et al.*, 1981). The constituents of Specol are approved for animal use by the USA Food and Drug Administration. A w/o emulsion of Specol/TNP-KLH, was prepared by mixing TNP-KLH and Specol in ratio of 9/11 (v/v). TNP-KLH was added dropwise to the Specol while mixing on the vortex.

MΦ elimination

Large multilamellar liposomes encapsulating the drug dichloromethylene-diphosphonate (Cl_2MDP-liposomes) were prepared as described earlier (Van Rooijen and Sanders, 1994). Cl_2MDP was a gift of Boehringer Mannheim GmbH, Mannheim, Germany. Cl_2MDP-liposomes were used to locally eliminate MΦ in the popliteal lymph node (PLN). Two days before immunization 50 µl PBS (control as described by van Rooijen and Sanders, 1994) or 50 µl Cl_2MDP-liposomes were injected subcutaneously

at the upper site of the right hind foot. MΦ elimination in the PLN was confirmed by acid phosphatase staining as described by Claassen *et al.* (1988).

Immunization of mice

On day 0 mice were immunized by injection of 50 µl FCA/TNP-KLH, 50 µl Specol/TNP-KLH or 50 µl TNP-KLH without an adjuvant subcutaneously at the upper site of the right hind foot. Physiologically there is no difference between footpad injection and injection at the upper site of the foot. Injection at the upper site is preferred since it is less painful because the animal is not walking on the injection site and minimal infections occur. A second injection followed on day 35 by injection (right hind foot) of TNP-KLH (50 µg), macrophages were not eliminated before secondary immunization and no adjuvants were used in any of the animals at this point in time. Blood samples were taken under ether anaesthesia by orbital puncture, on day -4, two times per week during the first 2 weeks after primary and secondary injection, and weekly thereafter. On day 75 animals were bled. Serum samples were stored at -20°C until required for analysis.

Detection of serum TNP-IgG antibody levels

TNP-specific IgG antibodies were determined in mice serum samples using a direct enzyme-linked immunosorbent assay (ELISA) as described earlier (Claassen *et al.*, 1987). In short, plates were coated with ovalbumin (OVA)-TNP (5 µg/ml), blocked with 0.2% gelatine in PBS, followed by diluted serum samples. Subsequent steps were goat anti-mouse IgG antibodies conjugated to alkaline phosphatase (2 µg/ml; Kirkegaard & Perry Laboratories, Gaithersburg, MD) and the substrate *p*-nitrophenylphosphate (PNPP; Sigma, St. Louis, MO) 1 mg/ml dissolved in DEA with $MgCl_2$. The reaction was stopped by adding NaOH after 30 min. The absorbance was measured at 405 nm with a microplate reader (Bio-Rad Laboratories, Richmond, CA). The amount of anti-TNP antibodies was determined by preparation of a standard curve with an anti-TNP monoclonal antibody (mAb) of mouse origin. From this curve, at 1 : 1600 dilution, the amount of anti-TNP antibodies was calculated and expressed as Arbitrary Units (AU).

Isotype-specific ELISA

TNP-specific IgG1, IgE, IgG2a and IgM were determined in mice serum by isotype-specific ELISA as described previously (Van Ommen *et al.*, 1994). The detection limits for the different isotypes were 0.2 ng/ml, 1 ng/ml, 0.3 ng/ml and 0.2 ng/ml, respectively. Purified TNP-specific mAb of the appropriate isotypes were used for the standard curves.

Determination of affinity distributions

The affinity distributions of serum IgG1 anti-TNP antibodies were determined in a TNP-specific isotype specific competitive ELISA (Pathak *et al.*, 1997) essentially as described by Rizzo *et al.* (1992). Briefly, serum samples were diluted and incubated overnight (4°C) with increasing concentrations of the monovalent hapten N-ε-DNP-L-lysine HCl (DNP-lys; 10^{-12} to 10^{-4} M in PBS; Sigma). The mixtures were then transferred to ELISA plates coated with TNP-KLH (0.5 µg/ml). After incubation for 3 h the plates were washed and the amount of TNP-specific antibody was established by using biotinylated goat anti-mouse IgG1 (0.5 µg/ml; Southern Biotechnology, Birmingham, AL), HRP conjugated streptavidin (Southern Biotechnology) and the substrate 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulphonic acid) (ABTS; A-1888; Sigma). In this type of ELISA, the high-affinity antibodies are inhibited from binding to the plates by low concentrations of free hapten. As the free hapten concentration increases by increments, lower affinity antibodies are inhibited until theoretically, at sufficiently high hapten concentrations, all TNP-specific antibodies are inhibited from binding. Sera with high affinity will have relatively more and/or higher bars in the right side of the diagram.

Immunohistochemical staining

The presence of MΦ and interdigitating cells in the draining PLN was studied 2 days after injection of 50 µl PBS or Cl_2MDP-liposomes, on the day of immunization. PLN were removed and frozen in liquid nitrogen. Cryostat sections of PLN (-20°C; 8 µm) were fixed in acetone containing 0.02% H_2O_2 for 10 min. at room temperature and then air dried. Acid phosphatase activity was demonstrated by incubation with naphthol-AS-BI-phosphate (N-2250; Sigma) for 30 min. at 37°C (Claassen *et al.*, 1988). Rat anti-mouse mAb, NLDC-145, which recognizes interdigitating cells in lymphoid organs of the mouse (Kraal *et al.*, 1986), was a kind gift of Prof. Dr. G. Kraal, Free University, Amsterdam, the Netherlands. After fixation cryostat sections were incubated with optimally titrated NLDC-145 mAb diluted in PBS containing 0.1% bovine serum albumin (BSA) overnight under high humidity at 4°C. After washing in PBS slides were incubated with a 1 : 150 dilution of rabbit anti-rat immunoglobulin conjugated to HRP (Dakopatts, Copenhagen, Denmark) in PBS containing 1% BSA and 1% normal mouse serum for 60 min. at room temperature. After washing in PBS, HRP activity (red colour) was demonstrated with the substrate, 3-amino-9-ethylcarbazole (AEC; A-5754; Sigma) as described by Claassen *et al.* (1988). Slides were rinsed with PBS, counterstained with haematoxylin and mounted in glycerin-gelatin.

Statistical evaluation

Antibody responses were analysed by the two-sided Student's *t*-test for comparison of two empirical means in a normally distributed population. A probability of 0.05 or less was considered significant.

Results

Elimination of MΦ in PLN

Delemarre *et al.* (1990) showed that a single footpad injection of Cl_2MDP-liposomes resulted in complete depletion of lymph node MΦ lining the subcapsular sinus and in the medulla a few days after injection. This depletion is of major importance for the validation of our study. We confirmed MΦ depletion by acid phosphatase staining on day 0, the moment of immunization. This is two days after Cl_2MDP-liposomes or PBS injection. Popliteal lymph nodes (PLN) of control animals (PBS treated) showed large numbers of acid phosphatase-positive cells (mainly MΦ) which were located lining the subcapsular sinus (subcapsular sinus MΦ), in the cortex (cortical MΦ) and in the medulla (medullary MΦ) (Fig. 1a). Subcapsular sinus MΦ and medullary MΦ were eliminated from the PLN two days after injection of Cl_2MDP-liposomes at the upper site of the hind foot (Fig. 1b). Macrophages in the cortex (main T cell area) or in the follicles (main B cell area) were not affected (red spots in Fig. 1b) as found before by Delemarre *et al.* (1990).

Demonstration of interdigitating cells

Interdigitating cells (IDC) are non-phagocytic cells which are not affected by Cl_2MDP-liposome injection (Delemarre *et al.*, 1990). Since IDC play a crucial role in antigen presentation, the presence of these cells was confirmed with IDC-specific mAb NLDC-145. Similar numbers of IDC were found in the para/deep cortex unit in the PLN of mice 2 days after PBS injection (Fig. 1c) and Cl_2MDP-liposome injection (Fig. 1d).

Increased TNP-specific IgG antibodies after MΦ elimination in TNP-KLH induced immune responses

TNP-KLH is immunogenic without an adjuvant, depending on the injected dose. In preliminary experiments, we determined a dose of TNP-KLH (50 µg) which was high enough to induce a detectable IgG response and low enough to be stimulated by an adjuvant (data not shown). To investigate the role of macrophages in antibody responses we injected mice with Cl_2MDP-liposomes to eliminate MΦ before primary injection of TNP-KLH. As described by van Rooijen and Sanders (1994) control mice were injected with PBS instead of Cl_2MDP-liposomes. TNP-specific IgG titres were

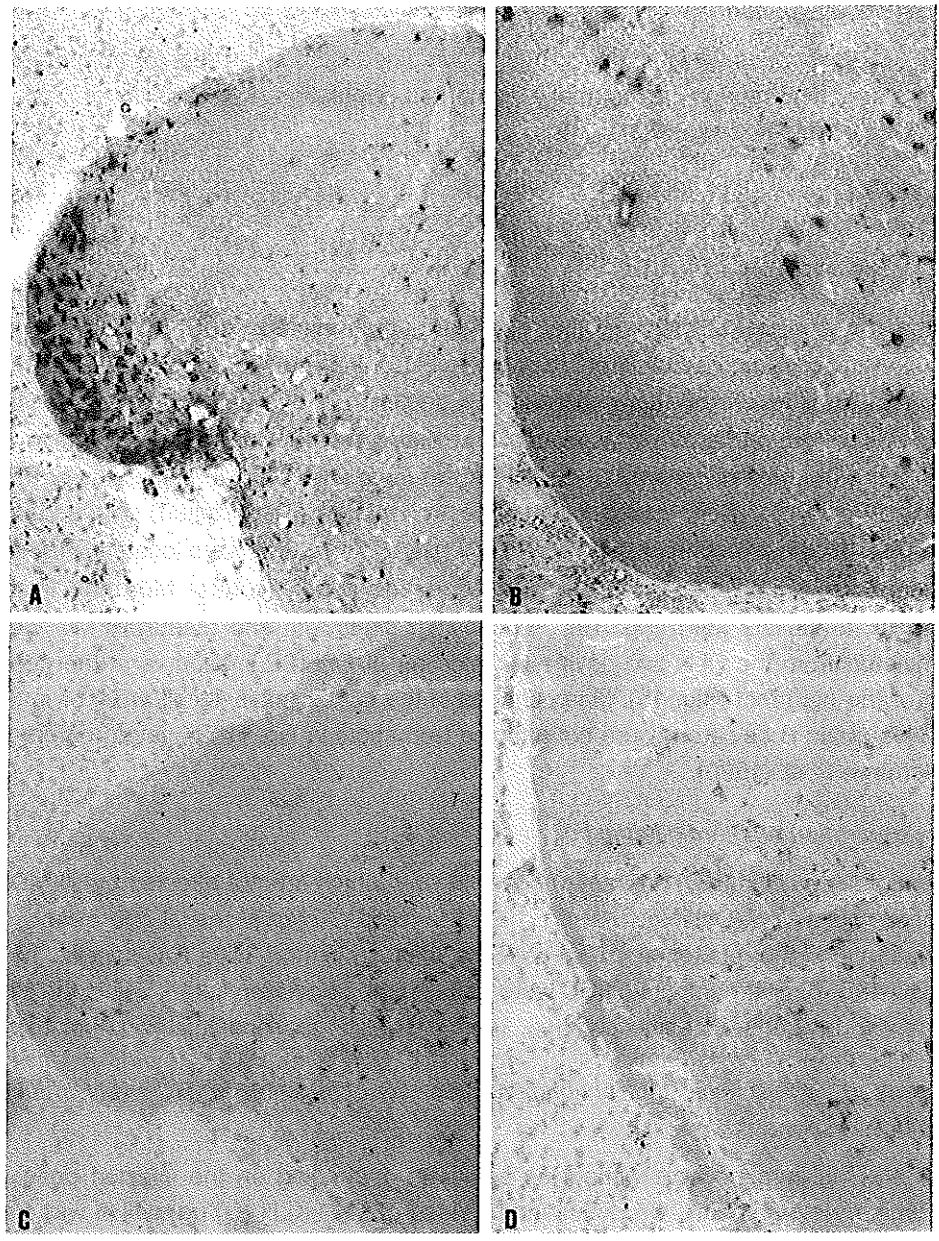

Figure 1 Localization of acid phosphatase positive cells (macrophages) in the PLN (a, b) and NLDC-145-positive cells (interdigitating cells) in the para/deep cortex unit of the PLN (c, d). Mice were injected with 50 µl PBS (a, c) or 50 µl Cl_2MDP-liposomes (b, d) and 2 days later PLN were collected. This is the moment animals in experimental groups were immunized. Note the absence of macrophages in the subcapsular sinus of the PLN of mice depleted of macrophages by injection of Cl_2MDP-liposomes (b) compared to PBS injected mice (a).

consistently higher when MΦ were eliminated before TNP-KLH injection compared to control animals (Fig. 2a). Note that the *y*-axis has a log scale.

Involvement of macrophages in FCA/TNP-KLH induced TNP-specific IgG responses

To investigate whether the presence of MΦ influences the efficacy of FCA, we locally eliminated macrophages by Cl_2MDP-liposome injection before primary FCA/TNP-KLH immunization. Control animals received PBS instead of Cl_2MDP-liposomes. Evaluating antibody responses after FCA/TNP-KLH immunization (Fig. 2b) we observed higher TNP-specific IgG antibody responses after primary immunization when MΦ were eliminated compared to control animals. Secondary immunization (TNP-KLH only, no adjuvants) of depleted animals resulted in a booster effect while antibody responses were significantly enhanced to 200 AU in control mice ($p < 0.05$), suggesting a role for macrophages in secondary immune response when FCA is used as an adjuvant.

To study the enhancing effect of FCA, we compared antibody responses in Fig. 2 (a) (TNP-KLH immunization without adjuvant) and Fig. 2 (b) (immunization with TNP-KLH emulsified in FCA). MΦ elimination before primary immunization resulted in similar (primary) anti-TNP IgG antibody responses after FCA/TNP-KLH injection and TNP-KLH injection without an adjuvant. Secondary immunization of these animals resulted in almost fourfold higher antibody responses after FCA/TNP-KLH immunization compared to TNP-KLH immunization without an adjuvant. PBS injection (control animals) before immunization with FCA/TNP-KLH resulted in (not significantly) higher primary and significantly higher ($p < 0.05$) secondary TNP-specific IgG antibody responses compared to TNP-KLH immunization without an adjuvant, showing an immune-stimulating effect of FCA as expected.

Involvement of macrophages in Specol/TNP-KLH induced TNP-specific IgG responses

To investigate whether the presence of MΦ influences efficacy of Specol (a less aggressive adjuvant than FCA) we locally eliminated macrophages by Cl_2MDP-liposome injection before Specol/TNP-KLH immunization. Control mice received PBS instead of Cl_2MDP-liposomes (Van Rooijen and Sanders, 1994). Comparing antibody responses between macrophage eliminated and control animals after Specol/TNP-KLH immunization (Fig. 2c) we observed consistently higher anti-TNP antibody responses in depleted animals. This suggested that MΦ do not play a major role in enhancing anti-TNP responses when Specol is used as an adjuvant but the presence of MΦ rather have a suppressive effect. In depleted animals primary immunization with Specol/TNP-KLH resulted in five times higher antibody responses than those observed after injection of TNP-KLH without an adjuvant or injection of FCA/TNP-KLH.

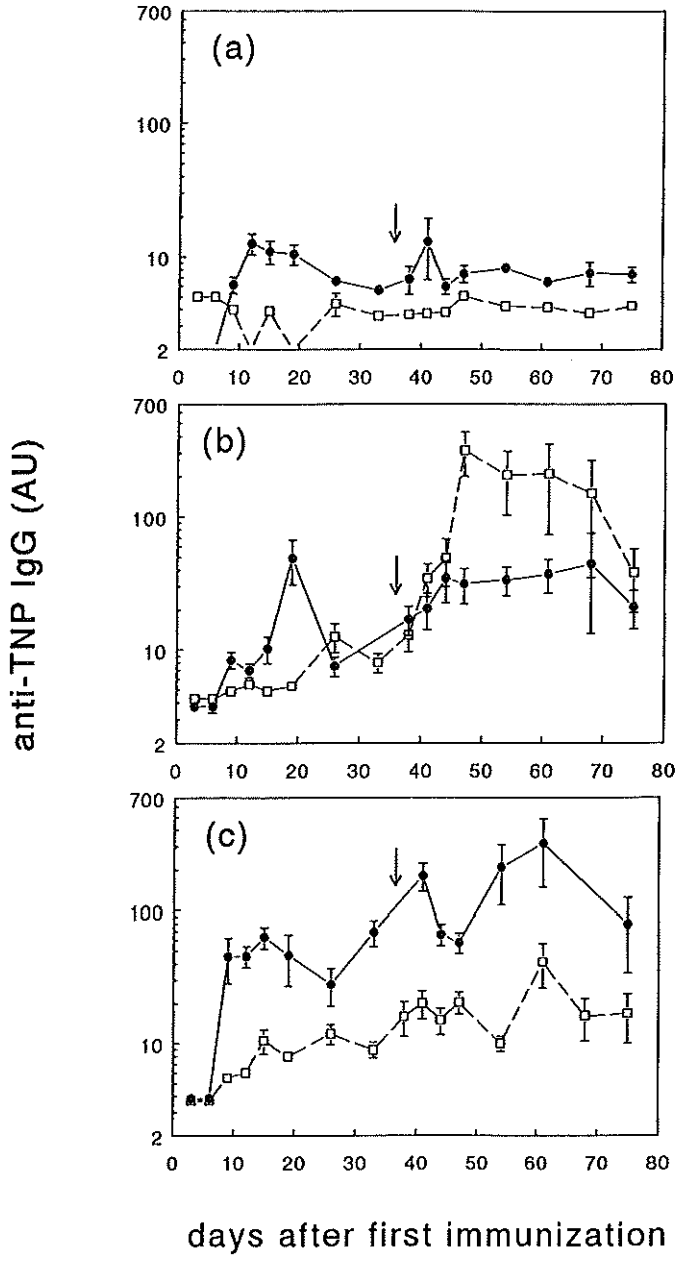

Figure 2 Total TNP-specific IgG antibody responses (Arbitrary Units; AU) in macrophage-depleted (—●—) and control animals (-- □ --) after immunization (day 0) with TNP-KLH without an adjuvant (a), FCA/TNP-KLH (b) or Specol/TNP-KLH (c). The arrows indicate the secondary immunization with PBS/TNP-KLH (day 35). Before immunization (day -2) control animals were injected with PBS and MΦ-depleted animals with Cl_2MDP-liposomes. Values are expressed as ^{10}log of the serum titre as detected in ELISA and AU are calculated as described in Materials and Methods (arithmetic mean ± SD; n = 5).

Effect of macrophage elimination before TNP-KLH immunization on isotype of TNP-specific antibodies

We showed that macrophage elimination before TNP-KLH immunization resulted in higher TNP-specific IgG antibody levels. We next studied the effect of macrophage elimination on isotype distribution. We determined TNP-specific antibody levels of the IgM, IgG1, IgG2a and IgE isotype in serum at selected time points. Antibody levels of these isotypes were identical in MΦ-depleted and control animals (data not shown).

Effect of macrophage elimination on affinity maturation of TNP-KLH induced TNP-specific IgG1 responses

To investigate whether the absence of macrophages influenced affinity maturation, we compared the relative affinity distribution of TNP-specific IgG1 antibodies of control and macrophage-depleted mice. Minimal differences in affinity distribution between MΦ-depleted and control groups in primary responses were observed (data not shown). On day 15 sera from both MΦ-depleted and control animals contained a major fraction (38%) of antibodies that were inhibited only at the high free hapten concentration of 10^{-4} M DNP-lys (low affinity). Affinity increased during the time after immunization: at day 26 of the secondary response the serum showed a smaller fraction of the lowest affinity antibodies (10^{-4}), along with a population of antibodies of higher affinity (10^{-5} to 10^{-7} M DNP-lys).

Effect of macrophage elimination on affinity maturation of adjuvant/TNP-KLH induced TNP-specific IgG1 responses

Macrophage elimination did not influence affinity maturation during immune responses induced by TNP-KLH immunization without the use of an adjuvant. We next studied whether macrophage elimination affected affinity maturation when the immune response was induced by TNP-KLH emulsified in adjuvant (FCA or Specol). Affinity of TNP-specific IgG1 antibodies at day 9 and day 26 after secondary immunization in macrophage-depleted and control groups are shown in Fig. 3 (FCA/TNP-KLH (Fig. 3a) and Specol/TNP-KLH (Fig. 3b)).

The histograms clearly show that affinity maturation occurred after secondary immunization in macrophage-depleted and control groups as well as in FCA/TNP-KLH and Specol/TNP-KLH groups. At day 9 of the secondary response serum contained a major fraction (around 35%) of antibodies that were inhibited mainly at the high free hapten concentration of 10^{-5} M DNP-lys (low affinity). Affinity maturation occurred thereafter as seen from the emergence of higher affinity subpopulations (more and higher bars at the right hand side of the graph; at 10^{-6} and 10^{-7} M DNP-lys) at day 26 of the secondary response. When affinity distributions of macrophage-depleted and control groups were compared, similar pictures emerged (Fig. 3). This was also the case when immunizations with the two adjuvants (FCA and Specol) are compared.

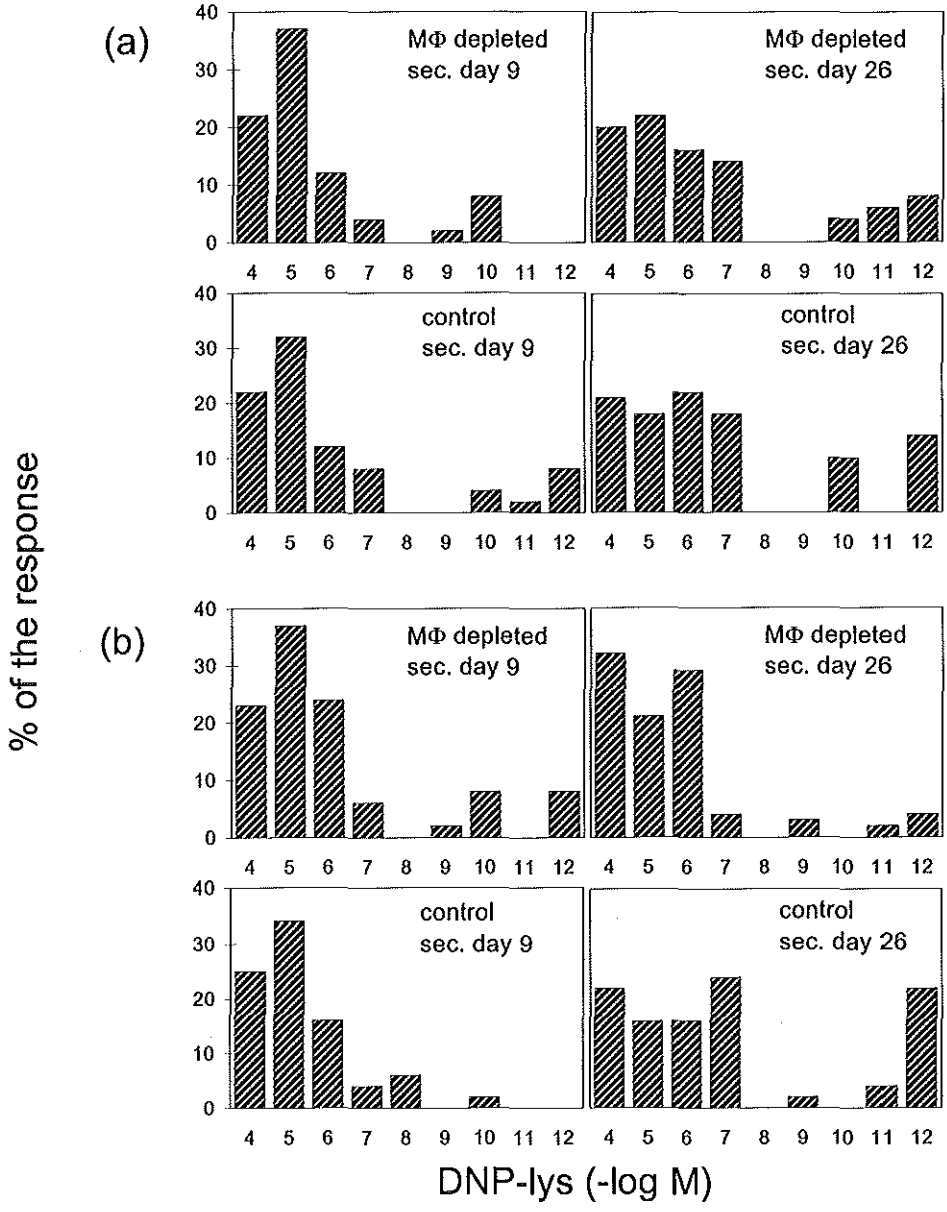

Figure 3 Relative affinity distributions of serum TNP-specific IgG1 antibodies in adjuvant/TNP-KLH immunized mice. Mice were injected with Cl_2MDP-liposomes (MΦ-depleted) or PBS (control) on day -2, immunized with FCA/TNP-KLH (a) or with Specol/TNP-KLH (b) on day 0, and secondarily immunized with 50 µg of TNP-KLH on day 35. Data are shown from sera collected on day 9 (sec. day 9) and day 26 (sec. day 26) after secondary immunization. Relative affinity was determined in a competitive inhibition ELISA. The abscissa indicates the concentration of free DNP-lys (-log M) used for inhibition. Affinity increases to the right, higher affinity is more and higher bars in the right side of the graph. The ordinate shows the percentage of antibody present in each affinity subgroup.

This shows that macrophage depletion does not change normal affinity maturation and that FCA and Specol induce similar affinity maturation patterns.

Discussion

This study shows that local macrophage depletion by administration of Cl_2MDP-liposome injection enhances TNP-specific IgG antibody responses induced by TNP-KLH immunization. Isotype distribution and affinity maturation are not affected by macrophage depletion.

We studied two adjuvants, FCA and Specol, which differ at major points. FCA is a, relatively variable animal unfriendly, water-in-mineral oil emulsion containing *Mycobacteria butyricum* (Stewart-Tull, 1995a). Specol is a standardized (constituents FDA approved) oil without any particulate matter. The bystander effect (help in T cell priming; Wright *et al.*, 1987) of the particulate matter in FCA (which is never used in the secondary immunization; cf FIA) is clearly dependent on macrophages as we here also show. Free antigen, administered subcutaneously, is phagocytosed by medullary MΦ (Fossum, 1980). *In vitro* studies demonstrated that preprocessing of particulate antigens by MΦ was crucial for the generation of immune responses (Van Rooijen, 1992; Wright *et al.*, 1987). MΦ have a specialized phagocytic and lysosomal function in processing particulate antigens while having a scavenging but not APC function for soluble antigens (Claassen *et al.*, 1991). By eliminating MΦ, immunization may be more effective because less antigen will be phagocytosed and therefore more antigen will come in contact with other APC such as for example interdigitating cells (IDC), which are extremely potent (Steinman, 1991). Using a specific mAb (NLDC-145) we confirmed the presence of IDC in the draining PLN after Cl_2MDP-liposome injection. MΦ elimination may result in more soluble TD antigen being available for IDC leading to a better induction of an immune response. Alternatively one might envisage that elimination of MΦ (and the subsequent release of mediators etc.) in itself results in adjuvanticity. However, in an adjuvant-free system Delemarre *et al.* (1991) did not observe higher primary antibody responses after MΦ depletion when immunizing animals in the footpad with 50 µg TNP-KLH, the number of antibody-forming cells (AFC) increased (but not significantly) and the peak of the AFC response was delayed, arguing against the latter view. The present results also show that the mechanism of action of oil emulsions as adjuvant is not exclusively dependent on macrophages in the draining lymph nodes.

Consequently, when an antibody response is required to (small amounts of) a soluble antigen, elimination of MΦ combined with Specol may be a way to induce an effective antibody response with minimal side effects.

Acknowledgements

We thank D. Kegler, P. van Schaaik, H. Strootman for technical assistance with the animals, H. Oostermeijer for ELISA testing and Dr. J. Laman for critically reading the manuscript.

Chapter 9

A rapid and simple method to localize oil based adjuvants *in vivo*

P.P.A.M. Leenaars, H.H.A. Oostermeijer, C.F.M. Hendriksen and E. Claassen, *J. Immunol. Methods, in press*

Abstract

In this study we show that water-in-oil and oil-in-water emulsions, often used as immunological adjuvants, can conveniently be labelled and detected with fluorescent carbocyanine dyes. The method is rapid and simple and analysis can be carried out with routine fluorescent microscopy equipment on frozen tissue sections. It enables kinetic studies of *in vivo* routing of hydrophobic adjuvants. After subcutaneous administration of labelled oil emulsion we observed the emulsion in draining lymph nodes. Within the nodes the emulsion was located in macrophages of the marginal sinus mainly. After intraperitoneal administration we observed the bulk of fluorescently labelled emulsion associated with the capsules of spleen and liver. Only 10-15% of the material was found in the marginal zone macrophages of the spleen (where antigens normally localize) and minimal amounts were seen in the phagocytic Kupffer cells of the liver. Double labelling of oil and antigen revealed that oil and aqueous phase (= antigen) do not separate but colocalize in the spleen. Intraperitoneal administration of emulsions, and subsequent capsule association, did not interfere with "normal" marginal zone localization of antigen (soluble or particulate) injected by the intravenous or intraperitoneal route thereafter. The results indicate a second depot, in addition to the depot at the site of injection, of antigen containing oil emulsions associated with the capsules of spleen and liver. This new method of oil and antigen localization provides a new tool to study tissue, cellular and subcellular distribution of oil emulsions in relation to their immune-stimulating effects and chemicophysical composition.

Introduction

Adjuvants are employed in immunological research and vaccines to enhance the immune response (Stewart-Tull, 1995b). Oil emulsions are efficient adjuvants and thus often used for this purpose, especially in laboratory animals. The mechanisms by which oil emulsions influence immunological responses remain obscure (Cox and Coulter, 1997). Suggested modes of action include: depot function at the site of injection, protection of antigen (Ag) from degradation, non specific stimulation of macrophages, cytokine release and vehicle (transport/routing) function (cf: Gupta and Siber, 1995; Cox and Coulter, 1997; Leenaars *et al.*, 1997; Allison and Byars, 1992).

In addition to this, injection site and composition of the adjuvant determines tissue and cellular localization of the antigen (Ag) in lymphoid organs, factors which in turn dictate the outcome of an immunization. Ag localization studies (in adjuvant free systems), using monoclonal antibodies or radioactive labelling methods (Van den Eertwegh *et al.*, 1992, Van Rooijen *et al.*, 1989) revealed specific localization patterns of different types of Ag, associated with different antibody levels and isotypes (cf. Laman and Claassen, 1996; Cox and Coulter, 1997). However, to our knowledge no simple method for the study of localization patterns of antigen containing emulsions is available.

Lipophilic carbocyanine dyes have been successfully used for membrane labelling of living neurones, lymphocytes, low density lipoproteins, viruses, bacteria, liposomes and iscoms (reviewed in: Claassen, 1992; 1996; Claassen and Van Iwaarden, 1997). This type of label is very stable (both in terms of fluorescence and membrane integration) and can not diffuse over any aqueous phase, which makes it extremely suitable for long term tracking studies *in vivo*. The hydrophobic character of oil makes it a potential candidate for labelling with carbocyanine dyes.

When studying oil emulsions used as adjuvant, two phases (oil and water) need to be labelled separately since oil phase may locate different from aqueous phase (usually containing the Ag). Localization of oil and Ag depends on route of injection. To stimulate draining lymph nodes, adjuvant/Ag is often injected subcutaneously. For a systemic stimulation, intravenous injection is the most suitable route, however, oil emulsions are not tolerated well when injected intravenously. A similar systemic stimulation can be achieved by intraperitoneal injection as shown for soluble TI-2 antigens (Van den Eertwegh *et al.*, 1992) or particulate liposomes (cf. Claassen, 1996). In daily practice, oil based adjuvants are preferentially given subcutaneously, intraperitoneally or by the intramuscular route (long term depot and lymph node stimulation).

From a recent study we found that in some cases macrophages play a suppressive and in other cases rather a stimulating role in the immune response induced by antigen or antigen containing oil emulsions. Furthermore, and more

importantly, it became clear that the efficient immune-stimulating effect of oil emulsions was only partly macrophage dependent (Leenaars *et al.*, 1997). To investigate which other mechanisms and macrophages are involved (or bypassed) in the uptake and processing of such emulsions a method for *in situ* localization of these compounds was deemed necessary.

In the present study oil emulsions were labelled with carbocyanine dye (oil phase) and amine active probe (aqueous phase = Ag/carrier), thereby allowing the study of their localization patterns in lymphoid organs *in situ*.

Materials and methods

Animals

Male BALB/c mice, aged 10-12 weeks, were obtained from Harlan, Zeist, the Netherlands and kept in polycarbonate cages, at 20-22°C, relative humidity of 50-80% and with a 12 h day/night cycle. Mice were given acidified water (pH 3) and fed pelleted mouse food (Hope Farms, Woerden, the Netherlands) *ad libitum*. Experiments were performed under the auspices of the Dutch Veterinary Inspection according to the law on Animal Experiments.

Chemicals and antigens

DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; D-282), DiO (3,3'-dioctadecyloxacarbocyanine perchlorate; D-275), DiA (4-)4-dihexadecylamino-styryl)-N-methylpyridinium iodide; D-3883) and AMCA (6-((7-amino-4-methyl-coumarin-3-acetyl)amino)hexanoic acid, succinimidyl ester; AMCA-X,SE; A-6118) were obtained from Molecular Probes, Leiden, the Netherlands. Bovine serum albumin (BSA; A-8022) was obtained from Sigma, St. Louis, MO. Ficoll-AECM83 was a kind gift from Van Oudenaren (Erasmus University Rotterdam, cf. Koch *et al.*, 1982; Van den Eertwegh *et al.*, 1992). All other reagents were analytical grade or better.*Adjuvants*

Freund's Incomplete Adjuvant (FIA; Difco Laboratories Detroit, MI) is an oil emulsion consisting of mineral oil (85%) and emulsifier (15%). FIA emulsion was prepared by emulsifying FIA with an equal volume of PBS using glass syringes and a double hub connector. Specol (ID-DLO, Lelystad, the Netherlands) is an oil emulsion consisting of a mineral oil (90%) and emulsifiers (10%) (Bokhout *et al.*, 1981; Boersma *et al.*, 1992). Specol emulsion was prepared by mixing PBS and Specol in ratio of 9/11 (v/v). PBS was added dropwise to Specol while mixing on the vortex. Multilamellar liposomes were prepared and labelled as described earlier and recently reviewed (for protocols see: Claassen and Van Iwaarden, 1997).

Fluorochrome labelling of emulsions and antigens

To investigate the effects caused by integration of the different carbocyanine dyes (DiI, DiA, and DiO) into the emulsion, we determined the dose dependency of emulsion stability and fluorescence intensity. Stock solutions of all three dyes were made in pure ethanol (2.5 mg/ml, alternatively DMSO can be used). For the fluorescence labelling of FIA different amounts of dye-stock (2.5 mg/ml) were added to FIA while vigorously mixing on the vortex. To check fluorescent labelling of oil phase in water-in-oil emulsions, FIA-dyes were mixed with PBS (in a 1 : 1 ratio) and studied with an Olympus (Tokyo) Vanox fluorescence microscope. DiI fluorescence was observed as red with green light (excitation filter BP545), DiO fluorescence as green with blue light (using FITC optics, excitation filter BP490 + EY455) and DiA fluorescence as red with green light (excitation filter BP545). Fluorescence intensity and the stability of the emulsion were studied 30 min. after addition of dye labels. The stability of the emulsion was studied by determining the (unwanted) formation of micelles.

Besides localization of oil phase of emulsion, we studied localization of the aqueous phase (containing the Ag) of the emulsion by labelling carriers with the water-soluble fluorescence AMCA. Stock solution of AMCA was made in N,N-dimethylformamide (DMF; BDH Laboratory supplies, Poole, England, UK) (10 mg/ml). AMCA was conjugated to BSA and Ficoll as described in manufacturer's protocol 'conjugation with amine reactive probes' (MP 0143 01/26/96) of Molecular Probes, Leiden. In short, the carrier (BSA or Ficoll) was dissolved in 0.1 M bicarbonate (10 mg/ml). While vortexing the carrier, AMCA stock was added. This product was incubated for 1 h with continuous stirring. The reaction was stopped by adding freshly prepared hydroxylamine in a final concentration of 0.15 M. This product was incubated for 1 h. Hydroxylamine and unreacted AMCA were separated from the conjugate product on a gel filtration column equilibrated with PBS. The first fluorescent band to elute is the carrier/AMCA-conjugate used in our experiments. This product was stored in the freezer (-20°C). To check AMCA fluorescence in DMF and carrier/AMCA conjugates, we mixed them with FIA and observed blue label in the aqueous phase with UV light (excitation filter UG1). Double labelled water-in-oil emulsions (aqueous phase, AMCA labelled BSA and oil phase, FIA labelled with DiI) were checked for fluorescence after mixing 1:1 on a vortex. We observed a red layer (oil phase) with green light and blue droplets (aqueous phase) with UV-light.

Liposomes were labelled with addition of carbocyanine dye DiI (1-50 µg/ml, 2.5 mg lipid) and liposomes were separated from unentrapped dye with flotation centrifugation after the addition of an equal volume of lymphoprep as previously described (cf. Claassen and Van Iwaarden, 1997).

Experimental design

To study localization of oil emulsion in draining lymph nodes, mice were injected subcutaneously in the upper site of the hind foot with 50 µl DiI labelled FIA mixed with PBS (FIA-DiI/PBS; ratio 1 : 1), killed after 6 h and draining popliteal lymph nodes were collected. To study localization of oil emulsion in murine spleens, mice were injected intraperitoneally (i.p.) with 0.1 ml FIA-DiI/PBS and killed after 6 h. To study localization of oil phase as well as aqueous phase, mice were injected intraperitoneally with 0.1 ml double labelled water-in-oil emulsion (FIA-DiI/BSA-AMCA) and killed 3 h later. To study possible interference of oil localization in capsule on Ag clearance in the spleen, mice were injected with 0.2 ml FIA-DiI/PBS and after 3 h labelled antigen (either fluorescently labelled ficoll or liposomes) was given by the intravenous (i.v.) or i.p. route. Mice were killed 3 or 24 h later. Spleens and liver were removed from i.p. injected mice and immediately frozen in liquid nitrogen and stored at -70°C till further use.

Fluorescence microscopy of tissues

Cryostat sections of 8 µm were cut of frozen tissues (spleen, lymph node and liver) at -20°C. Sections were examined and photographed immediately after cryo-sectioning with an Olympus (Tokyo) Vanox fluorescence microscope. DiI fluorescence was observed as red with green light (excitation filter BP 545) and AMCA fluorescence as blue with UV optics (excitation filter UG1).

Results

Fluorescent labelling of oil emulsions and liposomes

To investigate whether labelled oil can be used efficiently in water-in-oil and oil-in-water emulsions, we prepared emulsions by mixing the labelled oil with PBS and studied these emulsions using fluorescence microscopy. Using green light, red background of labelled oil (FIA) and black droplets of unlabelled aqueous phase can be observed in DiI labelled water-in-oil emulsion (Fig. 1A). This is the other way around in oil-in-water emulsion (Fig. 1B), red droplets are DiI labelled oil phase and black background is unlabelled aqueous phase. To evaluate fluorescence intensity of DiI labelled emulsions (Fig. 1A and 1B), we compared them with DiI labelled liposomes (Fig. 1C; cf Claassen, 1992). The fluorescence intensity of DiI labelled oil emulsions is clearly less intense than that observed with DiI labelled liposomes, but still more than enough for localization studies *in vivo*.

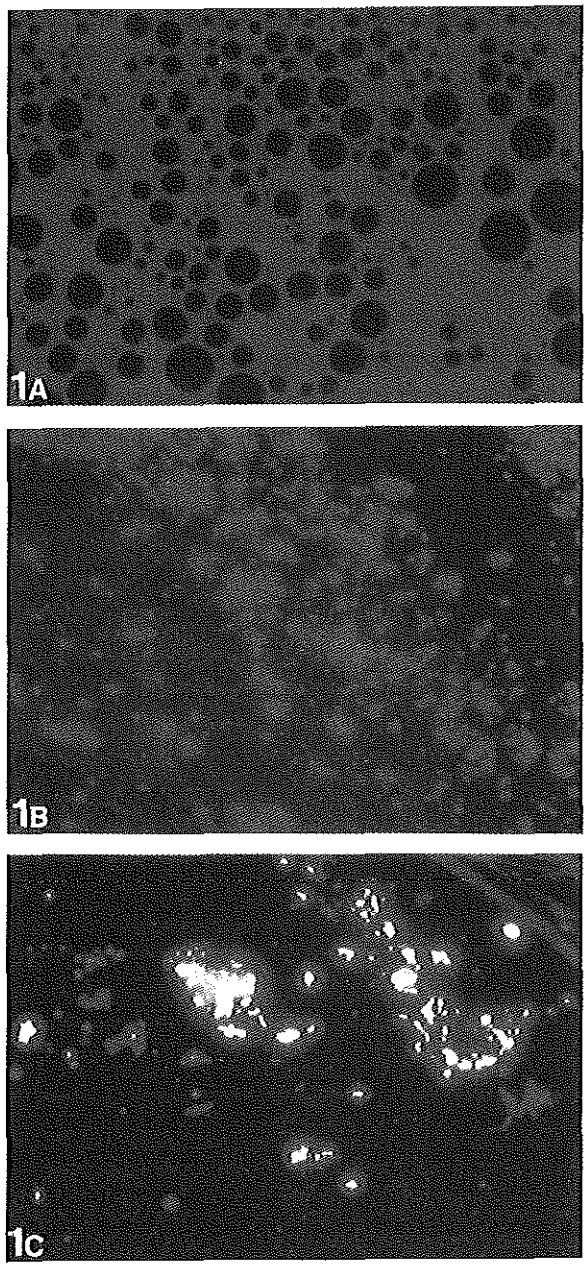

Figure 1 Fluorescent labelling of adjuvant products. DII fluorescence is red in: (A) oil phase (Freund's incomplete adjuvant) of water-in-oil emulsion, (B) oil phase (Specol) in oil-in-water emulsion, and (C) liposomal membrane.

Stability of fluorescence intensity and micelle formation

In view of differential effects of different carbocyanine compounds on stability of biomembranes (including lymphocytes) observed before (Claassen, 1992) we compared different dyes. For fluorescent labelling of oil emulsions we used DiI, DiO and DiA in concentrations ranging from 0-156 µg/ml. We studied the suitability of these dyes to label oil by determining: (i) the stability of the fluorescence intensity and (ii) amount of micelle formation. Fig. 2 shows these two parameters as a function of concentration of carbocyanine dye used for labelling oil (FIA).

When DiI is used for labelling (Fig. 2a) fluorescence intensity is strong at a concentration of 12 µg fluorochrome per ml oil, while no micelle formation revealed at any concentration used during the time of the experiment. When DiA was used for labelling (Fig. 2b) the fluorescence intensity was acceptable at 25 µg/ml and micelles were formed at 31 µg DiA per ml oil. When DiO was used for labelling (Fig. 2c), fluorescence intensity was acceptable at a concentration of 46 µg/ml but at this concentration of DiO, some micelle formation was already observed. Consequently, DiI is preferred for labelling oil emulsions (12 µg DiI per ml oil) and used in this study for localization experiments *in vivo*.

3.3 Localization of adjuvant in spleen after systemic administration

We studied the localization of oil phase in spleen and liver of mice after intraperitoneal injection of 0.1 ml FIA-DiI mixed with PBS. Fig. 3A shows 2/3 of a total spleen section, 6 h after FIA-DiI/PBS administration. Almost all fluorescent material is observed at the periphery of the spleen associated with the splenic capsule and not taken up by any particular cell. A minor fraction of the fluorescent label is found within the body of the spleen, the minimal label that is found here is associated with the marginal zone (note semi-circular patterns). After i.p. administration of DiI labelled emulsions, label was also observed in the capsule of the liver and only minimal amounts were seen in the Kupffer cells.

To compare this localization pattern of oil emulsions with other adjuvant products, we labelled liposomes with DiI and administered these intravenously or intraperitoneally and collected spleen and liver. Almost all labelled liposomes were seen in the body of the spleen, i.e. in the marginal zone macrophages (M) and on follicular cells (F) of the spleen (Fig 3B). Relatively few liposomes are taken up by the red pulp macrophages (R) and none by T-cells in the PALS (P). No liposomal material was associated with the capsule. After i.v. or i.p. administration of DiI labelled liposomes, label was observed mainly in Kupffer cells of the liver. Macrophages were identified by acid phosphatase staining as described before (Van Rooijen *et al.*, 1989).

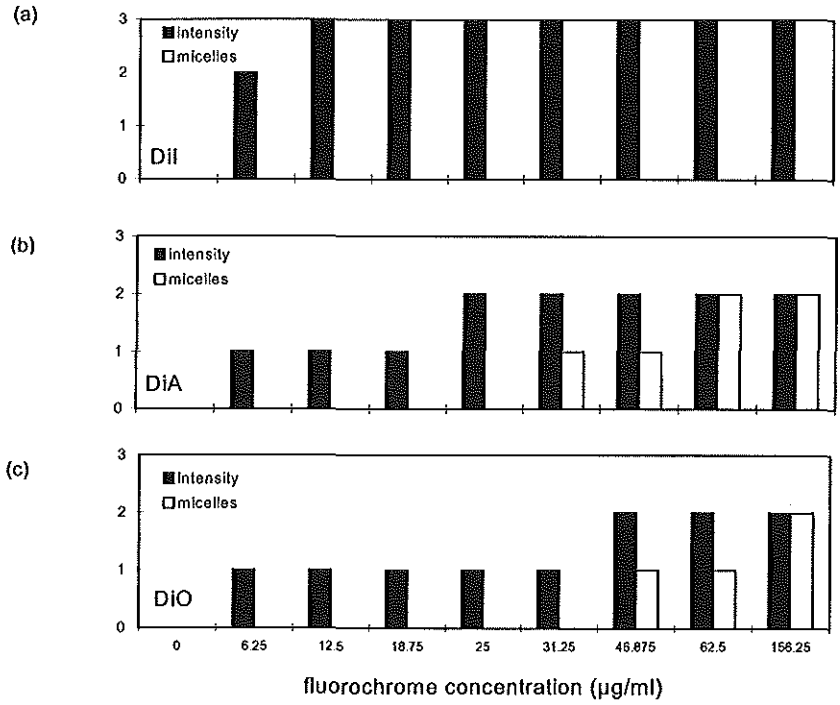

Figure 2 Fluorescence intensity (intensity) and emulsion stability (micelles) as function of concentration of carbocyanine dye used for labelling oil emulsion. Oil phase of water-in-oil emulsion was labelled utilizing DiI (a), DiO (b) or DiA (c). Fluorescence intensity expressed as 0 = no fluorescence observed; 1 = minimal fluorescence; 2 = acceptable fluorescence; 3 = strong fluorescence. Emulsion stability expressed as the amount of micelle formation; 0 = no micelle formation; 1 = micelles are formed; 2 = abundant micelle formation.

Double-fluorescent labelling studies

To study localization of oil phase as well as Ag phase, mice were injected intraperitoneally with 0.1 ml double labelled water-in-oil emulsion (FIA-DiI/BSA-AMCA). We observed minimal separation of Ag and oil phase in de spleen 6 h after administration. The major fraction of fluorescent material is observed in/under the splenic capsule, while minimal fluorescent label is found in the spleen. The minimal label that is found here is associated with the marginal zone (M) and oil and aqueous phase are not separated (Fig. 4A). To investigate whether this newly observed and typical localization pattern of oil phase of emulsions associated with the capsule of the spleen interferes with 'normal' clearance of the Ag by marginal zone macrophages of the spleen, mice were injected intraperitoneally with 0.1 ml FIA-DiI/PBS before 0.1 ml fluorescently labelled Ag (Ficoll-AMCA) was injected.

Fig. 4B shows localization of Ficoll-AMCA in a marginal zone macrophage of the spleen (AMCA fluorescence is blue (arrow). It was clearly shown that the normal localization of Ficoll-AMCA in the marginal zone was not changed by previous administration of the adjuvant.

In addition to the soluble Ag (Ficoll) we also used a particulate Ag (liposomes) in a pulse chase manner to demonstrate the effect of an earlier administration of a water in oil adjuvant on the capacity of splenic macrophages to take up the Ag. As Figure 6 shows the water in oil emulsion does not interfere with the normal follicular localization and uptake by splenic macrophages of liposomes (irrespective of the route of administration).

Localization of DiI labelled oil in popliteal lymph node after sc injection

To check localization of the oil emulsion in primary draining lymph node after sc injection, we administered DiI-FIA/PBS s.c. in the upper site of the hind foot and studied the popliteal lymph node (PLN). A tangential section through the marginal sinus shows that fluorescent label is found in the marginal sinus macrophages 6 h after administration (Fig. 5A), creating a web like pattern of marginal sinus macrophages which have taken up oil in varying amounts.

Efficacy of trapping of labelled oil by marginal sinus macrophages is clearly shown when observing a cross section through the PLN (Fig. 5B). No label was found in the follicles (F) while minimal label was found in the interfollicular T-cell areas. Increasing amounts of label can be observed in deep cortex and medulla, the major part of the fluorescent material stays in the marginal sinus. After s.c. administration of oil emulsion, DiI label eventually also localized associated with capsule of spleen and liver as found for the i.p route (data not shown).

Discussion

This study shows that carbocyanine dyes can efficiently be used to label oil, water-in-oil and oil-in-water emulsions to enable kinetic studies on *in vivo* routing of oil adjuvants, hereby adding a new tool to the immunotechnological arsenal.

A major new finding is that oil emulsions localize preferentially in association with the capsules of spleen and liver after intraperitoneal administration. It is not clear which cells, if any, are associated with this peculiar way of localization or how the oil emulsion exactly arrives at this site. In double labelling studies, in which we labelled both oil and Ag (in aqueous phase), we observed the majority of oil and Ag associated with the capsule and only minimal amounts in the marginal zone and follicles, indicating that no phase separation occurred. It seems that this small amount of Ag is effective in inducing an immune response. This is in agreement with earlier functional studies (Leenaars *et al.*, 1995) where we established much higher antibody responses to peptides after administration of peptides emulsified in oil than peptides mixed with PBS.

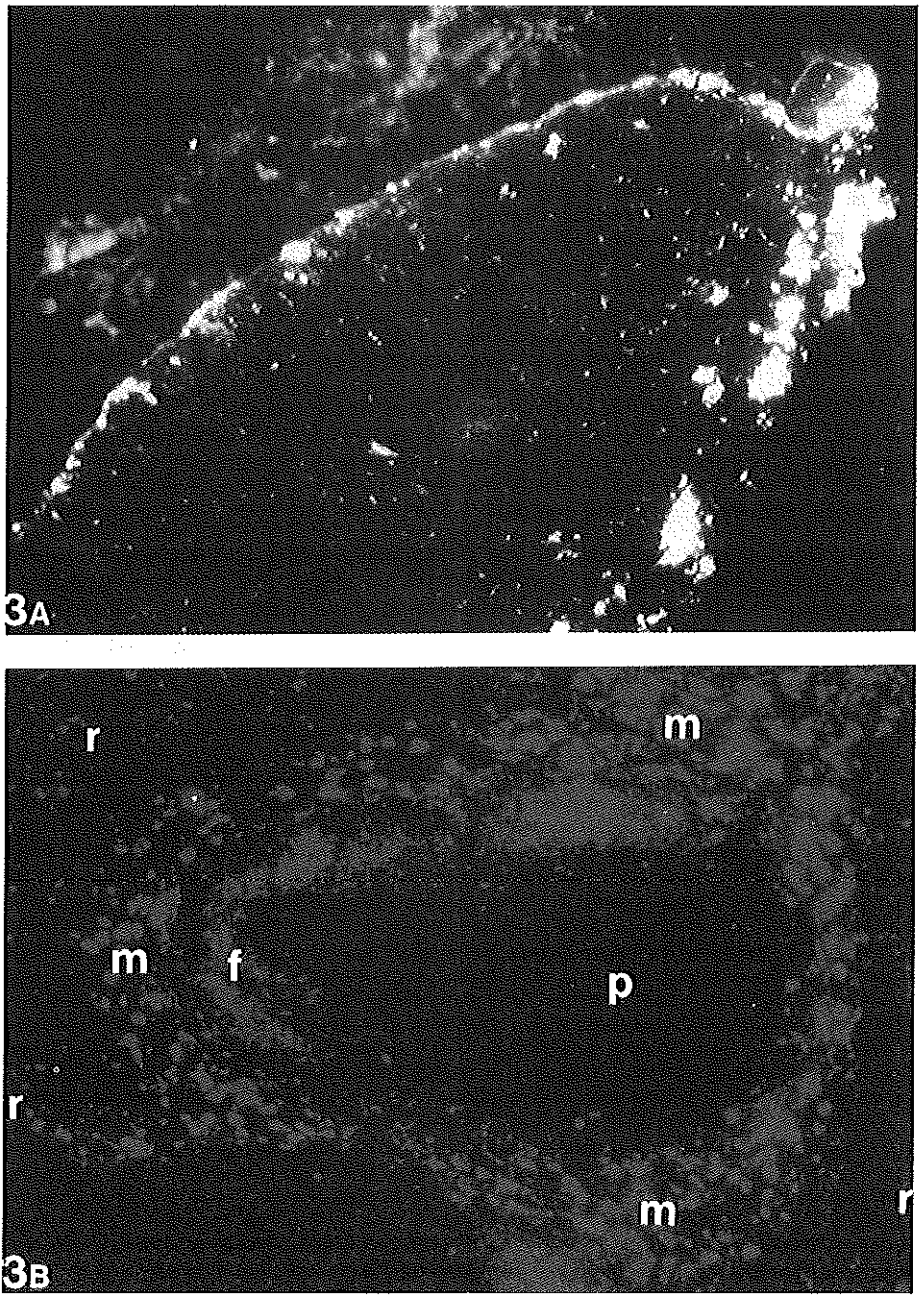

Figure 3 Fluorescence microscope image of an 8 µm spleen cryo-section. (A) 6 h after i.p. administration of FIA-DiI/PBS. (B) 24 h after i.v. administration of DiI-liposomes. DiI fluorescence is found: (A) mainly directly under splenic capsule not associated with cells after FIA-DiI/PBS and (B) in a ring like structure in the marginal zone macrophages (M) and on follicular cells (F) after DiI-liposomes. Magnification: (A): 2/3 of spleen section; x20, (B): 1/10 of spleen section; x100.

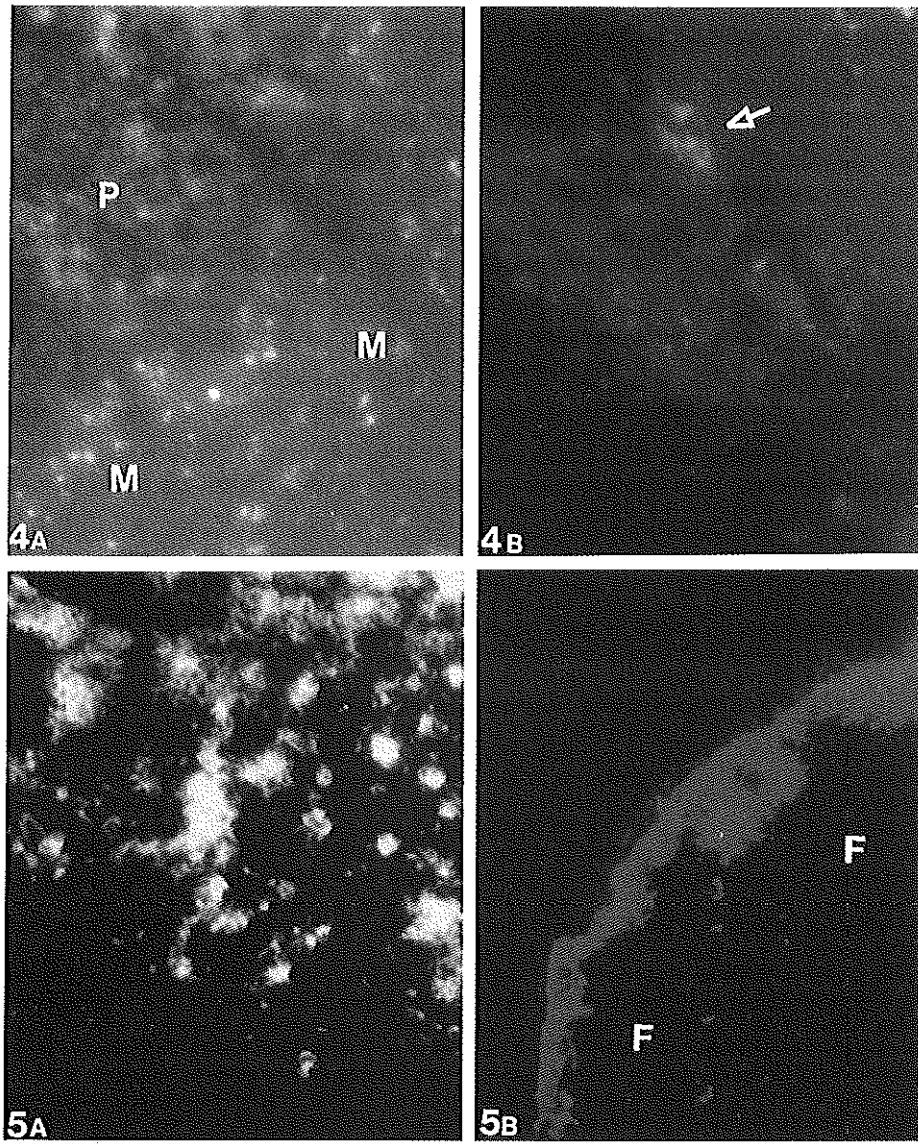

Figure 4 No separation of oil and Ag -> Fluorescence microscopic image of an 8 µm spleen cryo-section. (A) 3 h after i.p. administration of FIA-DiI mixed with BSA-AMCA. P = pals; M = marginal zone. DiI fluorescence is yellow; AMCA fluorescence is blue, note mostly double staining. (B) 24 h after i.p. administration of FIA-DiI/PBS and 21 h after i.v. administration of Ficoll-AMCA. AMCA fluorescence is blue (arrow). Magnification: 4A = x400; B = x800.

Figure 5 Fluorescence microscopic image of an 8 µm popliteal lymph node cryo-section, 6 h after s.c. administration in upper site of hind foot of FIA-DiI/PBS. (A) Tangential section, showing web like structure through macrophages in marginal sinus; DiI fluorescence is yellow with blue light. (B) cross section, showing efficiency of trapping labelled oil in marginal sinus macrophages; F = follicles; DiI fluorescence is red with green light. Magnification: A = x400; B = x200.

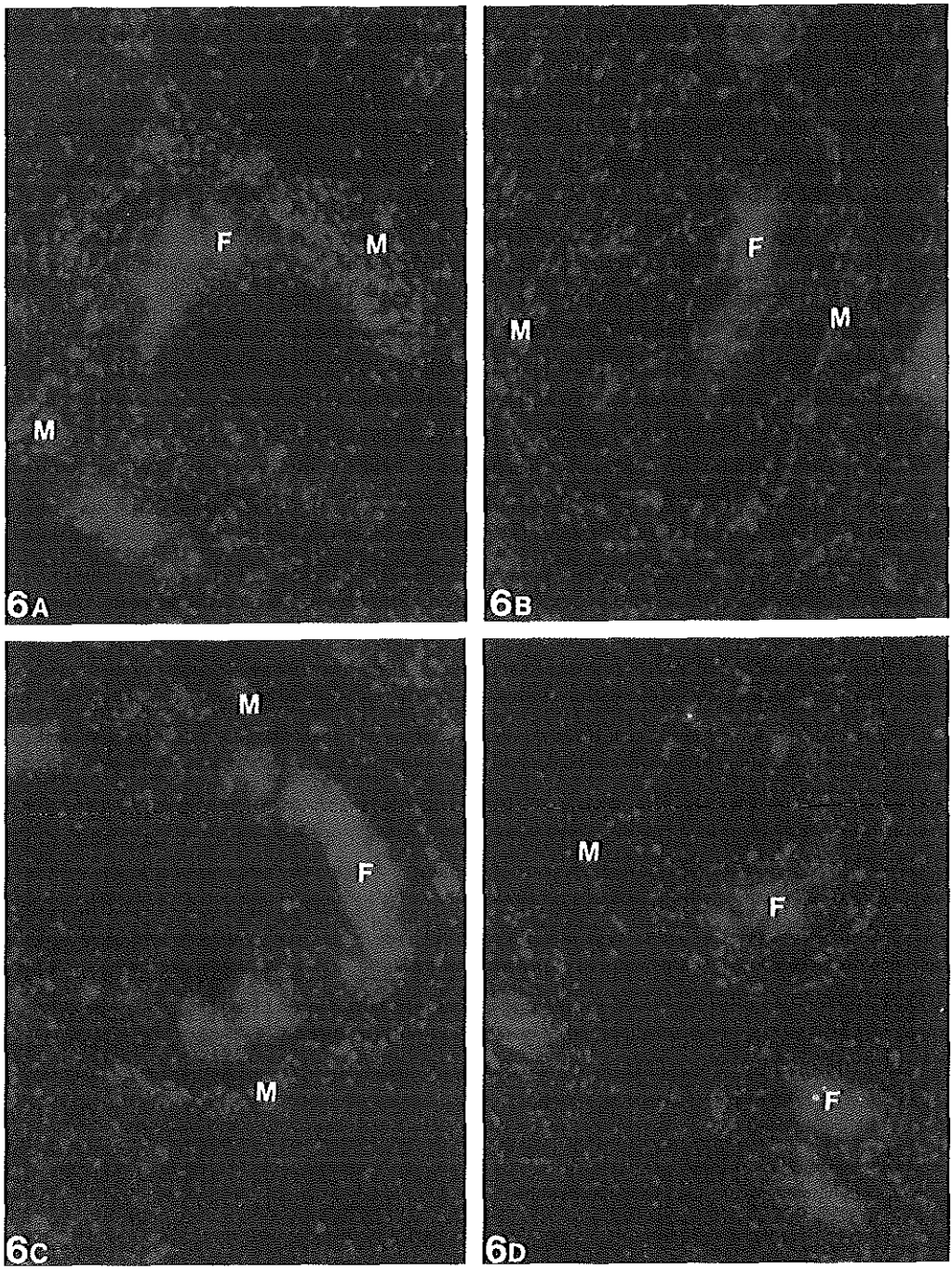

Figure 6 Animals as under 3B. Sections showing normal liposomal localization patterns at 21 h after liposomes (i.v. in A and B; i.p. in C and D) and 24 h after sham (PBS) or Freund's incomplete adjuvant treatment via the i.p. route.

Localization of oil associated with the capsule after administration of oil emulsions may function as a second depot, besides the depot at the site of administration, and herewith stimulate splenic antigen-presenting cells by continuous release of Ag. These results can very well explain the efficacy of oil emulsions in inducing immune responses, i.e. by keeping the bulk of the Ag away from the suppressive marginal zone macrophages (Leenaars *et al.*, 1997) and releasing Ag over time for uptake by follicular cells of the spleen or interdigitating cells in the interfollicular T-cell areas of the lymph node. The fact that the efficacy of bacteria containing oil emulsions as adjuvants was only partly macrophage dependent could also be explained by this macrophage independent mechanism.

Preparation of emulsions can be done in many different ways with as many different results. Important aspects of emulsions are stability, reproducibility, droplet size and charge. The localization pattern can possibly change when the emulsion is prepared differently, since physicochemical characteristics may change, in a similar fashion as described for liposomes (Claassen, 1996). The method described here can be employed for every combination and the results found, apply to the particular adjuvant used in this study. Nevertheless, the advantages of carbocyanins are evident and similar as we found before for the labelling of lymphocytes, bacteria, viruses, iscoms or liposomes (reviewed in Claassen, 1996). Furthermore, some emulsions can be labelled after they are formed, as shown with liposomes (Claassen, 1992), enabling retrospective localization studies with only those emulsions that need further study. Advantages of the use of DiI are stability (both of integration and of fluorescence), ease of use, minimal interference with physicochemical properties, no modification of proteins (as for e.g. FITC and TRITC), low price and the use of normal laboratory equipment. From the present results it has become clear that this new method enables the study of oil containing (or otherwise hydrophobic) adjuvant emulsions. Use of this method for other adjuvants (also those commercially available, for a complete listing and details see: Vogel and Powell, 1995) should elucidate the relation between efficacy and tissue/cellular localization and aid in educated design of hydrophobic and emulsion type adjuvants. Finally, in the design of adjuvants which are both efficient and have low intrinsic toxicity (i.e. side effects; Claassen and Boersma, 1992; Gupta *et al.*, 1993), the histopathological studies relating emulsions with local tissue reactions, will also be facilitated by this method.

Chapter 10

General discussion

General discussion

The main goal of the studies described in this thesis was to generate data on FCA and alternative adjuvants to analyse and expand recommendations on the use of adjuvants in laboratory animals and herewith reduce pain and distress in immunized animals. Additional goals of the studies were to increase insight into the correlation between cytokine profiles and antibody production and the mode of action of some selected adjuvants.

Immunizations of laboratory animals are widely performed in biomedical research, for instance, to prepare specific polyclonal antibodies, produce specific B cells for developing monoclonal antibodies, evaluate potential antigens for vaccine development or conduct fundamental immunological research. In immunization experiments, adjuvants (Latin: *adjuvare* = to help) may be used to induce an adequate immune response to an antigen. Adjuvants can influence the magnitude and the quality of the induced immune response (e.g. isotype of antibodies or cytokine profiles of T cells). In laboratory animals, Freund's complete adjuvant (FCA) is often used for this purpose because it induces effective immune responses to a broad range of antigens. However, besides satisfactory immune responses, severe side effects can be induced by FCA. These side effects resulted in restrictions on the use of FCA in order to reduce pain and distress in immunized laboratory animals. In the Netherlands, these restrictions are formulated in the Code of Practice for the immunization of laboratory animals (Veterinary Public Health Inspectorate, 1993), recommending restricted use of FCA. To adequately select an alternative to FCA, it is necessary to know efficacy and side effects of alternative adjuvants. However, comparative information on immune-potentiating properties and side effects of alternative adjuvants is often lacking. This thesis focusses on the use of adjuvants for the generation of polyclonal antibodies. To obtain data on efficacy and side effects of FCA and alternative adjuvants, we performed comparative studies in rabbits and mice and studied antibody responses and adverse effects induced by FCA and alternative adjuvants. Consequently, emphasis was also put on those references in literature where adjuvants were actually compared (APPENDIX A and B), as opposed to a mere description of their effects. In addition, cytokine production after adjuvant injection and the role of macrophages in adjuvant efficacy were studied.

In this general discussion, first antibody responses and side effects induced by five commercially available, potentially interesting adjuvants (FCA, Specol, RIBI, TiterMax, Montanide ISA50) are discussed, followed by other factors such as type of antigen, route of injection, animal species used, influencing the efficacy and side effects. Next, the predictive value of cytokine profiles for eventual antibody responses and the mode of action of water-in-oil emulsions are discussed. Thereafter, the degree of pain and distress induced by adjuvants is assessed and

alternative methods to produce antibodies are described. Finally, recommendations on the use of adjuvants for immunization procedures are given.

Antibody responses and side effects induced by adjuvants

To select a proper alternative to FCA, adjuvant induced antibody responses and side effects have to be evaluated in one and the same experiment. In several evaluation studies, FCA and alternative adjuvants were compared for their properties to induce antibody responses and clinical, behavioural and/or pathological changes in rabbits and mice. To mimic normal laboratory problems in evoking immune responses, the antigens used were mainly weak immunogens (e.g. synthetic peptides, autoantigen, glycolipid) as opposed to 'model antigens' such as carrier-hapten conjugates which are frequently used in comparative studies (e.g. Bennett *et al.*, 1992). Antigens were always given at a relatively low dose so as to visualize maximal adjuvant effects. FCA is universally applicable (Gupta *et al.*, 1993) and therefore used as 'gold standard' i.e. alternative adjuvants are compared with FCA on their antibody responses and side effects. Since minimal clinical and behavioural changes were observed after adjuvant/antigen injection, side effects will concern mainly pathological changes after injection. The meaning of pathological changes in the induction of pain and distress will be discussed later in this chapter in section 'Assessment of pain and distress induced by adjuvant'. The results of our comparative studies are discussed below per adjuvant. Emphasis is put on FCA, Specol, RIBI, TiterMax and Montanide ISA50 because these products are commercially available and easy to use.

Freund's complete adjuvant

FCA used in this thesis consists of 85% mineral oil (Bayol F), 15% Arlacel , and 0.5 mg/ml heat-killed *Mycobacterium butyricum* (Difco Laboratories, Detroit, MI; Freund *et al.*, 1937; Freund, 1951; Stewart-Tull, 1995a). Commercially available FCA may differ in the added amount and type of Mycobacteria; e.g. Difco Laboratories also has FCA containing 1 mg/ml heat-killed *Mycobacterium tuberculosis* H-37. The comparison of *M. butyricum* versus *M. tuberculosis* was not part of this study. Secondary injection was performed using FIA. FA (FCA in primary; FIA in secondary injection) emulsified with aqueous antigen solution (1 : 1) results in water-in-oil (w/o) emulsions.

antibody responses

The overall applicability of FA to induce polyclonal antibodies to a wide range of antigens was confirmed in all our studies. Antibody responses after FA/antigen injection were also comparable or higher than any other tested adjuvant. In mice, exceptions were the induction of antibodies to autoantigen. Antibody responses were (4 times) higher after s.c. injection of autoantigen (MBP) coupled to

RV-ISCOMs, than MBP emulsified in FA. The longer period between primary and secondary immunization with RV-ISCOMs/MBP may explain these higher levels than FA. After i.p. injection of TiterMax/synthetic peptide, peptide specific IgG antibodies levels recognizing the native protein were higher than those observed after FA/synthetic peptide injection.

side effects

In rabbits, no clinical or behavioural changes, indicative for pain and distress, were observed. In mice weight-loss, piloerection and hunched posture were observed 1 to 3 days after primary injection, which might be an indication of distress. These changes were most extensive in FA injected mice when compared with injection of other adjuvants. The pathological changes observed after administration of FA in rabbits and mice were at least moderate and often severe. Generally, gross and histopathological lesions after FA/antigen injection had highest severity grade compared with other adjuvants injected with the same antigen. An exception was injection of RIBI (s.c. or i.m.) in rabbits which resulted in more severe lesions with all three antigens tested, as compared with FA lesions. The observed pathological changes after FA injection (e.g. granulomatous lesions with exudative nature; intestinal adhesions) confirm data of other authors who studied lesions after injection of FCA (Broderson, 1989; Toth *et al.*, 1989; Deeb *et al.*, 1992; Smith *et al.*, 1992).

conclusion

FA is a very effective adjuvant in inducing antibody responses in rabbits and mice. Generally, the induced pathological lesions are severe, but clinical and behavioural changes were minimal in rabbits and only observed the first 1-3 days after FA injection in mice.

Specol

Specol (ID-DLO, Lelystad, the Netherlands; Bokhout *et al.*, 1981; Boersma *et al.*, 1992; Vogel and Powell, 1995) consists of a mineral oil (Marcol 52) and two emulsifiers (Span 85 and Tween 85). Preparing Specol and aqueous antigen, in a 5 : 4 ratio, results in a water-in-oil emulsion.

antibody responses

Immunization of rabbits or mice with Specol/antigen resulted in specific antibody responses comparable to those induced by Montanide ISA50/antigen and FA/antigen. This confirms data of Boersma *et al.* (1993) who described that for the induction of peptide specific antibodies in rabbits and mice, Specol leads to good results in almost all cases. Zegers *et al.* (1993) found Specol to be effective in producing anti-peptide antibodies that recognize the native protein in rabbits and mice. In mice, we only observed native protein cross-reactive antibodies when the Specol/peptide was i.p. injected and not when s.c. injected. Specol did not induce

antibodies to autoantigen (MBP), confirming that Specol has limited properties to induce T cell-mediated immunity. This is supported by the finding that Specol/synthetic peptide injection in mice resulted mainly in peptide specific antibodies of the IgG1 isotype (cf Boersma *et al.*, 1992).

side effects

No clinical and behavioural changes, indicative for pain or distress, were observed in rabbits immunized with Specol emulsions. In mice, we observed weight loss, piloerection and hunched posture the first 2 days after injection of Specol emulsions. These changes were less extensive than after FA injections. The pathological changes observed after injection of Specol emulsions were mostly minimal to moderate except for lesions observed after injection of Specol combined with measles virus, mumps virus or *Mycoplasma pneumoniae*. Lesions were then marked to severe and sometimes comparable to those after FA injection. The particulate character of these antigens may play a role in the severity of lesions. Particulate antigen may be released from the Specol emulsion slower than soluble antigens resulting in longer contact of particulate antigen with attracted cells, such as macrophages and neutrophils, resulting in more damage. Not all particulate antigens (e.g. Rubella-virus), however, were shown to induce severe pathological changes when emulsified in Specol. Bokhout *et al.* (1981) observed no tissue necrosis and only slight degeneration of muscular tissue after i.m. injection of Specol without antigen, while when antigen was added, lesions included granulomas of 1 cm. This again indicates that the (type of) antigen in Specol influences the induction of side effects.

conclusion

Specol induces comparable antibody titres to FA and Montanide ISA50, except for antibody responses to autoantigens, while the severity of lesions induced by Specol is variable, depending on the antigen. Generally, the severity of Specol lesions was comparable to Montanide ISA50 lesions and far less severe than FA lesions.

RIBI

RIBI adjuvant system (RIBI ImmunoChem Research Inc., Hamilton, MT; Ribi *et al.*, 1982: Masihi *et al.*, 1986; Rudbach *et al.*, 1995) consists of highly purified microbial components contained in a metabolizable oil (squalene) and emulsifier (Tween 80). Two formulations of RIBI adjuvant were used in our experiments as recommended by the manufacturer; for rabbits, the RIBI containing three microbial components, 0.5 mg/ml MPL (monophosphoryl lipid A), 0.5 mg/ml TDM (synthetic trehalose dicorynomycolate) and 0.5 mg/ml CWS (cell wall skeleton) and for mice, the RIBI containing 0.5 mg/ml MPL and 0.5 mg/ml TDM, were used. When RIBI is prepared with aqueous antigen solution, an oil-in-water emulsion is formed.

antibody responses

Immunization of rabbits with RIBI, combined with synthetic peptide, resulted in low antibody titres when compared to FA. Antibodies were only detectable after secondary injection. These findings confirm data of Johnston *et al.* (1991) and Smith *et al.* (1992) who observed low antibody responses in rabbits even though they used carrier-peptide antigens i.e. BSA/peptide and protein/polymer, respectively. Also Bennett *et al.* (1992) observed low antibody responses after injection of a carrier-peptide (BSA/LHRH) combined with RIBI in rabbits. Bennett *et al.* (1992) used RIBI which contains MPL and TDM and can therefore formally not be compared with our experiments. When RIBI/*Mycoplasma pneumoniae* was injected (s.c. or i.m.) in rabbits antibody responses were intermediate and transient compared to FA injections. Mallon *et al.* (1991) used RIBI containing MPL and TDM in rabbits and observed intermediate antibody titres after s.c. injection with a subunit enzyme (rat liver microsomal epoxide hydrolase). Deeb *et al.* (1992) found transient antibody responses after i.m. injection of synthetic polypeptide combined with RIBI compared to FA in rabbits.

When comparing immunological properties of RIBI with those of FA in mice, injection of synthetic peptide emulsified in RIBI induced low antibody levels compared with FA injection. Our findings are in contrast with those of Lipman *et al.* (1992) and Geerligs *et al.* (1989) who i.p. injected mice with RIBI combined with bovine gamma globulin/hapten and BSA/synthetic peptide respectively and observed specific antibody levels comparable to FA injection. This was also observed in mice after s.c. injection of a protein toxin (Hewetson *et al.*, 1993) or fusion protein (Daly and Long, 1996) in combination with FA or RIBI. The type of antigen used (carrier-hapten in the former two and proteins in the latter two studies) may explain these higher antibody levels found. Kenney *et al.* (1989) observed intermediate antibody titres when RIBI/human serum albumin was injected in mice. Relative, to IgG1, we observed high levels of IgG2a antibodies were observed after secondary i.p. injection of RIBI/synthetic peptide. This confirms the expected induction of IgG2a antibodies by MPL and TDM (Van de Wijgert *et al.*, 1991; Rudbach *et al.*, 1995). RIBI might therefore be useful to induce cell-mediated immunity (Johnson and Tomai, 1990).

side effects

In rabbits and mice minimal clinical and behavioural changes, which might be indicative for pain and distress, were observed. After RIBI/antigen injection in rabbits, lesions were more severe (with all three antigens) than those found after injection of FA/antigen, both after s.c. and i.m. injections. Johnston *et al.* (1991) injected BSA-peptide combined with RIBI or FA in rabbits and observed that RIBI and FA induced comparable pathological changes. In contrast, Deeb *et al.* (1992) found lesions after RIBI injection that were less severe than those found after FA

injection in rabbits. The severity of the RIBI lesions in our rabbit studies exceeding those induced by FA; this may be explained by the volume injected (2x0.5 ml). The manufacturer of RIBI recommends 1 ml divided in ten inoculation sites per rabbit. As we wanted to perform all inoculations in a similar manner, the recommended volume of 1 ml RIBI was divided in two inoculation sites (as approved by the manufacturer). Another explanation may be the way in which we prepared the RIBI/antigen emulsions. In mice however, we used the same preparation protocol (described by the manufacturer) and observed minimal side effects. These findings in mice are in contrast with findings of Lipman *et al.* (1992) who injected RIBI and FCA in mice and observed pathological lesions which were comparable for both adjuvants but FCA lesions were more extensive than RIBI lesions. It is not clear why these considerable differences in pathological changes between rabbits and mice occur after RIBI injection. The fact that the RIBI used in rabbits contained, besides MPL and TDM, also CWS may play a role.

conclusion

RIBI has only limited value in rabbits and mice for induction of antibody responses. Pathological changes in rabbits after RIBI injection, exceeded those induced by FA. In mice pathological changes were minimal after RIBI injection.

TiterMax

TiterMax (CytRx Corporation, Norcross, GA; Hunter *et al.*, 1981; Hunter and Bennett, 1986; Hunter *et al.*, 1995) consists of a metabolizable oil (squalene), emulsifier (Sorbitan Monooleate 80) and a patented block copolymer CRL-8941. When TiterMax is mixed 1 : 1 with aqueous antigen, a water-in-oil emulsion is formed.

antibody responses

Intramuscular injection of TiterMax/antigen in rabbits resulted in low antibody responses while injection via the s.c. route resulted in intermediate antibody responses. Smith *et al.* (1992) also observed low titres when TiterMax/antigen was i.m. injected. The high antibody titres claimed for TiterMax in rabbits (Bennett *et al.*, 1992) were not observed in our study. Subcutaneous injection of mice with TiterMax/synthetic peptide resulted in low antibody levels while after i.p. injection, these levels were high. In mice, we subcutaneously injected the recommended amount of 50 µl TiterMax/antigen per mouse while intraperitoneally 0.2 ml TiterMax/antigen was injected. This may be an explanation for the difference in response after the s.c. and i.p. route of injection. Zhou and Afshar (1995) also observed low antibody titres after s.c. injection with TiterMax. However, Bennett *et al.* (1992) observed comparable antibody levels when injecting carrier-hapten with TiterMax or with FA. Also, Sjölander *et al.* (1996) and Daly and Long (1996) observed comparable antibody titres and isotype distribution of antibodies (IgG1 and

IgG2a) after s.c. injection of influenza virus micelles or fusion protein, respectively, in combination with TiterMax or FA. Subcutaneous injection of whole killed parasite (Ten Hagen *et al.*, 1993) combined with TiterMax or FA resulted in comparable IgG1 levels while IgG2a levels were lower when TiterMax was used. The high antibody levels observed by the authors above after s.c. injection of TiterMax combined with antigen, as opposed to our findings, may be explained by the type of antigen used. In our studies, we used a synthetic peptide while in the studies described above, relatively immunogenic particulate or carrier-hapten antigens were used. After i.p. injection with TiterMax/synthetic peptide, peptide specific IgG1 antibody levels were comparable to those observed after FA injection while antibodies of the IgG2a isotype were 4 times higher after TiterMax than after FA injection. These high IgG2a antibody levels are in accordance with the ability of TiterMax to induce cell-mediated immunity (Kast *et al.*, 1993). Efficacy of TiterMax in T cell-mediated diseases was shown by Roberge *et al.* (1992) and Shenoy and Christadoss (1993) who successfully induced experimental autoimmune uveoretinitis and autoimmune myasthenia gravis, respectively, using TiterMax. However, induction of adjuvant arthritis in susceptible rats was not successful (Hunter *et al.*, 1995). The level of anti-peptide antibodies that recognize the native protein, was higher after i.p. injection of TiterMax/antigen than of FA/antigen.

side effects

No clinical or behavioural changes, indicative for pain and distress, were observed in rabbits. In mice, piloerection and hunched posture was observed the first days after primary and secondary immunization with TiterMax, especially after i.p. injection. These changes were as severe as those observed after i.p. injection of FA. Pathological changes after TiterMax injection in rabbits were less severe than those observed after FA injection. Upon clinical observation, no lesions were observed by Smith *et al.* (1992) after i.m. injection of TiterMax in rabbits while after i.m. injection of FA small granulomatous lesions were observed. Bennett *et al.* (1992) studied i.m. lesions after TiterMax injection histologically and observed mild reactions. In mice, we observed pathological changes after injection (i.p. or s.c.) of TiterMax which were as severe as those observed after FA injection. This was not reported by other authors. Hunter and Bennett (1984) described that the nonionic block polymer surfactants differ in the generation of inflammation. The combination of our antigen (synthetic peptide) with TiterMax may have changed its inflammatory effects, and this may also explain the differences between lesions found in rabbits and mice.

conclusion

TiterMax was not shown to be an effective adjuvant for antibody production to weak immunogens in rabbits. In mice, s.c. injection of TiterMax was not effective while i.p. injection of TiterMax resulted in very high IgG2a and native-protein cross-reactive antibody levels. The pathological changes in rabbits observed after TiterMax

injection were less severe than after FA while in mice lesions of comparable severity were found.

Montanide ISA50

Oils of the Montanide series (Seppic, Paris, France; Ganne *et al.*, 1994) are diverse, some of them are biodegradable. In this study, we used Montanide ISA50 which consists of 85% mineral oil and 15% emulsifier (mannide oleate). After mixing Montanide ISA50 with aqueous antigen in a 1 : 1 ration, a water-in-oil emulsion is formed.

antibody responses

Injection of Montanide ISA50/antigen in rabbits resulted in specific antibody responses comparable with those observed after FA/antigen injection. This was also observed by Johnston *et al.* (1991) who used BSA-peptide as an antigen. In mice, Montanide ISA50/synthetic peptide immunization resulted in antibody responses comparable to (s.c. injected) or even exceeding (i.p. injected) those induced by FA. These findings confirm the observations by Jones *et al.* (1990) who used carrier-synthetic peptide as antigen in mice. Montanide ISA50 immunizations in mice resulted mainly in antibodies of the IgG1 isotype while minimal IgG2a antibodies were found. Antibodies of the IgG1 isotype are commonly observed after injection of water-in-mineral oil emulsions (Allison and Byars, 1991). This suggests that Montanide ISA50 is not very effective in inducing cell-mediated immunity. Minimal anti-peptide antibodies recognizing native protein were induced by Montanide ISA50/synthetic peptide injection.

side effects

Injection of Montanide ISA50 emulsions in rabbits and mice resulted in lesions which were less severe than those observed after FA injection. When Montanide ISA50 was s.c. injected in rabbits, combined with HIV-peptide conjugated to tetanus toxoid, exudative lesions were found at the site of injection. These exudative lesions may be explained by the tetanus toxoid component in the antigen. Johnston *et al.* (1991) injected Montanide ISA50/BSA-peptide in rabbits and observed comparable pathological changes as those observed after FA injection. In mice, Montanide ISA50 injection (s.c.) resulted in comparable pathological changes as FA injection but lesions were less extensive.

conclusion

Montanide ISA50 was shown to be an effective adjuvant for the induction of antibodies in rabbits and mice while it induced variable lesions, depending on the antigen, the severity of lesions was comparable to Specol lesions and less severe than FA lesions

Other studied adjuvants

Immune-stimulating complexes (iscoms) are used as an experimental adjuvant (Morein *et al.*, 1987; Barr and Mitchell, 1996) and are not (yet) commercially available. Basically, ISCOM is formed from cholesterol, saponin, phospholipid and is termed ISCOM matrix or empty ISCOMs. When viral envelope proteins (e.g. rabies, measles, influenza) are incorporated into the ISCOM matrix, it is called ISCOMs. In our studies, ISCOMs with incorporated rabies virus glycoprotein (RV-ISCOMs) were used. Other antigens can to be coupled to the RV-ISCOMs to induce effective immune responses (Lövgren and Larsson, 1994). Since ISCOMs are a relatively small structure (40 nm), it is therefore ill-adapted for coupling of particulate antigens. We studied a synthetic peptide and an autoantigen (MBP) coupled to RV-ISCOMs. Immunization of these RV-ISCOMs with coupled antigen was shown to be very effective in inducing antigen specific antibody responses. This confirms findings of Lövgren *et al.* (1987) who coupled biotin as an antigen to influenza virus ISCOMs. In our studies, anti-MBP antibody responses exceeded those found after FA/MBP injection. Using another autoantigen (PLP-peptide) coupled to RV-ISCOMs in SJL/j mice, we induced high peptide specific antibody levels but we were not able to induce experimental autoimmune encephalomyelitis in these mice as opposed to FCA/PLP-peptide (unpublished data). At necropsy minimal gross and histopathological lesions were found after injection of RV-ISCOMs with coupled antigen. RV-ISCOMs is a very interesting adjuvant for the induction of antibody responses to small antigens. It can not easily replace FCA at this moment since it is not commercially available.

The experimental Lactobacillus adjuvant was not effective in inducing antibody responses. Boersma *et al.* (1994) showed that peptides need to be coupled to Lactobacillus to generate acceptable levels of responsiveness. This makes it less useful as ready to use adjuvant to replace FA. We confirmed that Lactobacillus is not pathogenic upon administration, but observed severe pathological lesions when it was injected in combination with *Mycoplasma pneumoniae*, suggesting a role for antigen/adjuvant interactions in the induction of pathological changes.

Gerbu and DDA induced no and moderate lesions, respectively, after injection in rabbits while weak immune responses were found. Quil A was only injected in combination with *Mycoplasma pneumoniae* in mice. This resulted in severe pathological changes, probably due to combination with this antigen. Antibody titres after Quil A/ *Mycoplasma pneumoniae* injection could not be used since the antigen induced antibodies without adjuvant.

Other factors influencing immunization result

Besides the adjuvants, many other factors influence the outcome of an immunization, e.g. type of antigen, injection volume and route, and animal species (Chapter 2). The influence of these factors on our data is discussed below.

As described in Chapter 2, the antigen greatly influences the immunological and pathological result of an immunization (Constant *et al.*, 1995; HayGlass and Stefura, 1991) depending on e.g. its source, chemical nature and size. Van Ommen *et al.* (1994) used the model antigen, TNP-KLH, in combination with adjuvant and speculated that the KLH part of the antigen was responsible for the outcome of the immunization and not the adjuvant. To allow discrimination between the potency of the alternative adjuvants, we generally used weak immunogens such as synthetic peptides and included a control group (injection of antigen only) in each experiment. Consequently, we found that *M. pneumoniae* induced an antibody titre without adjuvant in mice while in rabbits antibody titres were low. In rabbits and mice, *M. pneumoniae* induced severe lesions with all adjuvants tested while minimal lesions without adjuvant. The interaction between adjuvant and antigen may have caused the severe pathological changes.

The injection route affects the nature and intensity of the response, probably due to distinct types of antigen-presenting cells that encounter the antigen (Hsieh *et al.*, 1992; De Becker *et al.*, 1994). When an adjuvant is added to the antigen, adjuvant/antigen micelles may be formed, depending on the adjuvant. These micelles will be handled by a different type of antigen-presenting cells (Laman and Claassen, 1996) showing the importance of adjuvants in inducing an immune response. Minimal differences in antibody responses were observed between the injection routes used in rabbits (s.c. and i.m.) or in mice (s.c. and i.p.). An exception was TiterMax; antibody levels were high after i.p. injection of TiterMax while low after s.c. injection. In contrast, Bennett *et al.* (1992) found that the s.c. route of injection was superior to i.p. route in mice. The difference in injection volume, between s.c. and i.p. injections in our study, may explain our findings.

The induction of pathological lesions, however, depends on the route of injection. Comparing s.c. and i.m. injection route for induced pathological changes in rabbits, lesions with comparable extent were found after s.c. and i.m. injection. The i.m. route, however, is more prone to injection error than the s.c. route since it is not as easy to verify the location of injection, i.m. lesions are also more difficult to monitor clinically and i.m. lesions are supposed to be more painful. Therefore, the s.c. route is preferred to the i.m. route in rabbits. In mice, s.c. and i.p. injection route were used. The severity of the lesions induced after these two routes is completely different. As opposed to s.c. injection where adjuvant/antigen is localized at one place, i.p. injections effect the extensive surface of the peritoneum and the inoculate is in close contact with various vital organs. Additionally, Walvoort (1991) studied the effectiveness of i.p. injections by different biotechnicians and showed variability in

effectiveness between biotechnicians and sometimes damage of organs. The s.c. route should therefore be preferred to the i.p. route.

Different animal species and also different strains of animals, particularly mice, respond differently to adjuvant/antigen injection (Gupta and Siber, 1995). After injection of the same antigen (*M. pneumoniae*) in rabbits and mice, *M. pneumoniae* was found to be weakly immunogenic in rabbits, i.e. no antibody response without an adjuvant, while injection of *M. pneumoniae* without an adjuvant in mice resulted in antibody responses almost comparable to those induced by adjuvant/*M. pneumoniae* injection. BALB/c mice were used in the immunological studies described in this thesis. An inquiry under researchers in the Netherlands showed that BALB/c mice are most frequently used in immunization experiments to induce specific B cells (unpublished data). BALB/c mice are Th2 biased and consequently, immunological results may be different when, for example, C57BL/6 mice, a Th1 biased strain, are used. Besides differences in immunological response, different animal species or strains may tolerate an adjuvant/antigen mixture differently. We observed differences in the induction of pathological changes between rabbits and mice, injected with the same adjuvants e.g. lesions after TiterMax injection in rabbits were mild while in mice severe pathological changes were found. Differences between mice strains are described by Boersma *et al.* (1992), in C3H/He and BALB/c mice, no or only mild irritation was observed upon i.p. injection of Specol while, NZB and C57BL/6 mice showed chronic mild to severe peritonitis.

The preparation method of the emulsion can influence the immunogenic properties of antigen/adjuvant emulsions since it induces morphological changes in the emulsion structure e.g. droplet size (Bomford *et al*, 1992; Audibert and Lise, 1993). It is therefore important to pay attention to the preparation of proper water-in-oil emulsions. To test stability of water-in-oil emulsions a drop emulsion was put on the surface of water; when the emulsion is stable, the drop does not spread.

The number of injection sites also has influence on the outcome of an immunization. We injected 0.5 ml water-in-oil emulsion at one site and compared this with the same volume spread over 2 or 4 injection sites. This resulted in comparable antibody responses, suggesting that dividing antigen over more sites does not always improve the antibody response. In contrast, Bennett *et al.* (1992) injected mice s.c. at 2 or 4 sites and observed higher antibody titres after injection at 4 sites. They, however, used mice, different injection volumes, and another adjuvant. Since we observed pathological changes at all injection sites, injection at one site is suggested.

Predictive value of cytokine profiles induced by adjuvants

Murine T helper (Th) cell clones can be divided into different subsets based on their lymphokine profile (Mosmann *et al.*, 1986; Cherwinski *et al.*, 1987). Th1 cells produce IFN-γ and IL-2, whereas Th2 cells secrete IL-4 and IL-5. Several studies

(reviewed by Finkelman *et al.*, 1990; Mosmann and Coffman, 1989; Mosmann and Sad, 1996) showed that cytokines such as IL-4 and IFN-γ direct the quality of immune responses after infection and vaccination. IL-4 promotes an immune response dominated by antibodies of the IgG1 and IgE isotypes (Snapper and Mond, 1988; Swain *et al.*, 1990) while IFN-γ promotes antibodies of the IgG2a isotype (Stevens *et al.*, 1988; Finkelman *et al.*, 1988b). The regulation of isotype immunoglobulin production by adjuvants has been described *in vivo* (Hadjipetrou-Kourounakis and Möller, 1984; Kenney *et al.*, 1989; Karagouni and Hadjipetrou-Kourounakis, 1990). Adjuvants can also modulate the immune response to different T helper cells (Cox and Coulter, 1997). Street *et al.* (1990) described the important role of adjuvants in determining which Th cell subsets (Th1 or Th2) will be activated *in vivo*, and consequently, which cytokines are produced. Because cytokines play a crucial role in mediating immune responses, many of the activities induced by adjuvants may be mediated through cytokines. Van den Eertwegh *et al.* (1993) showed a peak in CD40L expressing cells (activated T cells) and KLH specific antibody forming cells 4-5 days after secondary i.v. injection of KLH and suggested that CD40L expression on activated T cells plays role in the specific antibody production. Grun and Maurer (1989) observed preferential stimulation of phenotypically different T helper cell subsets and found corresponding IgG1 and IgG2a antibodies in serum after injection of FCA and Alum in mice.

Using immunohistochemical techniques, we studied the number of activated T cells and cytokine producing cells in murine spleen sections 5 days after secondary i.p. injection of SP215 combined with FA, Specol, RIBI, TiterMax or Montanide ISA50. These data were correlated with antibody levels and isotype distribution in serum, 5 days after secondary immunization. Significantly higher numbers of activated T cells and cytokine producing cells (IL-4 and IFN-γ) were observed in spleen sections 5 days after secondary injection of RIBI compared with any of the other adjuvants while RIBI injection resulted in lowest antibody levels. TiterMax/SP215 injection resulted in very high peptide-specific IgG2a antibody levels (7 times higher than FA/SP215 injection) while no increased numbers of IFN-γ producing cells were found. Injection of Montanide ISA50 combined with SP215 resulted in, relatively to IgG2a antibody levels, very high SP215-specific IgG1 antibody levels while the number of IL-4 producing cells was not significantly increased. Data of our study suggest that specific antibody titres and isotype distribution in serum not necessarily correlate with the numbers of activated T cells and cytokine producing cells in spleen sections 5 days after secondary immunization. Studying cytokine production at more time points and using other cytokine detection methods may provide information on when these levels are indicative, if at all, for isotype distribution in serum. The number of activated T cells and cytokine producing cells was also determined after injection of adjuvant without antigen. No significant differences in cytokine profiles (IL-4 and IFN-γ) were

observed between adjuvant injection with or without antigen. This indicates that this cytokine production was not antigen specific stimulated but rather due to non-specific immune stimulation by the adjuvant. This may be explained by the type of antigen used in our study. The antigen studied, SP215, is a weak immunogen (synthetic peptide), the influence of which may be overruled by the non-specific immune-stimulating effects of the adjuvants. Van Ommen *et al.* (1994) showed that the antigen (model antigen, TNP-KLH) has a major impact on the outcome of an immunization. They injected TNP-KLH in combination with Alum or FCA and observed comparable antibody responses independent of the adjuvant used. They speculated that the KLH part of the antigen was responsible for the type of immune response while the adjuvant enhanced this response without changing the type of response.

The predictive value of cytokine profiles for isotype distribution of antibodies was also studied in the initial phase of the immune response. To obtain data on cytokine production following primary injection of FCA/SP215, we used immunohisto-chemistry and *in vitro* cell culturing of lymphoid cells but cytokine profiles could not be measured because these levels were below the detection limits of our system. Using a strong immunogen (TNP-KLH), increased numbers of IFN-γ producing cells were found in murine spleen sections 4 hours after FCA/TNP-KLH injection (unpublished data). Jotwani *et al.* (1994) injected a strong immunomodulator (LPS) and found IFN-γ responses in the bloodstream 6 hours later. To analyse whether injection of the weak immunogen (SP215) in FCA results in detectable cytokine production, we stimulated spleen cells *in vitro* with SP215 at 5 and 14 days after i.p. injection of FCA/SP215, and studied whether proper antigen specific proliferation or cytokine production could be found. Our data indicated that early antigen specific cytokine production and proliferation of spleen cell cultures is not easily detectable after i.p. injection employing a weak immunogen. *In vitro* antigen specific stimulation of lymph node cells resulted in significantly enhanced IFN-γ levels at 14 days after s.c. injection of FCA/SP215, FIA/SP215 or Specol/SP215. IL-4 production in these culture was below the detection limit. The ability of these three adjuvants to induce SP215 specific IgG1 and IgG2a antibodies was studied in serum 14 days after injection. IgG1 levels were significantly higher after Specol/SP215 injection while IgG2a levels were similar in all groups. A remarkable finding was that no IL-4 production was detectable *in vitro* while considerable IgG1 antibody levels were observed in serum. This indicates that IL-4 production by lymph node cells *in vitro* does not necessarily correlate with IgG1 production *in vivo* at 14 days after s.c. immunization. Probably a very small amount of IL-4 is enough to induce the isotype switch from IgM to IgG1 (Le Gros *et al.*, 1990).

In our testsystem, cytokine profiles were not shown to have a predictive value for antibody responses to weak immunogens emulsified in oil adjuvants. Fox (1992) used a peptide antigen and could not find differential effects of adjuvants on Th1

and Th2 cells while differences in antibody titres and isotypes were observed after injection of different adjuvants. Difficulties to identify a consistent relationship between systemic cytokine production and antigen specific antibody isotypes in serum induced by different adjuvants were also described by Valensi *et al.* (1994). From our results it appeared that isotype distribution in serum can give more information on immunization result than cytokine profiles. More studies are needed to elucidate the correlation between cytokine profiles and isotype distribution *in vivo*.

In vivo behaviour of water-in-oil emulsions

Water-in-oil (w/o) emulsions comprise microdroplets of water stabilized by surfactant in a continuous oil phase. The mechanism of action of w/o emulsions is poorly known (Cox and Coulter, 1997). W/o emulsions appear to improve and prolong antigen presentation by: depot function at the site of injection, protection of antigen from degradation, enhanced uptake of antigen by macrophages, activation of macrophages, and vehicle (transport/routing) function (Waksman, 1979). The preparation method of emulsions influences its structure (e.g. droplet size) which determines the antigen-presenting properties and thereby its efficacy (Harding *et al.*, 1991; Bomford, 1992; Ten Hagen *et al.*, 1993). One way in which water-in-oil emulsions are thought to enhance the immune response is the conversion of the antigen to particulate matter. Since particulate matter is taken up almost exclusively by macrophages (Van Rooijen, 1992), this conversion would enhance the uptake of antigen by these cells. We studied the role of macrophages in adjuvant efficacy of w/o emulsions. Macrophages were eliminated *in vivo* by injection of dichloro-methylene-diphosphonate (Cl_2MDP) containing-liposomes (Van Rooijen and Sanders, 1994) before injection of adjuvant/antigen preparations. Elimination of macrophages before injection of antigen combined with Specol or without adjuvant, resulted in increased antigen specific antibodies. Antibody responses were decreased, when macrophages were eliminated before FCA/antigen injection. This confirms that (pre)processing of particulate matter (FCA contains Mycobacteria) is dependent on macrophages. The importance of macrophages to process an antigen for the induction of an immune response, depends upon the type of antigen. *In vitro* studies demonstrated that pre-processing of particulate antigens by macrophages was crucial for the generation of immune responses (Wright *et al.*, 1987; Van Rooijen, 1992). Delemarre *et al.* (1991) showed this phenomenon *in vivo*. Soluble antigens are processed and presented to the immune system by other types of antigen-presenting cells, such as B cells and dendritic cells (Ibrahim *et al.*, 1995). We used TNP-KLH, which is a soluble antigen and therefore does not need macrophages (Laman and Claassen, 1996). By eliminating macrophages, immunization may be more effective because less antigen will be phagocytosed and therefore more antigen will come in contact with professional antigen-presenting cells such as interdigitating cells, which are extremely potent (Steinman, 1991;

Girolomoni and Ricciardi-Castagnoli, 1997). Our data showed that macrophages indeed play a role in the induction of the immune response with w/o emulsions, but that this can be a suppressive role.

This suppressive role of macrophages was confusing in the light of macrophage involvement in adjuvant efficacy of w/o emulsions. To investigate which other mechanisms and macrophages are involved (or bypassed) in the uptake and processing of such emulsions, a method for *in situ* localization of these compounds was developed. The oil phase of w/o emulsions was labelled with a lipophilic fluorescent dye (DiI). This enabled kinetic studies on *in vivo* routing of w/o adjuvants. After i.p. administration of labelled oil emulsion, localization of oil was associated with the capsule of the spleen. This may function as a second depot, besides the depot at the site of administration, and herewith stimulate splenic antigen-presenting cells by continuous release of antigen. These results can explain the efficacy of oil emulsions in inducing immune responses, i.e. by keeping the bulk of the antigen away from the suppressive marginal zone macrophages (Leenaars *et al.*, 1997) and releasing antigen over time for uptake by follicular cells of the spleen or interdigitating cells in the interfollicular T cells areas of the lymph node. It can be concluded that efficacy of w/o emulsions is not exclusively dependent on macrophages.

Assessment of pain and distress induced by adjuvants

In the Dutch guidelines for immunization of laboratory animals (Veterinary Public Health Inspectorate, 1993), the use of FCA is classified as causing (very) severe distress. Under the USDA Guidelines, experiments are classified as Type C (some pain or distress of short duration) when FCA is injected intramuscularly and Type E (significant pain or distress) when FCA is injected in the footpad, intradermally or intraperitoneally. To reduce pain and distress in immunized animals, the use of FCA in immunization experiments was restricted in the Netherlands. The reduction of pain and distress in immunized animals was a clear motive for the studies underlying this thesis. The clinical signs and behavioural changes to recognize pain, distress and discomfort in laboratory animals have been described extensively by Morton and Griffiths (1985), Wallace *et al.* (1990) and Bateson (1991). Using these criteria, no indications for pain or distress were found in rabbits injected with FCA or any other adjuvant/antigen combination. Johnston *et al.* (1991) did not observe evidence of weight decrease or behavioural changes suggestive of chronic pain in rabbits after FCA/antigen injection. Smith *et al.* (1992) injected rabbits s.c. and did not find indication of pain on palpation of inoculation site. At day 7, 14, 21 and 28 after primary and 7 and 14 days after secondary injection of adjuvant/antigen (s.c. or i.m.) in rabbits, we measured creatinine kinase levels and did not observe a clear increase within a group of animals. Deeb *et al.* (1992) observed elevated creatinine kinase levels in rabbits on day 2 after i.m. injection of FCA/antigen or RIBI/PBS. This

might be indicative for muscle inflammation or necrosis and therefore associated with discomfort. The 7 days interval, between i.m. injection and blood sampling, may explain why we did not observe elevated creatinine kinase levels. Deeb *et al.* (1992) also observed muscle quivering upon palpation of the inoculation site two days after injection of FCA or RIBI emulsions, while some FCA injected rabbits showed muscle quivering through day 35. They did not observe differences in weight gain, body temperature and heart rate between the groups.

To study the induced pain and distress after immunization more extensively, we used non-aversive behavioural tests in mice as recommended by Manser (1992). The behavioural tests included: (1) primary observation test, (2) activity measurement and (3) LABORAS. Using these additional tests and general criteria for pain and distress, only indications for acute pain and distress, 1 to 3 days after injection of FCA, were found and no indications for prolonged severe pain and distress. Toth *et al.* (1989) also demonstrated that mice were temporarily impaired after i.p. injection of FCA/BSA (0.5 ml). These immunized mice developed unkempt haircoats, hunched postures, mild diarrhoea, decreased food intake and decreased body weights and returned to an apparently normal condition within one week. The injected volume (0.5 ml) of FCA may explain why these changes were present for one week, as opposed to the 1-3 days we found, to return to normal condition. Jansen van 't Land and Hendriksen (1995) observed decreased locomotion activity using an automatic system to measure locomotion activity during day and night. They injected mice i.p. with 0.1, 0.2, or 0.5 ml FCA and found a dose-dependent reduction in locomotion activity. When 0.5 ml FCA was i.p. injected, activity was decreased until day 5 after injection. Body weight was also found to be decreased until day 5 in the 0.5 ml FCA injected group. When the maximum volume allowed in the Dutch guidelines (0.2 ml) was injected, Jansen van 't Land and Hendriksen (1995) observed significantly decreased activity in FCA injected animals the first two days after injection.

In accordance with Manser (1992), pathological changes were studied, resulting in obvious differences between adjuvant regimes without the need of invasive procedures during the animal's lifetime. These pathological changes can be indicative for pain, however, minimal clinical and behavioural changes were observed. This enigmatic finding may be explained in two ways; (1) the available methods to study clinical and behavioural changes to monitor pain and distress, are not adequate or (2) no severe prolonged pain and distress is induced as expected based on the pathological changes observed.

A problem in recognizing signs of pain in rabbits may be that rabbits do not readily exhibit pain signs, rabbits may be masking their suffering instinctively (Wallace *et al.*, 1990; Griffiths, 1991). In mice, behavioural measures indicated that the animals were temporarily impaired immediately after immunization. The availability of automated devices such as LABORAS makes it possible to monitor

subtle changes in the behavioural pattern of mice (locomotion, immobility, climbing, grooming, eating and drinking), providing adequate possibilities to detect severe pain and distress.

Rabbits and mice were injected in accordance with recommendations given in the Dutch guidelines for immunization of laboratory animals (Veterinary Public Health Inspectorate, 1993), e.g. volume, injection route, and FCA injection only in primary immunization. This may explain why no prolonged impairment after injection of FCA was observed. Careful control of injection quantity and site selection was also suggested by Amyx (1987) to eliminate most undesirable effects of FCA.

Based on analogy principle with humans, the pathological changes, observed after injection of FCA in laboratory animals, are suggested to be painful (Stafleu *et al.*, 1992). However, literature in which pain in humans is described after injection of FCA (Chapel and August, 1976; Hughes *et al.*, 1970) concerns papers in which lesions after FCA injection were painful in humans only when these persons were tuberculin-sensitized. This may indicate that the reaction was a result of repeated exposure to Mycobacteria (Stills and Bailey, 1991). Wanstrup and Christensen (1965) injected mice weekly with FCA (s.c. 0.05-0.2 ml) and observed decreased activity, dullness of fur, loss of hair in mice. We did not use FCA twice in the same animal. The assumption that FCA injection induces severe pain and distress may be based on studies prior to 1970 since these FCA studies often were completed with acid-treated mineral oil, while nowadays the production is done by the single or double hydrogenation process which results in a far less toxic product (Stewart-Tull, 1995a).

The next question is whether pathological changes found in humans, not induced by FCA but comparable to those found in laboratory animals after injection of FCA (e.g. peritonitis, adhesions), are painful. Based on the analogy principle with humans this may be expected. The amount of pain induced by lesions strongly depends on the location and extent. Walvoort (1991) showed that only a small number of pathological changes found in humans and in animals do correlate with respect to induced pain. This suggests that the pathological changes are not necessarily painful, but does not exclude the possibility that they are.

Finally, we have to answer the question whether lesions induced by injection of FCA are painful in laboratory animals during a prolonged period. This question can not be answered since no indications of prolonged impairment were found. The development of lesions may have been painful since inflammatory mediators are released, which may lead to pain sensation. The first 1-3 days after i.p. injection of FCA, indications of pain were found, probably due to acute peritonitis.

It can be argued that since there is no proof that the pathological changes observed after adjuvant injection do not induce pain, the benefit of the doubt should go to the animal (Morton and Griffiths, 1985), i.e. the adjuvant that induces the least pathological changes should be preferred. This implies the importance of

pathological studies when assessing side effects of adjuvants. Since, some alternative adjuvants also induce severe pathological changes, it may be disputed whether it is valid to claim that FCA is more harmful than alternative adjuvants. In rabbits, for example, pathological changes after RIBI/antigen injection were found to be more severe than those induced after FA/antigen injection. This concept has consequences for the selection of an alternative adjuvant. This will be discussed below in 'adjuvant selection for immunization'.

Alternative methods to produce antibodies

Antibody production requires the use of live animals. In this thesis, we studied the possibilities to reduce pain and distress in animals used for antibody production by careful selection of the adjuvant used. Below, other ways to reduce pain and distress in the production of antibodies are described.

Antibody production in the egg yolk (IgY) of chickens can be an alternative *in vivo* technique for production of polyclonal antibodies in mammals (McCafferty *et al.*, 1990; Svedson Bollen *et al.*, 1996). The current status of immunoglobulin (Ig) Y production in the chicken is reviewed in Schade *et al.* (1996) and ALTEX (1996). The IgY structure and function were reviewed by Warr *et al.* (1995). Scientifically, IgY atibodies are interesting because of the phylogenetic difference between chickens and mammals. This results in less cross-reactivity in assays of mammalian proteins. The use of chickens for antibody production, as opposed to mammals, can represent both a refinement and a reduction in animal use. Refinement is achieved in that the second painful step, the collection of blood, is replaced by antibody extraction from egg yolk. It entails a reduction in the number of animals used because chickens produce larger amounts of antibodies than laboratory rodents. Major disadvantages of IgY technique to produce antibodies are: (1) the animals still have to be injected with adjuvant/antigen mixtures, and (2) standard immunochemical assays for IgY antibodies are rarely available. The utility of chicken egg yolk as a realistic alternative source of polyclonal antibodies in mammals has to be questioned.

Some authors (Ohlin and Borrebaeck, 1992; Wohlleben et al., 1996) described *in vitro* immunizations for the production of antibodies. This method replaces the animal for *in vitro* cell cultures to produce antibodies, but it has not been very successful till now because antibodies produced *in vitro* include antibodies of the IgM isotype mainly.

A very promising technique is the phage antibody display technology. This technology permits the selection of antibody fragments from large libraries constructed from the B cells of naive or immunized individuals (De Kruif *et al.*, 1996). To create large libraries, animals may still need to be immunized (albeit only once). Phage antibody display technology is used to produce Fab fragments of purified antigens. By mimicking the affinity maturation of antibodies *in vitro*, phage display

strategies have the potential to reduce or eliminate the use of animals in antibody production protocols (Peterson, 1996).

Adjuvant selection for immunization

In the Dutch guidelines for immunization of laboratory animals (Veterinary Public Health Inspectorate, 1993), drafted by experts, the use of FCA is restricted and the use of alternative adjuvants is encouraged to minimize pain and distress in immunized animals. We followed these guidelines when comparing alternative adjuvants with FA and observed, with a number of clinical parameters and behavioural tests, no indications of pain and distress in rabbits and exclusively indications of acute pain and distress (1-3 days) after injection of FA in mice. This indicates that it is important to follow the guidelines for immunization procedures. Minimal clinical and behavioural changes were observed, while, severe pathological changes were found after injection of FA and some alternative adjuvants. Consequently, the adjuvant that induces the least pathological changes combined with acceptable immune responses should be preferred (unless clinical or behavioural changes are found). The fact that minimal indications of severe pain and distress are obtained from clinical and behavioural studies stresses the importance of pathology studies when assessing side effects of adjuvants (cf Manser, 1992).

From our studies, it is clear that severe pathological changes were also induced by some alternative adjuvants, while antibody responses with these adjuvants were not always acceptable. It may therefore not always be advantageous to use an alternative to FCA. Immunization with alternative adjuvants of which little information is available, may result in low antibody titres and severe pathological changes. As a result, additional animals may be needed and pathological changes induced in addition. Consequently, when an adjuvant is needed for only a single experiment, using small numbers of animals, and no analogous data on alternative adjuvants are present, we suggest not to test alternative adjuvants, but to use FCA, i.e. when no data are available on experiments using the same type of antigen, using the same animal species and strain, and with the same immunization goal. Testing alternative adjuvants is suggested in recurrent immunization experiments in which FCA is traditionally used. The selection of an adjuvant for combination with an antigen depends on the combined data available on efficacy and side effects of the adjuvant/antigen combination of interest. Several studies, described in this thesis, were performed to generate data on FA and alternative adjuvants. From these studies it can be concluded that when no data are available for adequate adjuvant selection, Specol or Montanide ISA50 can be used in pilot experiments.

The pilot experiment should be performed taking the following into account:

- inclusion of FA in the experiment as 'gold standard'
- use of alternative adjuvant in accordance with guidelines for use of FCA

- clinical and behavioural parameters monitored every day during the experiment
- necropsy at the end of the experiment and scoring of pathological changes.

From the data obtained from the pilot study, the use of the alternative adjuvant in future research can be decided. The alternative adjuvant should only be used when satisfactory immunological results are obtained and the side effects are less severe than after FA injection.

Concluding remarks

The supposed severe side effects induced by FCA injection resulted in restrictions on its use and, consequently, in the need for alternative adjuvants. The selection of an alternative is not without problems since the combined information on efficacy and side effects of these adjuvants is often lacking. The studies described in this thesis were designed to generate data on FCA and alternative adjuvants. Comparative studies were performed in accordance with existing guidelines and data generated showed minimal clinical and behavioural changes indicative for prolonged severe pain. This implies that most severe side effects can be avoided by good biotechnical practice, including aseptic working, proper preparation of emulsion and injection technique. The use of additional behavioural tests for the assessment of pain and distress did not result in clear-cut differences with respect to unwanted side effects of the adjuvants tested. Pathological changes, however, were observed after adjuvant injection and are considered an essential parameter to study, also retrospectively, side effects of adjuvants. The antigen used and the route of injection were shown to greatly influence the induction of pathological changes. The subcutaneous route should be preferred over intramuscular (in rabbits) and intraperitoneal route (in mice).

The offensive attitude with respect to FCA and the suggested need to use alternative adjuvants, was not confirmed by the data underlying this thesis, i.e. severe pathological changes were also observed after injection of alternative adjuvants without effective immune responses and minimal indications of prolonged severe pain and distress after FA injection were demonstrated by the clinical and behavioural tests performed. To minimize the number of animals used and the additional risk of inducing pathological changes, the use of FA is suggested when a single experiment is performed and no adequate data on alternatives are available. For recurrent experiments, a pilot experiment is suggested, using Montanide ISA50 or Specol as an alternative, including FA as a gold standard, injecting alternative adjuvants in accordance with guidelines on the use of FCA, studying clinical and behavioural parameters, and evaluating pathological changes. More research is needed to elucidate the mechanism of action of adjuvants to predict immune responses and to develop a new generation of adjuvants, combining efficacy with minimal side effects.

References

Abrams, J.S., Roncarolo, M.G., Yssel, H., Andersson, U., Gleich, G.J. and Silver, J.E. (1992) Strategies of anti-cytokine monoclonal antibody development: immunoassay of IL-10 and IL-5 in clinical samples. Immunol. Rev. 127, 5-8.

Allison, A.C. and Gregoriadis, G. (1990) Vaccines: recent trends and progress. Immunol. Today 11, 427-429.

Allison, A.C. and Byars, N.E. (1986) An adjuvant formulation that selectively elicits the formation of antibodies of protective isotypes and cell-mediated immunity. J. Immunol. Methods 95, 157-168.

Allison, A.C. and Byars, N.E. (1991) Immunological adjuvants: desirable properties and side effects. Mol. Immunol. 28, 279-284.

Allison, A.C. and Byars, N.E. (1992) Immunological adjuvants and their mode of action. In: Vaccines: New Approaches to Immunological Problems, W.R. Ellis (Ed.), Butterworth-Heineman, Boston, pp. 431-449.

ALTEX (1996) Alternativen zu Tierexperimenten; Dottersack-Antikörper, Egg Yolk Antibodies, IgY-Technology 13 (Suppl.), pp. 1-88.

Alving, C.R. and Richardson, E.C. (1984) Mitogenic activities of lipid A and liposome associated lipid A: Effects of epitope density. Rev. Infect. Dis. 6, 493-496.

Amyx, H.L. (1987) Control of animal pain and distress in antibody production and infectious disease studies. J. Am. Vet. Med. Assoc. 191, 1287-1289.

Arnon, R. (1991) Synthetic peptides as the basis for vaccine design. Mol. Immunol. 28, 209-215.

Audibert, F.M. and Lise, L.D. (1993) Adjuvants: Current status, clinical perspectives and future prospects. Immunol. Today 14(6), 81-284.

Azuma, I., Sugimura, K., Taniyama, T., Yamawaki, M., Yamamura, Y., Kusumoto, S., Okada, S. and Shiba, T. (1976) Adjuvant activity of mycobacterial fractions: Immunological properties of synthetic N-acetylmuramyl dipeptide and related compounds. Infect. Immun. 14, 18-27.

Bangham, J.A. (1988) Data sieving hydrophobicity plots. Anal. Biochem. 174, 142-145.

Barr, I.G. and Mitchell, G.F. (1996) Iscoms (immunostimulating complexes): The first decade. Immunol. Cell Biol. 74, 8-25.

Bateson, P. (1991) Assessment of pain in animals. Anim. Beh. 42, 827-839.

Bennett, B., Check, I.J., Olsen, M.R. and Hunter, R.L. (1992) A comparison of commercially available adjuvants for use in research. J. Immunol. Methods 153, 31-40.

Bessler, W.G. and Jung, G. (1992) Synthetic lipopeptides as novel adjuvants. In: 44th Forum in Immunology. Res. Immunol. 143, 548-553.

Bevan, M.J. (1987) Antigen recognition. Class discrimination in the world of immunology. Nature 325, 192-194.

Boersma, W.J.A., Bogaerts, W.J.C., Bianchi, A.T.J. and Claassen, E. (1992) Adjuvant properties of stable water-in-oil emulsions: evaluation of the experience with Specol. In: 44th Forum in Immunology. Res. Immunol. 143, 503-512.

Boersma, W.J.A., Claassen, E., Deen, C., Gerritse, K., Haaijman, J.J. and Zegers, N.D. (1988) Antibodies to short synthetic peptides for specific recognition of partly denatured protein. Analyt. Chim. Acta 213, 187-197.

Boersma, W.J.A., Deen, C., Gerritse, K., Zegers, N.D., Haaijman, J.J. and Claassen, E. (1989) Anti-peptide antibodies as a subclass specific reagents: epitope mapping of human IgG2. Prot. Biol. Fluids 36, 161-165.

Boersma, W.J.A., Haaijman, J.J. and Claassen, E. (1993) Use of synthetic peptide determinants for the production of antibodies. In: Immunohistochemistry 2nd Edition, A.C. Cuello (Ed.), John Wiley & Sons, New York, pp. 1-77.

Boersma, W.J.A., Zegers, N.D., Van Den Bogaerdt, A., Leer, R.J., Bergmans, A., Pouwels, P.H., Posno, M. and Claassen, E. (1994) Development of safe oral vaccines based on *Lactobacillus* as a vector with adjuvant activity. Proc. ICHEM 2nd. Int. Congress, Biotech UK, Brighton, pp. 43-45.

Bokhout, B.A., Van Gaalen, C. and Van der Heijden, Ph.J. (1981) A selected water-in-oil emulsion: composition and usefulness as an immunological adjuvant. Vet. Immunol. Immunopathol. 2, 491-500.

Bomford, R. (1980) The comparative selectivity of adjuvants for humoral and cell-mediated immunity. I. Effect on the antibody response to bovine serum albumin and sheep red blood cells of Freund's incomplete and complete adjuvants, Alhydrogel, Corynebacterium parvum, Bordetella pertussis, muramyldipeptides and saponin. Clin. Exp. Immunol. 39, 426-434.

Bomford, R., Stapleton, M., Winsor, S., Mcknight, A. and Andronova, T. (1992) The control of the antibody-isotype response to recombinant human immunodeficiency virus gp120 antigen by adjuvants. AIDS Res. Hum. Retroviruses 8, 1765-1771.

Boraschi, D., Nencioni, L., Villa, L., Censini, S., Bossu, P. and Ghiara, P. (1988) *In vivo* stimulation and restoration of the immune response by the non-inflammatory fragment 163-171 of human interleukin-1b. J. Exp. Med. 169, 675-686.

Borras-Cuesta, F., Fedon, Y. and Petit-Camurdan, A. (1988) Enhancement of peptide immunogenicity by linear polymerization. Eur. J. Immunol. 18, 199-202.

Broderson, J.R. (1989) A retrospective review of lesions associated with the use of Freund's adjuvant. Lab. Anim. Sci. 39, 400-405.

Broom, D.M. and Johnson, K.G. (1993) Stress and animal welfare. Chapman & Hall, London, 211pp.

Buiting, A.M.J., Van Rooijen, N. and Claassen, E. (1992) Liposomes as antigen carriers and adjuvants *in vivo*. In: 44th Forum in Immunology. Res. Immunol. 143, 541-548.

Bulthuis, R.J.A., Bergman, A.F., Nijessen, S., Schlingmann, F., Tolboom, J., Remie, R., Van de Weerd, H.A., Van Loo, P.L.P., Baumans, V. and Van Zutphen, L.F.M. (1997) Automated behaviour classification: the LABORAS project. In: Proceedings of the 6th FELASA Symposium 1996, Basel, in press.

Byars, N.E., Allison, A.C., Harmon, M.W. and Kendal, A.P. (1990) Enhancement of antibody responses to influenza B virus haemagglutinin by use of a new adjuvant formulation. Vaccine 8, 49-56.

Byars, N.E., Nakano, G., Welch, M., Lehman, D. and Allison, A.C. (1991) Improvement of hepatitis B vaccine by the use of a new adjuvant. Vaccine 9, 309-318.

Campbell, J.B. and Peerbaye, Y.A. (1992) Saponin. In: 44th Forum in Immunology. Res. Immunol. 143, 526-530.

Canadian Council on Animal Care (1991) CCAC guidelines on acceptable immunological procedures. 315-350 Albert, Ottawa, Ontario, Canada, 2 pp.

Cantrell, J.L. and Finn, D.J. (1989) Role of novel adjuvants in specific active immunotherapy. Prog. Clin. Biol. Res. 288, 297-307.

Carayanniotis, G. and Barber, B.H. (1990) Characterization of the adjuvant-free serological response to protein antigens coupled to antibodies specific for class II MHC determinants. Vaccine 8, 137-144.

Carayanniotis, G., Vizi, E., Parker, R., Hodges, R.S. and Barber, B.H. (1988) Delivery of synthetic peptides by anti class II monoclonal antibodies induces specific adjuvant free IgG response *in vivo*. Mol. Immunol. 25, 907-911.

Cassell, G.H. and Brown, M.B. (1983) Enzyme-linked immunosorbent assay (ELISA) for detection of anti-mycoplasma antibody. In: Methods in Mycoplasmology, Vol. 1: Mycoplasma Characterization, S. Razin and J.G. Tully (Eds.), Academic Press, New York, pp. 457-461.

Casten, L.A. (1988) Enhanced T cell responses to antigenic peptides targeted to B cell surface immunoglobulin, Ia or class I molecules. J. Exp. Med. 168, 171-181.

Chapel, H.M. and August, P.J. (1976) Report on nine cases of accidental injury to man with Freund's complete adjuvants. Clin. Exp. Immunol. 24, 538-541.

Check, I.J., Bennett, B. and Hunter, R.L. (1990) TiterMax, research adjuvant; Technical background. CytRx Corporation, Norcross, GA, 28 pp.

Cherwinski, H.M., Schumacher, J.H., Brown, K.D. and Mosmann, T.R. (1987) Two types of mouse helper T cell clone. III. Further differences in lympokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays, and monoclonal antibodies. J. Exp. Med. 166, 1229-1244.

Chou, P.Y. and Fasman, G.D. (1978) Prediction of the secondary structure of proteins from their amino acid sequences. In: Adv. Enzymol. Vol. 47, A. Meisner (Ed.), Wiley & Sons, New York, pp. 46-148.

Claassen, E. (1991) The role of heterogeneous macrophage macrophage subsets in liposome targeting and immunomodulation. In: 41th Forum in Immunology. Res. Immunol. 143, 255-256.

Claassen, E. (1992) Post-formation fluorescent labelling of liposomal membranes; *in vivo* detection, localization and kinetics. J. Immunol. Methods 147, 231-240.

Claassen, E. (1996) Liposomes in *in vivo* immunology. In: Vesicles, M. Rosoff (Ed.), Surfactant series Vol. 62, Marcel Dekker Inc., New York, pp. 649-678.

Claassen, E. and Adler, L. (1988) Sequential double immunocytochemical staining for the *in situ* study of an auto-anti-allotype immune response in allotype suppressed rabbits. J. Histochem. Cytochem. 36, 1455-1461.

Claassen, E. and Boersma, W.J.A. (1992) Characteristics and practical use of new generation adjuvants as an acceptable alternative for Freund's complete adjuvant. In: 44th Forum in Immunology. Res. Immunol. 143, 475-582.

Claassen, E., Boorsma, D.M., Kors, N. and Van Rooijen, N. (1986a) Double-enzyme conjugates, producing an intermediate color, for simultaneous and direct detection of three different intracellular immunoglobulin determinants with only two enzymes. J. Histochem. Cytochem. 34(4), 423-428.

Claassen, E., de Leeuw, W., de Greeve, P., Hendriksen, C. and Boersma, W. (1992) Freund's complete adjuvant: an effective but disagreeable formula. In: 44th Forum in Immunology. Res. Immunol. 143, 478-483.

Claassen, E., Kors, N. and Van Rooijen, N. (1986b) Influence of carriers on the development and localization of anti-2,4,6-trinitrophenyl (TNP) antibody forming cells in the murine spleen. II. Suppressed antibody response to TNP-Ficoll after elimination of marginal zone cells. Eur. J. Immunol. 16, 492-497.

Claassen, E., Kors, N. and Van Rooijen, N. (1987) Immunomodulation with liposomes: the immune response elicited by liposomes with entrapped dichloromethylene-diphosphonate and surface-associated antigen or hapten. Immunology 60, 509-515.

Claassen, E., Kottenhagen, M.J., Pouwels, P.H., Posno, M., Boersma, W.J.A. and Lucas, C.J. (1994) Use of *Lactobacillus*, a GRAS (Generally Recognized As Safe) organism, as a base for a new generation of "oral" live vaccines. In: Recombinant and Synthetic Vaccines. G.P. Talwar and V.S.R. Kanury (Eds.), Narosa Publishers, New Delhi, pp. 407-412.

Claassen, E. and Van Iwaarden, F. (1997) Liposomes. In: Immunochemistry 2: A Practical Approach. A.P. Johnstone and M .Turner (Eds.), Oxford University Press, Oxford, pp. 53-70.

Claassen, E., Vos, Q., Ott, A., Schellekens, M.M. and Boersma, W.J.A. (1991) Role of the splenic marginal zone in the removal of, and immune response against, neutral polysaccharides. In: Lymphatic Tissues and *in vivo* Immune Responses. B. Imhof, S. Berrih-Aknin and S. Ezine (Eds.), Marcel Dekker Inc., New York. p. 885.

Claassen, E., Westerhof, Y., Versluis, B., Kors, N., Schellekens, M. and Van Rooijen, N. (1988) Effects of chronic injection of sphingomyelin-containing liposomes on lymphoid and non-lymphoid cells in the spleen. Transient suppression of marginal zone macrophages. Br. J. Exp. Pathol. 69, 865-875.

Claassen, E., Van Winsen, R., Posno, M. and Boersma, W.J.A. (1995a) New and safe "oral" live vaccines based on *Lactobacillus*. In: Advances in Mucosal Immunology, Part B, J. Mestecky, M.W. Russel, S. Jackson, S.M. Michalek, H.T. Hogenova, J. Sterzl (Eds.), Plenum Press, New York, pp. 1553-1558.

Claassen, I. and Osterhaus, A. (1992) The ISCOM structure as an immune-enhancing moiety: experience with viral systems. In: 44th Forum in Immunology. Res. Immunol. 143, 531-541.

Claassen, I.J.T.M., Osterhaus, A.D.M.E. and Claassen, E. (1995b) *In vivo* antigen detection after immunization with different presentation forms of rabies virus antigen: Involvement of marginal metallophilic macrophages in the uptake of iscoms. Eur. J. Immunol. 25, 1446-1452.

Constant, S., Sant'Angelo, D., Pasqualini, T., Taylor, T., Levin, D., Flavell, R. and Bottomly, K. (1995) Peptide and protein antigens require distinct antigen-presenting cell subsets for the priming of CD4+ T cells. J. Immunol. 154, 4915-4923.

Cox, J.C. and Coulter, A.R. (1997) Adjuvants - a classification and review of their modes of action. Vaccine 15, 248-256.

Dalsgaard, K. (1978) A study of the isolation and characterization of the saponin Quil-A. Acta Vet. Scand. 19, 1-40.

Daly, T.M. and Long, C.A. (1996) Influence of adjuvants on protection induced by a recombinant fusion protein against malarial infection. Infect. Immun. 64, 2602-2608.

De Becker, G., Sornasse, T., Nabavi, N., Bazin, H., Tielemans, F., Urbain., J., Leo, O. and Moser, M. (1994) Immunoglobulin isotype regulation by antigen-presenting cells *in vivo*. Eur. J. Immunol. 24, 1523-1528.

De Kruif, J., Van der Vuurst de Vries, A.R., Cilenti, L., Boel, E., Van Ewijk, W. and Logtenberg, T. (1996) New perspectives on recombinant human antibodies. Immunol. Today 17, 453-455.

Deeb, B.J., DiGiacomo, R.F., Kunz, L.L. and Stewart, J.L. (1992) Comparison of Freund's and Ribi adjuvants for inducing antibodies to the synthetic antigen (TG)-AL in rabbits. J. Immunol. Methods 152, 105-113.

Deen, C., Claassen, E., Gerritse, K., Zegers, N.D. and Boersma, W.J.A. (1990) A novel carbodiimide coupling method for synthetic peptides enhanced anti-peptide antibody responses. J. Immunol. Methods 129, 119-125.

Delemarre, F.G.A., Kors, N., Kraal, G. and Van Rooijen, N. (1990) Repopulation of macrophages in popliteal lymph nodes of mice after liposome mediated depletion. J. Leukoc. Biol. 47, 251-257.

Delemarre, F.G.A., Kors, N. and Van Rooijen, N. (1991) Elimination of spleen and of lymph node macrophages, and its difference in the effect on the immune response to particulate antigens. Immunobiology 182, 70-78.

Dyson, H.J., Cross, K.J., Houghton, R.A., Wilson, I.A., Wright, P.E. and Lerbner, R.A. (1985) The immunodominant site of a synthetic immmunogen has a conformational preference in water for a type II reverse turn. Nature 318, 480-483.

Eisenberg, D., Schwarz, E., Komaromy, M. and Wall, R.J. (1984a) Analysis of membrane and surface proteins sequences with a hydrophobic moment plot. J. Mol. Biol. 179, 125-142.

Eisenberg, D., Weiss, R.M. and Terwilliger, T.C. (1984b) The hydrophobic moment detects periodicity in protein hydrophobicity. Proc. Natl. Acad. Sci. USA 81, 140-144.

Emini, E.A., Hughes, J.V., Perlow, D.S. and Boger, J. (1985) Induction of hepatitis A virus neutralizing antibody by a virus specific synthetic peptide. J. Virol. 55, 836-839.

Fekadu, M., Shaddock, J.H., Ekstrom, J., Osterhaus, A., Sanderlin, D.W., Sundquist, B. and Morein, B. (1992) An immunestimulating complex (ISCOM) subunit rabies vaccine protects dogs and mice against street rabies challenge. Vaccine 10, 192-197.

Fieser, T.M., Tainer, J.A., Geysen, H.M., Houghten, R.A. and Lerner, R.A. (1987) Influence of protein flexibility and peptide conformation on reactivity of monoclonal antipeptide antibodies with a protein a-helix. Proc. Natl. Acad. Sci USA 84, 8568-8572.

Finkelman, F.D., Holmes, J., Katona, I.M., Uraban, J.F., Beckman, M.P., Park, L.S., Schooley, K.A., Coffman, R.L., Mosmann, T.R. and Paul, W.E. (1990) Lymphokine control of *in vivo* immunoglobulin isotype selection. Ann. Rev. Immunol. 8, 303-333.

Finkelman, F.D., Katona, I.M., Mosmann, T.R. and Coffman, R.L. (1988a) Interferon-γ regulates the isotypes of immunoglobulin secreted during *in vivo* humoral immune responses. J. Immunol. 140, 1022-1027.

Finkelman, F.D., Katona, I.M., Urban, J.F., Holmes, J., Ohara, J., Tung, A.S., Sample, J.V. and Paul, W.E. (1988b) IL-4 is required to generate and sustain *in vivo* IgE responses. J. Immunol. 141, 2335-2341.

Fossum, S. (1980) The architecture of rat lymph nodes. IV. Distribution of ferritin and colloidal carbon in the draining lymph nodes after foot-pad injection. Scand. J. Immunol. 12, 433-441.

Fox, B.S. (1992) Antibody responses to a cytochrome c peptide do not correlate with lymphokine production patterns from helper T cell subsets. Immunology 75, 164-169.

Francis, M.J., Hastings, G.Z., Syred, A.D., McGinn, B., Brown, F. and Rowlands, D. (1987) Non responsiveness to a foot and mouth disease virus peptide overcome by addition of foreign helper T cell determinants. Nature 330, 168-171.

Freund, J. (1951) The effect of paraffin oil and mycobacteria on antibody formation and sensitization: a review. Am. J. Clin. Pathol. 21, 645-656.

Freund, J. (1956) The mode of action of immunologic adjuvants. Adv. Tuberc. Res. 7, 130-148.

Freund, J., Casals, J. and Hosmer, E.P. (1937) Sensitization and antibody formation after injection of tubercle bacilli and parafin oil. Proc. Soc. Exp. Biol. Med. 37, 509-513.

Fry, J.N., Lisak, R.P., Manning, M.C. and Silberberg, D.H. (1976) Serological techniques for detection of antibody to galactocerebroside. J. Immunol. Methods 11, 185-193.

Ganne, V., Eloit, M., Laval, A., Adam, M. and Trouve, G. (1994) Enhancement of the efficacy of a replication-defective adenovirus-vectored vaccine by the addition of oil adjuvants. Vaccine 12, 1190-1196.

Garnier, J., Osguthorpe, D.J. and Robson, B. (1978) Analysis of accuracy and implications of simple methods for predicting the secondary structure of globular proteins. J. Mol. Biol. 120, 97-120.

Geerligs, H.J., Weijer, W.J., Welling, G.W. and Welling-Wester, S. (1989) The influence of different adjuvants on the immune respons to a synthetic peptide comprising amino acid residues 9-21 of herpes simplex virus type 1 glycoprotein D. J. Immunol. Methods 124, 95-102.

Germain, R.N. (1986) The ins and outs of antigen processing and presentation. Nature 322, 687-689.

Gerritse, K., Slierendregt, B., Deen, C., Fasbender, M.J., Boersma, W.J.A. and Claassen, E. (1993) *In situ* detection of self reactive epitope specific antibody forming cells in the central nervous system of EAE rhesus monkey. EOS-J. Immunol. Immuno pharmacology 13, 63-65.

Girolomoni, G. and Ricciardi-Castagnoli, P. (1997) Dendritic cells hold promise for immunotherapy. Immunol. Today 18(3), 102-104.

Greeve, de P., Duimel, P., Thuring, J., Canninga, P., Geurts van Kessel, P., Hein, B., Storm, P., Rijke, E., Bertens, A. and Van de Gulden, W. (1982) Bereiding van immuunsera (Production of antisera) (in Dutch). Biotechniek 21, 69-75.

Griffiths, P.H.M. (1991) Clinical assessment of pain, distress and discomfort. In: Animals in Biomedical Research; Replacement, Reduction and Refinement: Present Possibilities and Future Prospects. C.F.M. Hendriksen and H.B.W.M. Koëter (Eds.), Elsevier Science Publishers B.V., Amsterdam, the Netherlands. pp. 235-246.

Grun, J.L. and Maurer, P.H. (1989) Different T helper subsets elicited in mice utilizing two different adjuvant vehicles: the role of endogenous interleukin-1 in proliferative responses. Cell. Immunol. 121, 134-145.

Gupta, R.K. and Siber, G.R. (1995) Adjuvants for human vaccines - current status, problems and future prospects. Vaccine 13(14), 1263-1276.

Gupta, R.K., Relyveld, E.H., Lindblad, E.B., Bizzini, B., Ben-Efraim, S. and Gupta, C.K. (1993) Adjuvants - A balance between toxicity and adjuvanticity. Vaccine 11, 293-306.

Gustafson, G.L. and Rhodes, M.J. (1992) Bacterial cell wall products as adjuvants: early interferon-gamma as a marker for adjuvants that enhance protective immunity. In: 44th Forum in Immunology. Res. Immunol. 143, 483-488.

Haaijman, J.J., Coolen, J., Deen, C., Kroese, C.J.M., Zijlstra, J.J. and Radl, J. (1988) Monoclonal antibodies directed against human immunoglobulins: preparation and evaluation procedures. In: Reviews on Immunoassay Technology 1, S.B. Pal (Ed.), MacMillan, London, pp. 59-93.

Hackett, C.J., Hurwitz, J.L., Dietzschold, B. and Gerhard, W. (1985) A synthetic decapeptide of influenza virus hemagglutinin elicits helper T cells with the same fine recognition specificities as occur in response to whole virus. J. Immunol. 135, 1391-1394.

Hadjipetrou-Kourounakis, L. and Möller, E. (1984) Adjuvants influence the immunoglobin subclass distribution of immune responses *in vivo*. Scand. J. Immunol. 19, 219-225.

Harding, C.V., Collins, D.S., Kanagawa, O. and Unanue, E.R. (1991) Liposome-encapsulated antigens engender lysosomal processing for class II MHC presentation and cytosolic processing for class I presentation. J. Immunol. 147, 2860-2863.

HayGlass, K.T. and Stefura, B.P. (1991) Anti-interferon-gamma treatment blocks the ability of glutaraldehyde-polymerized allergens to inhibit specific IgE responses. J. Exp. Med. 173, 279-285.

Heath, A.W. and Playfair, J.H.L. (1990) Conjugation of interferon gamma to antigen enhances its adjuvanticity. Immunology 71, 454-456.

Heath, A.W. and Playfair, J.H.L. (1992) Cytokines as immununological adjuvants. Vaccine 10, 427-434.

Herbert, W.J. and Kristensen, F. (1986) Laboratory animal techniques for immunology. In: Handbook of Experimental Immunology in Four Volumes, Vol. 4: Applications of Immunological Methods in Biomedical Sciences, D.M. Weir (Ed.), Blackwell Scientific Publications, Oxford, pp. 133.1-133.36.

Hewetson, J.F., Rivera, V.R., Creasia, D.A., Lemley, P.V., Rippy, M.K. and Poli, M.A. (1993) Protection of mice from inhaled ricin by vaccination with ricin or by passive treatment with heterologous antibody. Vaccine 11(7), 743-746.

Hilgers, L.A.T. and Snippe, H. (1992) DDA as an immunological adjuvant. In: 44th Forum in Immunology. Res. Immunol. 143, 494-503.

Hong, T.H., Chen, S.T., Tang, T.S., Wang, S.C. and Chang, T.H. (1989) The production of polyclonal and monoclonal antibodies in mice using novel immunization methods. J. Immunol. Methods 120, 151-157.

Hopp, T.P. (1986) Protein surface analysis. Methods for identifying antigenic determinants and other interaction sites. J. Immunol. Methods 88, 1-18.

Hopp, T.P. and Woods, K.R. (1981) Prediction of protein antigenic determinants from amino acid sequences. Proc. Natl. Acad. Sci. USA 78, 3821-3824.

Horiuchi, N., Caulfield, P., Fisher, J.E., Goldman, M.E., McKee, R., Reagan, J.E., Levy, J.J., Nutt, R.F., Rodan, S.B., Schofield, T.L., Clemens, T.L. and Rosenblatt, M. (1987) Similarity of synthetic peptide from human tumor to parathyroid hormone *in vivo* and *in vitro*. Science 238, 1566-1568.

Hsieh, C.S., Heimberger, A.B., Gold, J.S., O'Garra, A. and Murphy, K.M. (1992) Differential regulation of T helper phenotype development by interleukins 4 and 10 in an alpha beta T cell receptor transgenic system. Proc. Natl. Acad. Sci. USA 89, 6065-6069.

Hughes, L.E., Kearney, R. and Tully, M. (1970) A study in clinical cancer immunotherapy. Cancer 26, 269-278.

Hunter, R.L. and Bennett, B. (1984) The adjuvant activity of nonionic block polymer surfactants. II. Antibody formation and inflammation related to the structure of triblock and actablock copolymers. J. Immunol. 133(6), 3167-3175.

Hunter, R.L. and Bennett, B. (1986) The adjuvant activity of nonionic block polymer surfactants. III. Characterization of selected biologically active surfaces. Scand. J. Immunol. 23, 287-300.

Hunter, R.L., Olsen, M.R. and Bennett, B. (1995) Copolymer adjuvants and TiterMax. In: The Theory and Practical Application of Adjuvants, D.E.S. Stewart-Tull (Ed.), John Wiley & Sons, Chichester, pp. 51-94.

Hunter, R.L., Strickland, F. and Kezdy, F. (1981) Studies on the adjuvant activity of nonionic block polymer surfactants. I. The role of hydrophile-lipophile balance. J. Immunol. 127, 1244-1250.

Ibrahim, M.A.A., Chain, B.M. and Katz, D.R. (1995) The injured cell: the role of the dendritic cell system as a sentinel receptor pathway. Immunol. Today 16, 181-186.

Irwin, S. (1968) Comprehensive observational assessment: Ia. A systematic, quantitative procedure for assessing the behavioural and physiological state of the mouse. Psychopharmacology Series (Berlin) 13, 222-257.

Jackson, S., Mestecky, J., Childers, N.K. and Michalek, S.M. (1990) Liposomes containing anti-idiotypic antibodies: an oral vacine to induce protective secretory immune responses specific for pathogens of mucosal surfaces. Infect. Immun. 58, 1932-1936.

Jacob, C.O., Grossfeld, S., Sela, M. and Arnon, R. (1986) Priming immune responses to cholera toxin induced by synthetic peptides. Eur. J. Immunol. 16, 1057-1062.

Jansen van 't Land, C. and Hendriksen, C.F.M. (1995) Change in locomotor activity pattern in mice: a model for recognition of distress? Lab. Animals 29, 286-293.

Jemmerson, R. and Hutchinson, R.M. (1990) Fine manipulation of antibody affinity for synthetic epitopes by altering peptide structure: antibody binding to looped peptides. Eur. J. Immunol. 20, 579-585.

Johnson, A.G., Gaines, S. and Landy, M. (1956) Studies on the O antigen of *salmonella typhosa*. V. Enhancement of antibody responses to protein antigens by the purified lipopolysaccharide. J. Exp. Med. 103, 225-246.

Johnson, A.G. and Tomai, M.A. (1990) A study of the cellular and molecular mediators of the adjuvant action of a nontoxic monophosphoryl lipid A. Adv. Exp. Med. Biol. 256, 567-579.

Johnston, B.A., Eisen, H. and Fry, D. (1991) An evaluation of several adjuvant emulsion regimens for production of polyclonal antisera in rabbits. Lab. Anim. Sci. 41(1), 15-21.

Jones, G.L., Spencer, L., Lord, R., Mollard, R., Pye, D. and Saul, A. (1990) Peptide vaccines derived from a malarial surface antigen: effects of dose and adjuvants on immunogenicity. Immunol. Lett. 24, 253-260.

Jones, P.D., Tha Hla, R., Morein, B., Lövgren, K. and Ada, G.L. (1988) Cellular immune responses in the murine lung to local immunozation with influenza A virus glycoproteins in micelles and immunostimulatory complexes (ISCOM). Scand. J. Immunol. 27, 645-652.

Jotwani, R., Tanaka, Y., Watanabe, K., Tanaka-Bandoh, K., Kato, N. and Ueno, K. (1994) Comparison of cytokine induction by Lipopolysaccharide of *Bacteroides fragilis* with *Salmonella typhimurium* in mice. Microbiol. Immunol. 38(9), 763-766.

Karagouni, E.E. and Hadjipetrou-Kourounakis, L. (1990) Regulation of isotype immunoglobulin production by adjuvants *in vivo*. Scand. J. Immunol. 31, 745-754.

Karplus, P.A. and Schulz, G.E. (1985) Prediction of chain flexibility in proteins. Naturwissenschaften 72, 212-223.

Kast, W.M., Brandt, R.M.P. and Melief, C.J.M. (1993) Strict peptide length is not required for the induction of cytotoxic T lymphocyte-mediated antiviral protection by peptide vaccination. Eur. J. Immunol. 23, 1189-1192.

Kaufmann, S.H.E. (1995) Immunity to intracellular microbial pathogens. Immunol. Today 16, 338-343.

Kenney, J.S., Hughes, B.W., Masada, M.P. and Allison, A.C. (1989) Influence of adjuvants on the quantity, affinity, isotype and epitope specificity of murine antibodies. J. Immunol. Methods 121, 157-166.

Kensil, C.R., Patel, U., Lennick, M. and Marciani, D. (1991) Separation and characterization of saponins with adjuvant activity form *Quillaja saponaria Molina* cortex. J. Immunol. 146, 431-437.

Kersten, G.F.A. (1990) Aspects of iscoms. Structural, pharmaceutical and adjuvant properties. PhD Thesis, State University Utrecht, the Netherlands.

Koch, G., Lok, B.D., Van Oudenaren, A. and Benner, R. (1982) The capacity and mechanism of bone marrow antibody formation by thymus-independent antigens. J. Immunol. 128, 1497-1501.

Kraal, G., Breel, M., Janse, M. and Bruin, G. (1986) Langerhans' cells, veiled cells and interdigitating cells in the mouse recognized by a monoclonal antibody. J. Exp. Med. 163, 981-997.

Kuziel, W.A. and Greene, W.C. (1990) Interleukin-2 and the IL-2 receptor: new insights into structure and function. The Society for Investigative Dermatology, 94(6) Supplement: 27S-32S.

Kyte, J. and Doolittle, R.F. (1982) A simple method for displaying the hydropathic character of protein. J. Mol. Biol. 157, 105-132.

Laman, J.D. and de Boer, M. (1997) Monoclonal antibodies for modulation of B cell activity. In: Immunology Methods Manual. I. Lefkovits (Ed.), Academic Press, London, pp. 1016-1028.

Laman, J.D. and Claassen, E. (1996) T cell independent and T cell dependent humoral immunity In: Cytokine Regulation of Humoral Immunity: Basic and Clinical Aspects. C.M. Snapper (Ed.), John Wiley & Sons, Chichester, pp. 23-72.

Lanzavecchia, A. (1990) Receptor-mediated antigen uptake and its effect on antigen presentation to class II-restricted T lymphocytes. Ann. Rev. Immunol. 8, 773-793.

Le Gros, G., Ben-Sasson, S.Z., Seder, R., Finkelman, F.D. and Paul, W.E. (1990). Generation of interleukin-4 producing cells *in vivo* and *in vitro*:IL-2 and IL-4 are required for *in vitro* generation of IL-4 producing cells. J. Exp. Med. 172, 921-929.

Leenaars, P.P.A.M., Hendriksen, C.F.M., Angulo, A.F., Koedam, M.A. and Claassen, E. (1994) Evaluation of several adjuvants as alternatives to the use of Freund's adjuvant in rabbits. Vet. Immunol. Immunopathol. 40, 225-241.

Leenaars, P.P.A.M., Hendriksen, C.F.M., Koedam, M.A., Claassen, I. and Claassen, E. (1995) Comparison of adjuvants for immunopotentiating properties and side effects in mice. Vet. Immunol. Immunopathol. 48, 123-138.

Leenaars, P.P.A.M., Hendriksen, C.F.M., Savelkoul, H.F.J., Van Rooijen, N. and Claassen, E. (1997) Increased adjuvant efficacy in stimulation of antibody responses after macrophage elimination *in vivo*. Immunology 90, 337-343.

Leenaars, P.P.A.M., Oostermeijer, H.H.A., Hendriksen, C.F.M. and Claassen, E. A rapid and simple method to localize oil based adjuvants *in vivo*. J. Immunol. Methods, in press.

Leenaars, P.P.A.M., Koedam, M.A., Wester, P.W., Baumans, V., Claassen, E. and Hendriksen, C.F.M. Assessment of side effects induced by injection of different adjuvant/antigen combinations in rabbits and mice. submitted.

Lees, A., Morris, S.C., Thyphronitis, G., Holmes, J.M., Inman, J.K. and Finkelman, F.D. (1990) Rapid stimulation of large specific antibody responses with conjugates of antigen and anti-IgD antibody. J. Immunol. 145, 3594-3600.

Liew, F.Y. (1985) New aspects of vaccine development. Clin. Exp. Immunol. 62, 225-241.

Lipman, N.S., Trudel, L.J., Murphy, J.C. and Sahali, Y. (1992) Comparison of immune response potentiation and *in vivo* inflammatory effects of Freund's and RIBI adjvuants in mice. Lab. Anim. Sci. 42, 193-197.

Lövgren, K., Kaberg, H. and Morein, B. (1990) An experimental subunit vaccine (iscom) induced protective immunity to influenza virus infection in mice after a single intranasal administration. Clin. Exp. Immunol. 82, 435-439.

Lövgren, K. and Larsson, M. (1994) Conjugation of synthetic peptides to carrier ISCOMs: Factors affecting the immunogenicity of the conjugate. J. Immunol. Methods 173, 237-243.

Lövgren, K., Lindmark, J., Pipkorn, R. and Morein, B. (1987) Antigenic preparation of small molecules and synthetic peptides conjugated to a preformed iscom as a carrier. J. Immunol. Methods 98, 137-143.

Lövgren, K. and Morein, B. (1988) The requirement of lipids for the formation of immunostimulating complexes (iscoms). Biotech. Appl. Biochem. 10, 161-172.

Lycke, N., Bromander, A.K., Ekman, L., Karlsson, U. and Holmgren, J. (1989) Cellular basis of immunomodulation by cholera toxin *in vitro* with possible association to the adjuvant function *in vivo*. J. Immunol. 142, 20-27.

Mallon, F.M., Graichen, M.E., Conway, B.R. and Landi, M.S. (1991) Comparison of antibody response by use of synthetic adjuvant system and Freund complete adjuvant in rabbits. Am. J. Vet. Res. 52(9), 1503-1506.

Manser, C.E. (1992) The assessment of stress in laboratory animals. RSCPA, 208 pp.

Martinon, F., Gras-Masse, H., Boutillon, C., Chirat, F., Deprez, B., Guillet, J.G., Gomard, E., Tartar, A. and Levy, J.P. (1992) Immunization of mice with lipopeptides bypasses the prerequisite for adjuvant: Immune response of Balb/c mice to human immuno-deficiency virus envelope glycoprotein. J. Immunol. 149, 3416-3422.

Masihi, K.N., Lange, W., Brehmer, W. and Ribi, E. (1986) Effects of muramyl dipeptide and trehalose dimycolate on resistance of mice to *Toxoplasma gondii* and *Acanthamoeba culbertsoni* infections. Int. Arch. Allergy Appl. Immunol. 81, 112-117.

McCafferty, J, Griffiths, A.D., Winter, G. and Chiswell, D.J. (1990). Phage antibodies: filamentous phage displaying antibody variable domains. Nature 348, 552-554.

McKercher, P.D. (1986) Oil adjuvants: Their use in veterinary biologics. In: Advances in Carriers and Adjuvants for Veterinary Biologists. R.M. Nervig, P.M. Gough, M.L. Kaeberle and C.A. Whetstone (Eds.) Ames: Iowa State University Press, pp. 1-7.

McLean, G.W., Owsiaka, A.M., Subak-Sharpe, J.H. and Marsden, H.S. (1991) Generation of antipeptide and anti-protein sera. effect of peptide presentation on immunogenicity. J. Immunol. Methods 137, 149-157.

Mirza, I.H., Wilkin, T.J., Cantarini, M. and Moore, K. (1987) A comparison of spleen and lymphe node cells as fusion partners for the raising of monoclonal antibodies after different routes of immunization. J. Immunol. Methods 105, 235-243.

Morein, B. (1990) The ISCOM: an immunostimulating system. Immunol. Lett. 25, 281-283.

Morein, B., Lövgren, K., Höglund, S. and Sundquist, B. (1987) The ISCOM: an immunostimulating complex. Immunol. Today 8(11), 333-338.

Morton, D.B. and Griffiths, P.H.M. (1985) Guidelines on the recognition of pain, distress and discomfort in experimental animals and an hypothesis for assessment. Vet. Record 116, 431-436.

Mosmann, T.R., Cherwinski, H., Bond, M.W., Giedlin, M.A. and Coffman, R.L. (1986) Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J. Immunol. 136, 2348-2357.

Mosmann, T.R. and Coffman, R.L. (1989) Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. Ann. Rev. Immunol. 7, 145-173.

Mosmann, T.R. and Sad, S. (1996) The expanding universe of T cell subsets: Th1, Th2 and more. Immunol. Today 17(3), 138-146.

Muzon, J. (1964) Effect of bacteria and abacterial products on antibody response. In: Advances in Immunology, Vol. 4, F.J. Dixon Jr and J.H. Humphrey (Eds.), Academic Press, New York, pp. 397-440.

Nair, S., Zhou, X., Huang, L. and Rouse, B.T. (1992) Class I restricted CTL recognition of a soluble protein delivered by liposomes containing lipophilic polylysines. J. Immunol. Methods 152, 237-243.

National Institute of Health (1988) NIH intramural recommendations for the research use of Freund's adjuvant. ILAR News 30(2), 9.

Neurath, A.R., Strick, N. and Girard, M. (1989) Hepatitis B virus surface antigen (HbsAg) as a carrier for synthetic peptides having an attached hydrophobic tail. Mol. Immunol. 26, 53-62.

Newman, W.J., Wu, J.Y., Gardner, B.H., Munroe, K.J., Leombruno, D., Recchia, J., Kensil, C.R. and Coughlin, R.T. (1992) Saponin adjuvant induction of ovalbumin specific CD8 cytotoxic T-lymphocyte responses. J. Immunol. 148, 2357-2362.

Nicklas, W. (1992) Aluminium salts. In : 44th Forum in Immunology. Res. Immunol. 143, 489-494.

Noelle, R.J., Roy, M., Shepherd, D.M., Stamenkovic, I., Ledbetter, J.A. and Aruffo, A. (1992) A 39 kDa protein on activated helper T cells binds CD40 and transduces the signal for cognate activition of B cells. Proc. Natl. Acad. Sci. USA, 89, 6550-6554.

Novotny, J., Handschuhmacher, M., Haber, E., Bruccoleri, R.F., Carlson, R.E., Fanning, W.B., Smith, J.A. and Rose, G.D. (1986) Antigenic determinants in proteins coincide with surface regions accessible to large probes (antibody domains). Proc. Natl. Acad. Sci. USA 83, 226-230.

Ohara, J. and Paul, W.E. (1985) Production of a monoclonal antibody to and molecular characterization of B cell stimulatory factor-1. Nature 315, 333-336.

Ohlin, M. and Borrebaeck, C.A.K. (1992) Production of human monoclonal antibodies. In: Methods of Immunological Analysis. R.F. Masseyeff, W.H.W. Albert and N.A. Staines (Eds.), VCH Verlagsgesellschaff GmbH, Weinheim, p. 298.

Parkman, P.D. and Meyer, H.M. (1966) Attenuated Rubella Virus. I. Development and laboratory characterization. New Engl. J. Med. 275, 569-274.

Partidos, C.D., Obeid, O.E. and Steward, M.W. (1992) Antibody responses to non-immunogenic synthetic peptides induced by co-immunization with immunogenic peptides. Immunology 77, 262-268

Pathak, S.S, Tibbe, G.J.M. and Savelkoul, H.F.J. (1997) Determination of antibody affinity and affinity distribution. In: Immunology Methods Manual. I. Lefkovits (Ed.), Academic Press, London, pp. 1077-1094.

Peeters, J.M., Hazendonk, T.G., Beuvery, E.C. and Tesser, G.I. (1989) Comparison on four bifunctional reagents for coupling peptides to proteins and the effect of the three moieties on the immunogenicity of the conjugates. J. Immunol. Methods 120, 133-143.

Peterson, N.C. (1996). Recombinant antibodies: Alternative strageties for developing and manipulating murine-derived monoclonal antibodies. Lab. Anim. Sci. 46, 8-14.

Phillips, N.C. and Emili, A. (1992) Enhanced antibody response to liposome associated protein antigens: preferential stimulation of IgG2a/b production. Vaccine 10, 151-158.

Prieto, I., Hervas-Stubbs, S., Garcia-Granero, M., Berasain, C., Riezu-Boj, J.I., Lasarte, J.J., Sarobe, P., Prieto, J. and Borras-Cuesta, F. (1995) Simple strategy to induce antibodies of distinct specificity: application to the mapping of gp120 and inhibition of HIV-I infectivity. Eur. J. Immunol. 25, 877-883.

Ribi, E., Amano, K., Cantrell, J., Schwartzman, S., Parker, R. and Takayama, K. (1982) Preparation and antitumor activity of nontoxic lipid A. Cancer Immunol. Immunother. 12, 91.

Rizzo, L.V., DeKruyff, R.H. and Umetsu, D.T. (1992) Generation of B cell memory and affinity maturation; induction with Th1 and Th2 cell clones. J. Immunol. 148, 3733-3739.

Roberge, F.G., Xu, D. and Chan, C.C. (1992) A new effective and non-harmful chemical adjuvant for the induction of experimental autoimmune uveoretinitis. Curr. Eye Res. 11(4), 371-376.

Rosendal, S. and Black, F.T. (1972) Direct and indirect immunofluorescence of unfixed and fixed mycoplasma colonies. Acta Path. Microbiol. Scand. Section B. 80, 616-622.

Rudbach, J.A., Johnson, D.A. and Ulrich, J.T. (1995) Ribi adjuvants: chemistry, biology and utility in vaccines for human and veterinary medicine. In: The Theory and Practical Application of Adjuvants, D.E.S. Stewart-Tull (Ed.), John Wiley & Sons, Chichester, pp. 287-313.

Ruedl, Ch., Albin, B., Bock, G., Wick, G., Wolf, H. and Albini, B. (1993) Oral administration of a bacterial immunomodulator enhances murine intestinal lamina propria and the peyer's patch lymphocyte traffic to the lung: possible implications for infectious disease prophylaxis and therapy. Int. Immunol. 5, 29-36.

Sarobe, P., Lasarte, J.J., Golvano, J., Gullon, A., Gullon, A., Aveira, M.P., Prieta, J. and Borras-Cuesta, F. (1991) Induction of antibodies against a peptide hapten and a class II presentable helper peptide. Eur. J. Immunol. 21, 155-160.

Scalzo, A.A., Elliott, S.L., Cox, J., Gardner, J., Moss, D.J. and Suhrbier, A. (1995) Induction of protective cytotoxic T cells to murine cytomegalovirus by using nonapeptide and a human-compatible adjuvant (Montanide ISA720). J. Virol. 69, 1306-1309.

Schade, R., Staak, C., Hendriksen, C., Erhard, M., Hugl, H., Koch, G., Larsson, A., Pollmann, W., Van Regenmortel, M., Rijke, E., Spielmann, H., Steinbusch, H. and Straughan, D. (1996) The production of avian (Egg Yolk) antibodies: IgY. ECVAM Workshop Report 21; ATLA 24, 925-934.

Shenoy, M. and Christadoss, P. (1993) Induction of experimental autoimmune myasthenia gravis with acetylcholine receptors using a nonionic block copolymer as adjuvant. Immunol. Invest. 22(4), 267-282.

Sjölander, A., Lövgren-Bengtsson, K., Johansson, M. and Morein, B. (1996) Kinetics, localization and isotype profile of antibody responses to immune stimulating complexes (Iscoms) containing human influenza virus envelope glycoproteins. Scand. J. Immunol. 43, 164-172.

Skea, D.L. and Barber, B.H. (1993) Studies of the adjuvant independent antibody response to immunotargeting. J. Immunol. 151, 3557-3568.

Smith, D.E., O'Brien, M.E., Palmer, V.J. and Sadowski, J.A. (1992) The selection of an adjuvant emulsion for polyclonal antibody production using a low-molecular-weight antigen in rabbits. Lab. Anim. Sci. 42(6), 599-601.

Snapper, C.M. and Mond, J.J. (1993) Towards a comprehensive view of immunoglobulin class switching. Immunol. Today 14 (1), 15-17.

Snippe, H., Verheul, A.F.M. and Hilgers, L.A.Th. (1992) Characteristics and use of new-generation adjuvants. In: 44th Forum in Immunology. Res. Immunol. 143, 574-576.

Speijers, G.J.A., Danse, L.H.J.C., Beuvery, E.C., Strik, J.J.T.W.A. and Vos, J.G. (1988) Local reactions of the saponin Quil A and a Quil A containing iscom measles vaccine after intramuscular injection of rats: a comparison with the effect of DTP-polio vaccine. Fundam. Appl. Toxicol. 10, 425-430.

Spitz, M. (1986) Single-shot intrasplenic immunization for the production of monoclonal antibodies. In: Methods in enzymology, Vol. 121, J.J. Langone and H.V. Vunakis (Eds.), Academic Press, New York, pp. 33-41.

Stafleu, F.R., Rivas, E., Rivas, T., Vorstenbosch, J., Heeger, F.R. and Beynen, A.C. (1992) The use of analogous reasoning for assessing discomfort in laboratory animals. Animal Welfare 1, 77-84.

Staruch, M.J. and Wood, D.D. (1983) The adjuvanticity of interleukin 1 *in vivo*. J. Immunol. 130, 2191-2194.

Steinman, R.M. (1991) The dendritic cell system and its role in immunogenicity. Ann. Rev. Immunol. 9, 271-296.

Stevens, T.L., Bossie, A., Sanders, R., Fernandez-Botran, R., Coffman, L., Mosmann, T.R. and Vitetta, E.S. (1988) Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. Nature 334, 255-258.

Stewart-Tull, D.E.S. (1995a) Freund-type mineral oil adjuvant emulsions. In: The Theory and Practical Application of Adjuvants. D.E.S. Stewart-Tull (Ed.), John Wiley & Sons, Chichester, pp. 1-19.

Stewart-Tull, D.E.S. (1995b) The theory and practical application of adjuvants. D.E.S. Stewart-Tull (Ed.), John Wiley & Sons, Chichester, 380 pp.

Stills, H.F. and Bailey, M.Q. (1991) The use of Freund's complete adjuvant. Lab Animal 20, 25-30.

Street, N.E., Schumacher, J.H., Fong, T.A.T., Bass, H., Fiorentino, D.F., Leverah, J.A. and Mosmann, T.R. (1990) Heterogeneity of mouse helper T cells. Evidence from bulk cultures and limiting dilution cloning for precursors of Th1 and Th2 cells. J. Immunol. 144, 1629-1639.

Svedson Bollen, L., Crowley, A., Stodulski, G. and Hau, J. (1996) Antibody production in rabbits and chickens immunized with human IgG; A comparison of titre and avidity development in rabbit serum, chicken serum and egg yolk using three different adjuvants. J. Immunol. Methods 191, 113-120.

Swain, S.L., Weinber, A.D., English, M. and Huston, G. (1990) IL-4 directs the development of Th2 like helper effectors. J. Immunol. 145, 3796-3806.

Tagliabue, A. and Boraschi, D. (1993) Cytokines as vaccine adjuvants: Interleukin 1 and its synthetic peptide 163-171. Vaccine 11, 594-595.

Takayama, K., Olsen, M., Datta, P. and Hunter, R.L. (1991) Adjuvant activity of nonionic block copolymers. - V. Modulation of antibody isotype by lipopolysaccharides, lipid A and precursors. Vaccine 9, 257-265.

Tam, J.P. and Zavala, F. (1989) Multiple antigen peptide. A novel approach to increase detection sensitivity of synthetic peptides in solid phase immuno-assays. J. Immunol. Methods 124, 53-61.

Ten Hagen, T.L.M., Verheul, A.F.M., Snippe, H. and Hunter, R.L. (1993) Role of adjuvants in the modulation of antibody isotype, specificity, and induction of protection by whole blood-stage Plasmodium yoelli vaccines. J. Immunol. 151, 7077-7085.

Thapar, M.A., Parr, E.L., Bozzola, J.J. and Parr, M.B. (1991) Secretory immune responses in the mouse vagina after parenteral or intravaginal immunization with an immunostimulating complex (ISCOM), Vaccine 9(2), 129-133.

Tiong, G.K.L., Gill, J.S., Lofthouse, S. and Nirdosh, K.P. (1993) Comparison of conventional adjuvants and 'adjuvant-free' monoclonal antibody targeting for stimulating antibody responses against a conjugate of luteinizing hormone releasing hormone and avidin. Vaccine 11(4), 425-430.

Toth, L.A., Dunlap, A.W., Olson, G.A. and Hessler, J.R. (1989) An evaluation of distress following intraperitoneal immunization with Freund's adjuvant in mice. Lab. Anim. Sci. 39, 122-126.

Vajdy, M. and Lycke, N.Y. (1992) Cholera toxin adjuvant promotes long-term immunological memory in the gut mucosa to unrelated immunogens after oral immunization. Immunology 75(3), 488-492.

Valensi, J.M., Carlson, J.R. and Van Nest, G.A. (1994) Systemic cytokine profiles in BALB/c mice immunized with trivalent influenza vaccine containing MF59 oil emulsion and other advanced adjuvants. J. Immunol. 153, 4029-4039.

Van Binnendijk, R.S., Van der Heijden, R.W.J., Van Amerongen, G., UytdeHaag, F.G.C.M. and Osterhaus, A.D.M.E. (1994) Viral replication and development of specific immunity in macaques after infection with different measles virus strains. J. Infect. Dis. 170, 443-448.

Van de Weerd, H.A. (1996) Environmental enrichment for laboratory mice: preferences and consequences. PhD Thesis, Utrecht University, the Netherlands.

Van de Wijgert, J.H.H.M., Verheul, A.F.M., Snippe, H., Check, I.J. and Hunter, R.L. (1991) Immunogenicity of S. pneumoniae type 14 capsular polysaccharide: influence of carriers and adjuvants on istotype distribution. Infect. Immun. 59, 2750-2757.

Van den Broek, F.A.R., Peters, M.A.W., Van Tintelen, G., Kaper, J., Van Beresteijn, E.C.H. and Beynen, A.C. (1994) The influence of intraperitoneal injection of Freund's adjuvant on some distress indicators in mice (in Dutch). Biotechniek 33(1), 9-11.

Van den Eertwegh, A.J.M., Boersma, W.J.A. and Claassen, E. (1992) Immunological functions and *in vivo* cell-cell interactions of T cells in the spleen. Crit. Rev. Immunol. 11(6), 337-380.

Van den Eertwegh, A.J.M., Laman, J.D., Schellekens, M.M., Boersma, W.J.A. and Claassen, E. (1992) Complement mediated follicular localization of T-independent type-2 antigens: the role of marginal zone macrophages revisited. Eur. J. Immunol. 22, 719-726.

Van den Eertwegh, A.J.M., Noelle, R.J., Roy, M., Shepherd, D.M., Aruffo, A., Ledbetter, J.A., Boersma, W.J.A. and Claassen, E. (1993) *In vivo* CD40-gp39 interactions are essential for thymus-dependent humoral immunity. I. *In vivo* expression of CD40 ligand, cytokines, and antibody production delineates sites of cognate T-B cell interactions. J. Exp. Med. 178, 1555-1565.

Van Denderen, J., Hermans, A., Meeuwsen, T., Troelstra, C., Zegers, N.D., Boersma, W., Grosveld, G. and Van Ewijk, W. (1989) Antibody recognition of the tumor-specific bcr-abl joining region in chronic myeloid leukemia. J. Exp. Med. 169, 87-98.

Van Denderen, J., Meeuwsen, T., Van der Plas, D., Zegers, N., Boersma, W., Grosveld, G. and Van Ewijk, W. (1990) Immunological characterization of the tumor specific bcr-abl junction in Philadelphia chromosome positive-acute lymphoblastic leukemia. Blood 76, 136-141.

Van der Meide, P.H., Borman, A.H., Beijaars, H.G., Dubbeld, M.A., Botman, C.A.D. and Schellekens, H. (1989) Isolation and characterization of monoclonal antibodies directed to rat interferon-gamma. Lymphokine Res. 8, 439-449.

Van Noort, J.M., Van Sechel, A., Boon, J., Boersma, W.J.A., Polman, C.H. and Lucas, C.J. (1993) Minor myelin proteins can be major targets for peripheral blood T cells from both multiple sclerosis patients and healthy subjects. J. Neuroimmunol. 46, 67-72.

Van Ommen, R., Vredendaal, A.E.C.M. and Savelkoul, H.F.J. (1994) Suppression of polyclonal and antigen-specific murine IgG1 but not IgE responses by neutralizing IL-6 *in vivo*. Eur. J. Immunol. 24, 1396-1403.

Van Ommen, R., Vredendaal, A.E.C.M., de Gooyer, M., Van Oudenaren, A. and Savelkoul, H.F.J. (1994) The effect of IFN-γ, alum and complete Freund adjuvant on TNP-KLH induced IgG1, IgE and IgG2a responses in mice. Mediat. Inflamm. 3, 387-392.

Van Regenmortel, M.H.V. (1986) Which structural features determine protein antigenicity. TIBS 11, 36-39.

Van Regenmortel, M.H.V. (1989) Structural and functional approaches to the study of antigenicity. Immunol. Today 10, 266-272.

Van Rooijen, N. (1992) Macrophages as accessory cells in the *in vivo* humoral immune response: from processing of particulate antigens to regulation by suppression. Semin. Immunol. 4, 237-245.

Van Rooijen, N., Claassen, E., Kraal, G. and Dijkstra, C.D. (1989) Cytological basis of immune functions of the spleen. Immunocytochemical characterization of lymphoid and non-lymphoid cells involved in the *'in situ'* immune response. Prog. Histochem. Cytochem. 19, 1-71.

Van Rooijen, N. and Sanders, A. (1994) Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. J. Immunol. Methods 174, 83-93.

Van Rooijen, N. and Van Nieuwmegen, R. (1980) Liposomes in immunology: evidence that their adjuvant effect results from surface exposition of the antigens. Cell. Immunol. 49, 402-407.

Van Wezel, A.L., Van Steenis, B., Hannik, C.A. and Cohen, H. (1978) New approach to the production of concentrated and purified inactivated polio and rabies tissue culture vaccines. Dev. Biol. Stand. 41, 159-168.

Verheul, A.F.M. and Snippe, H. (1992) Nonionic block polymer surfactants as immunological adjuvant. In : 44th Forum in Immunology. Res. Immunol. 143, 512-519.

Veterinary Public Health Inspectorate (1993) Code of Practice for immunization of laboratory animals. Working group on immunization procedures, Veterinary Public Health Inspectorate (VHI), Rijswijk, 34 pp.

Vogel, F.R. and Powell, M.F. (1995) A compendium of vaccine adjuvants and excipients. In: Vaccine Design: The Subunit and Adjuvant Approach. M.F. Powell and M.J. Newman (Eds.), Plenum Publishing Corp., NewYork, pp. 141-228.

Waksman, B.H. (1979) Adjuvants and immune regulation by lymphoid cells. Springer Semin. Immunopathol. 2, 5-33.

Wallace, J., Sanford, J., Smith, M.W. and Spencer, K.V. (1990) The assessment and control of the severity of scientific procedures on laboratory animals. Lab. Animals 24, 97-130.

Walvoort, H.C. (1991) Assessment of distress through pathological examination. In: Animals in Biomedical Research. C.F.M. Hendriksen and H.B.W.M. Koëter (Eds.), Elsevier Science Publishers, Amsterdam, the Netherlands, pp. 265-271.

Wanstrup, J. and Christensen, H.E. (1965) Granulomatous lesions in mice produced by Freund's adjuvant. Acta Pathol. Microbiol. Scand. 63, 340-354.

Warr, G.W., Magor, K.E. and Higgings, D.A. (1995) IgY: clues to the origins of modern antibodies. Immunol. Today 16(8), 392-398.

Warren, H.S., Vogel, F.R. and Chedid, L.A. (1986) Current status of immunological adjuvants. Ann. Rev. Immunol. 4, 369-418.

Westhof, E., Altschuh, D., Moras, D., Bloomer, A.C., Montdragon, A., Klug, A. and Van Regenmortel, M.H.V. (1984) Correlation between the segmental motility and the reaction of antigenic determinants in proteins. Nature 311, 123-126.

White, R.G. (1976) The adjuvant effect of microbial products on the immune response. Ann. Rev. Microbiol. 30, 579-600.

Wiedemann, F., Link, R., Pumpe, K., Jacobshagen, U., Schaefer, H.E., Wiesmüller, K.H., Hummel, R.P., Jung, G., Bessler, W. and Böltz, T. (1991) Histopathological studies on the local reactions induced by

complete Freund's adjuvant (CFA), bacterial lipopolysaccharide (LPS), and synthetic lipopeptide (P_3C) conjugates. J. Pathol. 164, 265-271.

Wiesmüller, K.H., Bessler, W.G. and Jung, G. (1983) Synthesis of the mitogenic S-(2,3-bis(palmitoloxy)propyl)-N-palmitoyl pentapeptide from the Escherichia coli lipoprotein. Hoppe Seyler's Z. Physiol. Chem. 364, 593-606.

Wohlleben, G., Gray, D. and Schimpl, A. (1996) *In vitro* immunization of naive mouse B cells: establishment of IgM secreting hybridomas specific for soluble protein or hapten from B cells cultured on CD40 ligand transfected mouse fibroblasts. Int. Immunol. 8, 343-349.

Wolf, H., Modrow, S., Motz, M., Jameson, B.A., Hermann, G. and Förtsch, B. (1988) An integrated family of amino acid sequence analysis programs. Comput. Appl. Biosci. 4, 187-191.

Woodard, L.F. (1990) Surface chemistry and classification of vaccine adjuvants and vehicles. Adv. Biotechnol. Processes 13, 281-306.

Woodard, L.F. and Jasman, R.L. (1985) Stable oil-in-water emulsions: preparation and use as vaccine vehicles for lipophilic adjuvants. Vaccine 3, 137-144.

Wright, M.D., Wood, P.R., Coia, G. and Cheers, C. (1987) Particulate antigens may be reprocessed after initial phagocytosis for presentation to T cells *in vivo*. Immunol. Cell. Biol. 65, 505-510.

Yasuda, T., Kanegasaki, S., Tsumita, T., Takakuma, T., Homma, J.Y., Inage, M., Kusumoto, S. and Shiba, T. (1982) Biological activity of chemically synthesized analogues of lipid A. Demonstration of adjuvant effect in hapten-sensitized liposomal system. Eur. J. Biochem. 124, 405-407.

Zegers, N.D., Boersma, W.J.A. and Claassen, E. (1992) Peptide induced memory (IgG) response, crossreactive with native proteins, requires covalent linkage of a specific B cell epitope with T cell epitope. In: Peptides 1992. H. Schneider and A.N. Eberle (Eds.), Elsevier Science (ESCOM) Publishers, Leiden, the Netherlands, pp. 836-837.

Zegers, N.D., Claassen, E., Gerritse, K., Deen, C. and Boersma, W.J.A. (1991) Detection of genetic variants of α_1-antitrypsin with site-specific monoclonal antibodies. Clin. Chem. 37(9), 1606-1611.

Zegers, N.D., Van Holten, C., Claassen, E. and Boersma, W.J.A. (1993) Peptide-induced memory (IgG) response, cross-reactive with native proteins, requires covalent linkage of a specific B cell epitope with a T cell epitope. Eur. J. Immunol. 23, 630-634.

Zhou, E. and Afshar, A. (1995) Comparison of Freund's adjuvant and TiterMax in inducing anti-idiotype to idiotype antibodies against pseudorabies virus antigens. Vet. Immunol. Immunopathol. 48, 113-122.

Summary/Samenvatting

Summary

In biomedical research, laboratory animals are immunized on large scale, e.g. for the induction of poly- and monoclonal antibodies or in vaccination experiments. To enhance and direct the immune response to an antigen, adjuvants (Latin: *adjuvare*, to help) are applied. Various substances (e.g. mineral salts, oil emulsions, bacterial products, saponins) have been recognized as an enhancer of the immune response. In laboratory animals, immunization procedures are frequently performed with Freund's complete adjuvant (FCA; water-in-oil emulsion with added heat-killed Mycobacteria) because of its overall applicability to induce immune responses. However, besides the intended stimulation of the immune response, FCA can induce undesirable side effects. In many countries (e.g. the Netherlands), guidelines restricting the use of FCA in laboratory animals are developed. This resulted in a need for alternative adjuvants. An overall alternative to replace FCA is not available. Adequate selection of an alternative adjuvant is difficult because sufficient combined information on antibody responses and side effects of these alternatives is usually lacking, and the mechanism of action of adjuvants is largely unknown, which makes it difficult to predict the outcome of an immunization.

The main goals of the studies presented in this thesis was to compare FCA and several commercially available adjuvants on immunostimulating properties and side effects, to provide a basis for recommendations on the use of adjuvants in immunization procedures and ultimately to reduce pain and distress in immunized animals. Emphasis is put on the use of adjuvants for the production of antibodies. Additional goals were to study the correlation between antibody production and cytokine profiles, and to increase insight into the mode of action of water-in-oil emulsions.

Chapter 1, the introduction of this thesis, describes the aim of the studies and gives a short introduction to the chapters.

In Chapter 2, adjuvants and other factors (e.g. type and amount of antigen, route of injection, animal species), influencing the outcome of an immunization, are described. Adequate selection of an adjuvant for antibody production is not possible from the literature overview in Chapter 2. To obtain information, comparative studies were conducted to evaluate FCA and alternative adjuvant (Specol, RIBI, TiterMax, Montanide ISA50, ISCOMs, Quil A, *Lactobacillus,* DDA, Gerbu) on their properties to induce antibody responses and side effects in rabbits (Chapter 3 and 5) and mice (Chapter 4, 5 and 6). In Chapter 5, special attention is paid to the assessement of side effects induced by adjuvants. The use of FCA and some alternative adjuvants resulted in severe pathological changes, however, indications of prolonged pain and distress, were not found. Based on the studies described in Chapter 3, 4, 5, and 6, it was concluded that Montanide ISA50 and Specol are possible alternatives to the use of FCA for antibody production, and that the subcutaneous injection route is

preferred above the intramuscular route in rabbits and the intraperitoneal route in mice.

Since many factors determine the outcome of an immunization, and the mechanism of action is not completely elucidated, prediction of the outcome of the immunization is difficult. The possibilities to predict the immunization result were studied by determining cytokine profiles and antibody responses after adjuvant/antigen injection (Chapter 6 and 7). No correlation between antibody responses and cytokine profiles could be demonstrated.

In Chapter 8, the role of macrophages in the mode of action of water-in-oil emulsions was studied by using a well-established macrophage elimination technique. Besides a stimulative role, macrophages were shown to play a suppressive role in the induction of the immune response with water-in-oil emulsions. This suppressive role of macrophages was confusing in the light of presumed macrophage involvement in adjuvant efficacy of water-in-oil emulsions. Consequently, a new method was developed to label the oil phase of water-in-oil emulsions. Oil distribution in lymphoid organs after injection of water-in-labelled oil emulsion was studied (Chapter 9). Oil was found to be mainly localized associated with the capsule of the spleen. This localization may function as a second depot and partly explain the efficacy of water-in-oil emulsions.

Chapter 10 presents a general discussion on the main points emerging from the experimental work. Finally, recommendations are given on the use and selection of adjuvants for immunization procedures in laboratory animals.

Samenvatting

In het biomedisch onderzoek worden proefdieren voor diverse doeleinden gebruikt, bijvoorbeeld voor de productie van antilichamen voor onderzoek, diagnostiek en therapie. Proefdieren zijn hiervoor nodig omdat op dit moment geen geschikte methoden zonder dieren voorhanden zijn. Om het ongerief bij proefdieren, veroorzaakt door het inspuiten van materiaal (immuniseren), te verminderen zijn richtlijnen opgesteld voor het uitvoeren van immunisaties. Speciale aandacht gaat hierbij uit naar de toepassing van adjuvantia. Adjuvantia (van het Latijnse woord *adjuvare* = helpen) worden gebruikt om het immuunsysteem te stimuleren en de immuunrespons te sturen en kunnen daardoor helpen de gewenste immuunrespons op te wekken. Vele stoffen hebben een adjuvant werking zoals: aluminium zouten, bacteriële producten en water-in-olie emulsies (water druppels in olie fase). De structuur en samenstelling van een adjuvant zijn bepalend voor de immuunstimulerende werking maar ook voor mogelijke bijwerkingen. Het werkingsmechanisme van adjuvantia is echter niet volledig bekend. Voor de productie van antilichamen in proefdieren wordt veelvuldig gebruik gemaakt van Freund's complete adjuvant (FCA). Dit adjuvant is een water-in-olie emulsie waaraan Mycobacteriën zijn toegevoegd. FCA is een zeer effectief adjuvant voor een groot aantal antigenen (= materiaal waartegen een immuunrespons wordt opgewekt), maar het kan ernstige bijwerkingen veroorzaken. Het gebruik van FCA wordt daarom sterk ontmoedigd in de bestaande richtlijnen voor immunisatie procedures. Het selecteren van een alternatief adjuvant is echter moeilijk omdat weinig informatie beschikbaar is over zowel effectiviteit als bijwerkingen van deze producten. De in dit proefschrift beschreven studies zijn uitgevoerd om informatie te verzamelen over FCA en alternatieve adjuvantia en op basis hiervan aanbevelingen te doen voor het gebruik van adjuvantia in proefdieren, met als uiteindelijk doel het ongerief te verminderen.

De immuunrespons in proefdieren wordt door vele factoren beïnvloed zoals type en hoeveelheid antigeen, injectieroute en gebruik van adjuvantia. Een overzicht van deze factoren wordt gegeven in Hoofdstuk 2. Om informatie over immuunresponsen en bijwerkingen te verkrijgen werden evaluatie studies uitgevoerd in konijnen en muizen. In deze studies werden FCA en alternatieve adjuvantia (Specol, RIBI, TiterMax, Montanide ISA50, ISCOMs, Quil A, *Lactobacillus*, DDA, Gerbu) vergeleken op basis van de geïnduceerde antilichaamproductie en de bijwerkingen (Hoofdstuk 3, 4, 5 en 6). FCA en sommige alternatieve adjuvantia blijken ernstige pathologische veranderingen te veroorzaken, echter, aanwijzingen voor ernstig ongerief op basis van klinische en gedragsveranderingen werden met de door ons gebruikte methoden niet waargenomen. Uit de experimenten, zoals beschreven in Hoofdstuk 3, 4, 5 and 6, kan onder andere geconcludeerd worden dat Montanide ISA50 en Specol alternatieven kunnen zijn voor het gebruik van FCA ten behoeve

van de productie van polyclonale antistoffen. Daarnaast wordt de voorkeur gegeven aan de subcutane toedieningsroute boven de intramusculaire route bij konijnen en de intraperitoneale route bij muizen.

Omdat vele factoren de uitkomst van een immunisatie bepalen, werd geprobeerd een model op te zetten om deze uitkomst te voorspellen. Cytokinen worden over het algemeen beschouwd als belangrijke regulatoren van de immuunrespons en zouden dan ook in een vroeg stadium informatie kunnen verschaffen over het immunisatie resultaat. De cytokine profielen en de antilichaamresponsen na immunisatie werden bestudeerd teneinde een relatie tussen deze twee parameters vast te stellen (Hoofdstuk 6 en 7). Het bleek zeer moeilijk om op basis van de cytokine profielen een uitspraak te doen over de geïnduceerde antilichaamresponsen.

Omdat de kennis omtrent de werkingsmechanismen van adjuvantia van belang kan zijn voor het efficiënter opwekken van antilichamen, werden deze mechanismen nader bestudeerd en daarbij werd de nadruk gelegd op water-in-olie emulsies (Hoofdstuk 8 and 9). Algemeen wordt aangenomen dat macrofagen ('veel etende cellen') een belangrijke stimulerende rol spelen bij de adjuvant werking van water-in-olie emulsies. De rol van macrofagen in de effectiviteit van water-in-olie emulsies werd onderzocht door macrofagen plaatselijk (in de lymfeklier) te verwijderen en vervolgens een adjuvant toe te dienen (Hoofdstuk 8). Uit de resultaten van deze studies kan worden geconcludeerd dat macrofagen een rol spelen in de effectiviteit van water-in-olie emulsies maar dat dit ook een onderdrukkende rol kan zijn. Om deze opvallende bevinding verder te onderzoeken werd een methode ontwikkeld om de olie fase van water-in-olie emulsies te labellen (Hoofdstuk 9). Na toediening van water-in-gelabelde olie emulsies werd de localisatie van de emulsie bestudeerd. Water-in-olie emulsie bleek zich met name in en rond het kapsel van de milt te bevinden en niet in de macrofagen. Dit suggereert dat een tweede depot van water-in-olie emulsie (naast dat op de plaats van toediening) wordt gevormd dat zorgt voor extra stimulatie van het immuunsysteem.

Aan het einde van het proefschrift worden de belangrijkste bevindingen uit de studies bediscussieerd en worden aanbevelingen gegeven voor het gebruik van adjuvantia in proefdieren (Hoofdstuk 10).

De algemene conclusie van het proefschrift is dat de gereserveerde houding die internationaal bestaat ten aanzien van het gebruik van FCA genuanceerd dient te worden. Weliswaar veroorzaakt FCA (ernstige) pathologische veranderingen maar dit leidt niet tot aanwijzingen voor blijvend ernstig ongerief, tenminste niet op grond van de door ons gebruikte modellen voor klinisch en gedragsonderzoek. Bij *lége-ártis* gebruik van FCA, konden slechts minimale klinische en gedragsveranderingen, die een indicatie zouden kunnen zijn van ongerief, worden waargenomen bij konijnen en niet meer dan een paar dagen na inspuiten bij muizen. Daarnaast geven sommige alternatieve adjuvantia, net als FCA, ernstige pathologische veranderingen, maar geen acceptabele antilichaamresponsen.

Aanbevelend wordt gesteld dat het gebruik van alternatieve adjuvantia niet wordt aangeraden als slechts één enkel experiment met een klein aantal dieren wordt uitgevoerd en niets bekend is over de te gebruiken adjuvant/antigeen combinatie. Dit om het aantal dieren dat gebruikt wordt voor immunisaties te beperken en te voorkomen dat ernstige pathologische veranderingen geïnduceerd worden zonder acceptabele immuunresponsen. Omdat het niet duidelijk is of de pathologische veranderingen door gebruik van een adjuvant ongerief veroorzaken, dient bij de keuze van een adjuvant de voorkeur gegeven te worden aan een adjuvant dat minimale pathologische veranderingen veroorzaakt én acceptabele immuunresponsen induceert. Het bestuderen van pathologische veranderingen is essentieel om de bijwerkingen van (alternatieve) adjuvantia te beoordelen. Het antigeen en de inspuitroute zijn mede bepalend voor de pathologische veranderingen.

Curriculum vitae

Philomena Petronella Antonia Maria Leenaars werd geboren op 23 oktober 1967 te Made. In 1986 behaalde zij het VWO diploma aan het Dongemond College te Raamsdonksveer. In datzelfde jaar begon zij met de studie Zoötechniek aan de Landbouwuniversiteit te Wageningen, alwaar zij in 1992 het doctoraal examen behaalde. Van november 1992 tot maart 1997 was zij, vanuit de vakgroep Immunologie (hoofd: Prof. Dr. R. Benner), Faculteit der Geneeskunde en Gezondheidswetenschappen van de Erasmus Universiteit Rotterdam gedetacheerd, eerst bij het Centraal Dierenlaboratorium van het Rijksinstituut voor Volksgezondheid en Milieu (RIVM) te Bilthoven en daarna bij de Divisie Immunologische en Infectieziekten van TNO Preventie en Gezondheid (TNO-PG) te Leiden. Onder begeleiding van Prof. Dr. E. Claassen (DLO-Instituut voor Dierhouderij en Diergezondheid, Lelystad) en Dr. C.F.M. Hendriksen (RIVM) werd het in dit proefschrift beschreven onderzoek uitgevoerd. Vanaf 1 mei 1997 is zij werkzaam als wetenschappelijk medewerkster bij het Centraal Dierenlaboratorium van het RIVM te Bilthoven.

List of publications

1. Leenaars, P.P.A.M., Hendriksen, C.F.M., Koedam, M.A. and Claassen, E. (1993) Evaluation in rabbits of several adjuvants as alternative to Freund's adjuvant. In: *Annual Scientific Report 1992.* National Institute of Public Health and the Environment (RIVM), Bilthoven, the Netherlands, pp. 159-162.

2. Leenaars, M., Hendriksen, C., Koedam, M. and Claassen, E. (1993) Onderzoek in konijnen naar alternatieven voor Freund's adjuvant (in dutch). *Histotechniek/cyto-visie* 1(3), 15-19.

3. Leenaars, P.P.A.M., Hendriksen, C.F.M., Angulo, A.F., Koedam, M.A. and Claassen, E. (1994) Evaluation of several adjuvants as alternatives to the use of Freund's adjuvant in rabbits. *Veterinary Immunology and Immunopathology* 40, 225-241.

4. Leenaars, P.P.A.M., Hendriksen, C.F.M., Koedam, M.A., Claassen, I. and Claassen, E. (1995) Comparison of adjuvants for immune potentiating properties and side effects in mice. *Veterinary Immunology and Immunopathology* 48, 123-138.

5. Leenaars, P.P.A.M., Claassen, E. and Hendriksen, C.F.M. (1996) Considering the side effects of adjuvant products in immunization procedures. *Lab Animal Magazine* September 1996, 40-43.

6. Leenaars, M., Claassen, E. and Boersma, W.J.A. (1997) Modulation of the humoral immune response; Antigens and antigen presentation. In: *Immunology Methods Manual,* I. Lefkovits (Ed.), Academic Press Ltd., London, pp. 989-1013.

7. Leenaars, M., Hendriksen, C.F.M., Koedam, M.A. and Claassen, E. (1997) Comparison of alternatives to Freund's complete adjuvant. In: *Ersatz- und Ergänzungsmethoden zu Tierversuchen; Forschung ohne Tierversuche 1996,* H. Schoffl, H. Spielmann and H.A. Tritthart (Eds.), Springer-Verlag/Wien, Austria, pp. 233-241.

8. Leenaars, P.P.A.M., Hendriksen, C.F.M., Savelkoul, H.F.J., Van Rooijen, N. and Claassen, E. (1997) Increased adjuvant efficacy in stimulation of antibody responses after macrophage elimination *in vivo. Immunology* 90, 337-343.

9. Leenaars, P.P.A.M., Nagelkerken, L., Hendriksen, C.F.M. and Claassen, E. (1997) Model to test efficacy of immunization procedures by early cytokine production in mice. In: *Animals Alternatives Welfare and Ethics*, L.F.M. van Zutphen and M. Balls (Eds.), Elsevier Science, Amsterdam, *in press.*

10. Leenaars, P.P.A.M., Oostermeijer, H.H.A., Hendriksen, C.F.M. and Claassen, E. A rapid and simple method to localize oil based adjuvants *in vivo. J. Immunol. Methods, in press.*

11. Leenaars, P.P.A.M., Koedam, M.A., Wester, P.W., Baumans, V., Claassen, E. and Hendriksen, C.F.M. Assessment of side effects induced by injection of different adjuvant/antigen combinations in rabbits and mice. *submitted.*

12. Leenaars, M., Koedam, M.A., Hendriksen, C.F.M. and Claassen, E. Immune responses and side effects of five different oil based adjuvants in mice. *submitted.*

APPENDIX A en B

APPENDIX A

Overview of results of studies in rabbits comparing FCA with Specol, RIBI, TiterMax and/or Montanide ISA50 on antibody responses and (in several cases) clinical, behavioural and pathological changes.

adjuvant	antigen	route (volume in ml)	Ab titre[a]	clinical/behavioural changes[b]	pathogical changes[c]	reference
FCA						
	- glycolipid	- sc (0.5)	-[d]	s	+	Leenaars *et al.*, 1994
		- im (0.5)	-	-	-	
	- subunit enzyme	- sc (5x0.2)	+	s.c. mass > 3cm	n.d.	Mallon *et al.*, 1991
	- synth. pept.	- sc (0.5)	+	-	+	Leenaars *et al.*, 1994
		- im (0.5)	+	-	+	
	- M. pneu.	- sc (0.5)	+	s	u, g, a	Leenaars *et al.*, 1994
		- im (0.5)	+	-	+	
	- synth. polypept. (Mw 240 kDa)	- im (2x0.25)	+	+ q, ck, b	m	Deeb *et al.*, 1992
	- pept./BSA	- im (2x0.4) + id (2x0.1)	+	-	deep tissue reaction r, s, u (< 1 cm)	Johnston *et al.*, 1991
	- TNP/HEA	- fp (2x0.04)	+	r, s	n.d.	Bennett *et al.*, 1992
	- LHRH/BSA	- im (0.5)	+	r, s	n.d.	Bennett *et al.*, 1992
	- protein/polymer	- sc (4x0.25)	+	-	g (9-32 cm^2), f, u	Smith *et al.*, 1992
	- synth .pept./ tetanus toxoid	- sc (4x0.1)	+	s		Leenaars *et al.*, subm.
	- BSA	- sc (0.1)	+	s	+	Leenaars *et al.*, subm.
		- im (0.5)	+	-	+	
	- virus (Rubella)	- sc (0.1)	+	s	+	Leenaars *et al.*, subm.
		- im (0.5)	+	-	+	
Specol						
	- glycolipid	- sc (0.5)	-	-	-	Leenaars *et al.*, 1994
		- im (0.5)	-	-	-	
	- synth. pept.	- sc (0.5)	+	-	-	Leenaars *et al.*, 1994
		- im (0.5)	+	-	-	
	- M. pneu.	- sc (0.5)	+	s	+/-	Leenaars *et al.*, 1994
		- im (0.5)	+	-	+/-	
	- synth. pept./tetanus-toxoid	- sc (4x0.1)	+	s	+	Leenaars *et al.*, subm.
	- BSA	- sc (0.1)	+	s	+	Leenaars *et al.*, subm.
		- im (0.5)	+	-	-	
	- virus (Rubella)	- sc (0.1)	+	-	-	Leenaars *et al.*, subm.
		- im (0.5)	+	-	-	
RIBI[e]						
III	- glycolipid	- sc (2x0.5)	-	s	++, g, u, a	Leenaars *et al.*, 1994
		- im (2x0.5)	-	-	++, g	

adjuvant	antigen	route (volume in ml)	Ab titre	clinical/behavioural changes	pathological changes	reference
RIBI						
II	- subunit enzyme	- sc (5x0.2)	+/-	s (2-3 cm)	n.d.	Mallon *et al.*, 1991
I	- subunit enzyme	- sc (5x0.2)	-	s (< 2 cm)	n.d.	
III	- synth. pept.	- sc (2x0.5)	-	-	++, g, a, u	Leenaars *et al.*, 1994
		- im (2x0.5)	-	-	++, g	
III	- M. pneu.	- sc (2x0.5)	+/-	s	+, g, u, a	Leenaars *et al.*, 1994
		- im (2x0.5)	+/-	-	+, g	
III	- synth. polypept. (Mw 240 kDa)	- im (2x0.25)	+/-	+/-	m (+/-)	Deeb *et al.*, 1992
III	- pept./BSA	- im (2x0.4) + id (2x0.1)	-	-	deep tissue reaction r, s, u (<1 cm)	Johnston *et al*, 1991
II	- LHRH/BSA	- sc (0.1) + ip (0.2) + im (2x0.2) + id (6x0.05)	-	mild transient	n.d.	Bennett *et al.*, 1992
III	- protein/polymer	- sc (0.1) + ip (0.2) + im (2x0.2) + id (6x0.05)	-	-	-	Smith *et al.*, 1992
TiterMax						
	- glycolipid	- im (0.08)	-	-	-	Leenaars *et al.*, 1994
		- sc (0.08)	-	-	-	
	- protein/polymer	- im (2x0.05)	-	-	-	Smith *et al.*, 1992
	- synth. pept.	- im (0.08)	-	-	+/-	Leenaars *et al.*, 1994
		- sc (0.08)	+/-	-	+/-	
	- M. pneu.	- im (0.08)	+/-	s	-	Leenaars *et al.*, 1994
		- sc (0.08)	+/-	-	+/-	
	- TNP/HEA	- im (2x0.04)	+	mild transient	n.d.	Bennett *et al.*,1992
Montanide ISA50						
	- pept./BSA	- im (2x0.4) + id (2x0.1)	+	-	deep tissue reaction r, s, u (<1 cm)	Johnston *et al.*, 1991
	- synth. pept./tetanus toxoid	- sc (4x0.1)	+	-	+	Leenaars *et al.*, subm.
	- BSA	- sc (0.1)	+	s	+/-	Leenaars *et al.*, subm.
		- im (0.5)	+	-	+/-	
	- virus (Rubella)	- sc (0.1)	+	-	+/-	Leenaars *et al.*, subm.
		- im (0.5)	+	-	+/-	

[a] antibody titre
[b] clinical and behavioural changes: b=body weight loss; ck = elevated creatinine kinase levels; q = muscle quivering; r=redness; s=swelling
[c] pathological changes: a=abscess; f=fistulous extension; g=granulomatous lesion; m=myositis; r=redness; s=swelling; u=ulceration;
[d] n.d. = not determined; - = not found; +/- = less than FCA; + = comparable to FCA; ++ = exceeding FCA
[e] RIBI I is RIBI containing MPL; RIBI II is RIBI containing MPL + TDM; RIBI III is RIBI containing MPL + TDM + CWS.

APPENDIX B

Overview of results of studies in mice comparing FCA with Specol, RIBI, TiterMax and/or Montanide ISA50 on antibody responses and (in several cases) clinical, behavioural and pathological changes.

adjuvant	antigen	route (volume in ml)	Ab titre[a]	clinical/behavioural changes[b]	pathological changes[c]	reference
FCA	- self antigen	- sc (0.2)	+/-[d]	b, s	g (>4 mm), fi	Leenaars *et al.*, 1995
		- ip (0.2)	+	b, pi	a, w, p	
	- synth. pept.	- sc (0.1)	+	b, s	n.d.	Leenaars *et al.*, 1995
	- synth. pept.	- sc (0.1)	+	pi, s	g (2-4 mm), fi	Leenaars *et al.*, subm.
		- ip (0.2)	+	pi, h	a, p	
	- idiotype	- sc (1)	+	n.d.	n.d.	Zhou and Afshar, 1995
	- fusion protein	- sc (2x0.1)	+	n.d.	n.d.	Daly and Long, 1996
	- BGG/hapten	- ip (0.2)	+	-	a, w	Lipman *et al.*, 1992
	- synth.pept/OVA	- ip (0.1)	+	n.d.	n.d.	Geerligs, *et al*, 1989
	- synth.pept./BSA	- ip (0.1)	+	n.d.	n.d.	Geerligs, *et al*, 1989
	- LHRH/BSA	- im (0.5)	+	r, s	n.d.	Bennett,1992
	- TNP/HEA	- fp (2x0.04)	+	r, s	n.d.	Bennett,1992
	- M. pneu.	- sc (0.1)	+	p, s	g (>4 mm), fi	Leenaars *et al.*, 1995
		- ip (0.2)	+	b, pi	w, p	
	- synth. pept./diphtheria toxoid	- ip (?)	+	n.d.	n.d.	Jones *et al.*, 1990
	- LHRH/avidin	- sc (0.2)	+/-	n.d.	n.d.	Tiong *et al.*, 1993
	- human serum albumin (HSA)	- ip (0.15) + fp (2x0.025)	+	n.d.	n.d.	Kenney *et al.*,1989
	- HSA	- sc (0.2)	+	n.d.	n.d.	Kenney *et al.*,1989
	- influenza virus micelles	- sc (0.1)	+	n.d.	n.d.	Sjölander *et al.*, 1996
	- whole killed parasite	- sc (0.05)	+	n.d.	n.d.	Ten Hagen *et al.*, 1993
	- toxic protein	-sc (?)	+	n.d.	n.d.	Hewetson *et al.*, 1993
Specol						
	- self antigen.	- sc (0.2)	-	b	-	Leenaars *et al.*, 1995
		- ip (0.2)	-	-	w, p	
	- synth. pept.	- sc (0.1)	+	b	-	Leenaars *et al.*, 1995
		- ip (0.1)	+	b	w, p	
	- synth. pept.	- sc (0.1)	+	-	+/-	Leenaars *et al.*, subm.
		- ip (0.2)	+	pi, h	a, p	
	- M. pneu.	- sc (0.1)	+	pi, s	g (>4 mm), fi	Leenaars *et al.*, 1995
		- ip (0.2)	+	pi, b	w, p	

adjuvant	antigen	route (volume in ml)	Ab titre	clinical/behavioural changes	pathological changes	reference
RIBI[e]						
II	- synth. pept.	- sc (0.1)	-	-	-	Leenaars *et al.*, subm.
		- ip (0.2)	-	h, pi	w, p (+/-)	
II	- fusion protein	- sc (2x0.1)	++	n.d.	n.d.	Daly and Long, 1996
II	- BGG/hapten	- ip (0.2)	++	-	a, w (+/-)	Lipman *et al.*, 1992
II	- synth.pept/OVA	- ip (0.1)	+	n.d.	n.d.	Geerligs *et al.*, 1989
	- synth.pept/BSA	- ip (0.1)	+	n.d.	n.d.	Geerligs *et al.*, 1989
II	- LHRH/BSA	- sc (0.2)	+/-	n.d.	mild transient	Bennett *et al.*, 1992
II	- TNP/HEA	- sc (2x0.1)	-	n.d.	mild transient	Bennett *et al.*, 1992
III	- LHRH/avidin	- sc (0.2)	+/-	n.d.	n.d	Tiong *et al.*, 1993
II	- HSA	- ip (0.15) + fp (2x0.025)	+/-	n.d.	n.d.	Kenney *et al.*, 1989
	- toxic protein	-sc (?)	+	n.d.	n.d.	Hewetson *et al.*, 1993
TiterMax						
	- synth. pept.	- sc (0.05)	-	pi, h, s	g (>4 mm), fi	Leenaars *et al.*, subm.
		- ip (0.2)	++	pi, h	a, p	
	- fusion protein	- sc (2x0.1)	++	n.d.	n.d.	Daly and Long, 1996
	- LHRH/BSA	- sc (0.04)	+	n.d.	mild transient	Bennett *et al.*, 1992
	- TNP/HEA	- sc (0.08)	++	n.d.	mild transient	Bennett *et al.*, 1992
		- fp (2x0.04)	+	n.d.	mild transient	
	- influenza virus micelles	- sc (0.05)	+	n.d.	n.d.	Sjölander *et al.*, 1996
	- whole killed parasite	- sc (0.05)	+	n.d .	n.d.	Ten Hagen *et al.*, 1993
Montanide ISA50						
	- synth. pept.	- sc (0.1)	++	s	g (2-4 mm), fi	Leenaars *et al.*, subm.
		- ip (0.2)	++	pi, h	w, p	
	- virus	- im	+	-	n.d.	Ganne *et al.*, 1994
	- synth. pept./diphtheria toxoid	- ip (?)	++	n.d.	n.d.	Jones *et al.*, 1990

[a] antibody titre
[b] clinical changes: b= body weight loss; h = hunched posture; pi = pilo-erection; s = swelling
[c] pathological changes: a=adhesions; fi=fibrosis; g=granuloma; n=necrosis; p=peritonitis; w=white plaques on organs
[d] n.d. = not determined; - = not found; +/- = less than FCA; + = comparable to FCA; ++ = exceeding FCA
[e] RIBI II is RIBI containing MPL + TDM; RIBI III is RIBI containing MPL + TDM + CWS.