QUANTITATIVE ASPECTS OF IMMUNOGLOBULIN PRODUCTION IN THE INTESTINE OF MICE

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KWANTITATIEVE ASPEKTEN VAN DE IMMUNOGLOBULINE PRODUKTIE IN DE DARM VAN MUIZEN

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aan mijn ouders

CONTENTS

VOORWOORD 9

1. GENERAL INTRODUCTION 11

2. INTRODUCTION TO THE EXPERIMENTAL WORK 29

3. ASSAYS FOR THE ISOLATION OF LYMPHOID CELLS AND SECRETIONS FROM THE INTESTINE 33
   - 3.1 Improved procedure for the isolation of functionally active lymphoid cells from the murine intestine 35
   - 3.2 Comparison of two methods for collecting murine intestinal secretions to detect antigen-specific antibodies 45

4. IMMUNOGLOBULIN-SECRETING CELLS IN THE INTESTINE 57
   - 4.1 Contribution of immunoglobulin-secreting cells in the murine small intestine to the total 'background' immunoglobulin production 59
   - 4.2 Background (spontaneous) immunoglobulin production in the murine small intestine before and after weaning 71
   - 4.3 Decrease of IgA-secreting cells in the intestine of mice during aging 83

5. ANTIBODY PRODUCTION IN THE INTESTINE 95
   - 5.1 Quantification of antigen-specific antibody-secreting cells in the small intestine and other lymphoid organs of mice after oral booster immunization 97
   - 5.2 Manipulation of intestinal immune responses against ovalbumin by cholera toxin and its B subunit in mice 111

6. GENERAL DISCUSSION 123

7. SUMMARY 135

SAMENVATTING 137

ABBREVIATIONS 139

DANKWOORD 141

CURRICULUM VITAE 142

VOORWOORD

Ieder gewerveld dier beschikt over een complex afweersysteem dat dient ter bescherming tegen infektie met pathogenen zoals bacteriën, virussen, parasieten en schimmels. Dit afweersysteem omvat zowel aspecifieke als specifieke mechanismen. Door een samenwerking van deze mechanismen wordt het binnendringen van pathogenen in het lichaam bemoeilijkt en worden pathogenen die toch in het lichaam doorgedrongen zijn, vernietigd.

Tot de aspecifieke afweermechanismen behoren o.a. mechanische barrières (huid, slijmvliezen, trilharen, hoestreflex, niezen etc.), biochemische faktoren (zuurgraad van de maag, lysozym, C-reactive protein, complementfaktoren, etc.), en cellen (granulocyten, macrofagen/monocyten, killer cellen).

Het specifieke afweermechanisme of immuunsysteem wordt gekenmerkt door twee eigenschappen: specificiteit en geheugen. Het immuunsysteem is in staat lichaamseigen en lichaamsvreemde stoffen van elkaar te onderscheiden. Lichaamsvreemde stoffen (antigenen) induceren een immuunrespons, lichaamseigen stoffen doen dat in principe niet.

Lymfocyten vormen de basis van het immuunsysteem. Deze cellen, die behoren tot de leukocyten of witte bloedcellen, laten zich op grond van hun funktionele eigenschappen onderscheiden in B- en T-lymfocyten.

Onder invloed van antigeen kunnen B-lymfocyten, die het antigeen door middel van een receptor op de celmembraan herkennen, differentiëren en prolifereren tot plasmacellen welke antilichamen (immunoglobulinen) secerneren (de humorale immuunrespons). Deze antilichamen hebben dezelfde specificiteit als de antigeenreceptor welke het antigeen als eerste herkende. Antilichamen spelen een belangrijke rol in de afweer tegen ziekteverwekkers. Ze zijn o.a. in staat om virussen en bacteriën onschadelijk te maken en toxinen te neutraliseren.

T-lymfocyten kunnen onder invloed van antigenen blasttransformatie ondergaan en overgaan tot uitscheiding van lymfokinen (de cellulaire immuunrespons). We onderscheiden verschillende typen T-lymfocyten. Zo kunnen cytotoxische T-cellen door direkte interaktie met b.v. virus geinfekteerde cellen, deze cellen vernietigen. Andere T-cellen produceren faktoren die fagocyterende cellen aanzetten tot het doden van intracellulair groeiende parasieten of produceren faktoren die een vertraagd type overgevoeligheidsreaktie teweegbrengen. In tegenstelling tot de humorale immuniteit, welke met serum van het ene naar het andere dier kan worden overgedragen, kan de cellulaire immuniteit alleen via cellen worden overgedragen.

T-lymfocyten zijn niet alleen verantwoordelijk voor de cellulaire immuniteit, maar spelen tevens een belangrijke regulerende rol bij zowel de humorale als cellulaire immuunrespons. Zo zijn, m.b.t. de meeste antigenen, T-helper cellen nodig om B-cellen te stimuleren tot antilichaamvorming, terwijl T-suppressor cellen de respons juist kunnen remmen.

Een deel van de door het antigeen gestimuleerde B- en T-lymfocyten differentiëert tot effektor cel. Een ander deel wordt kleine lymfocyt en blijft gedurende

lange tijd circuleren in het lichaam. Deze cellen zijn verantwoordelijk voor de tweede belangrijke eigenschap van het immuunsysteem: het geheugen. Bij een hernieuwd kontakt met het antigeen dat voor hun induktie verantwoordelijk was, zorgen deze kleine langlevende lymfocyten voor een snellere en sterkere immuunrespons. Van deze geheugenfunktie wordt gebruik gemaakt bij vaccinatie. Door een individu opzettelijk kennis te laten maken met een antigeen afkomstig van een ziekteverwekker, zodanig dat geen (of nauwelijks) ziekteverschijnselen optreden bij de gastheer, kan het individu later, ten tijde van een echte infektie, veel sneller en effektiever reageren. Net als de oorspronkelijke immuunrespons zelf, is ook deze geheugenrespons antigeenspecifiek.

Op basis van lokalisatie en wijze van expressie, is het immuunsysteem nog op een andere manier te benaderen. Het kan nl. verdeeld worden in een systemisch en een mucosaal immuunsysteem. Het systemische immuunsysteem, welke het "inwendige" van een individu beschermt, manifesteert zich o.a. door de aanwezigheid van antilichamen van de typen IgM en IgG in het serum. Het mucosale immuunsysteem (mucosa = slijmvlies) is gelokaliseerd aan de "uitwendige" slijmvlies oppervlakken van het lichaam en manifesteert zich o.a. in de uitscheiding van IgA antilichamen aan deze slijmvliezen en wel in de vorm van secretoir-IgA.

Expressie van mucosale immuniteit vindt plaats langs alle slijmvliezen, zoals die van het spijsverteringskanaal, de ademhalingswegen, traanklieren, urogenitaal stelsel, etc.. Het mucosale en systemische immuunsysteem worden tot op zekere hoogte onafhankelijk van elkaar gereguleerd. Uit het oogpunt van vaccinatie is het mucosale immuunsysteem bijzonder belangrijk. Pathogenen zullen doorgaans via de slijmvliezen proberen het lichaam binnen te dringen. Al aanwezige mucosale immuniteit zal dit kunnen voorkomen. Het ligt dus voor de hand dat vaccinatie vaak gericht zal zijn op het voorkomen van het binnendringen van pathogenen door induktie van mucosale immuniteit.

Behalve belangrijk als eerste lijn van immunologische bescherming, is induktie van mucosale immuniteit de enige manier van bescherming indien het infekties betreft die tot de mucosa beperkt blijven.

Het onderzoek dat in dit proefschrift wordt beschreven, was er vooral op gericht meer inzicht te verkrijgen in het belang van het mucosale immuunsysteem en daarnaast om de mogelijkheden te onderzoeken tot induktie van specifieke immuunresponsen aan de mucosa. Daartoe zijn eerst methoden ontwikkeld om kwantitatieve gegevens te verkrijgen m.b.t. het B-cel compartiment van de darm. Vervolgens zijn het B-cel compartiment in de darmwand, de ontwikkeling van dit compartiment en de faktoren die hieraan bijdragen, bestudeerd. Hiertoe is het totale aantal immunoglobuline secernerende cellen in de darm als funktie van leeftijd, wijze van huisvesting en antigeenbelasting bepaald en vergeleken met het aantal immunoglobuline secernerende cellen in perifere lymfoïde organen als milt, beenmerg en lymfklieren. Vervolgens is onderzocht of deze methoden tevens geschikt zijn voor bepaling van antigeen-specifieke immuunresponsen aan de mucosae, en zijn mogelijkheden onderzocht om de mucosale immuunrespons te stimuleren.

CHAPTER 1

GENERAL INTRODUCTION

1.1. The common mucosal immune system

Mucosal surfaces are continuously exposed to the environment and have developed a variety of protective mechanisms. These mechanisms, both immunological and nonimmunological, guard the host against pathogens and against damage resulting from absorption of foreign antigens such as toxins. Because immune responses that arise after systemic or mucosal exposure to antigen differ, the term "secretory immunity" is used to specify immunological protection at the mucosal surfaces. Early studies of Besredka (1) demonstrated that oral immunization with live Shigella bacteria resulted in local humoral immunity in the gut without inducing systemic humoral immunity. Also, Bull and McKee (2) showed that when antigen was introduced into the respiratory tract, a local immune response unaccompanied by serum antibody could be induced.

Later it was shown (3,4) that resistance to influenza virus in mice correlated with local secretory antibodies and, more important, that local antigen presentation was more effective in eliciting these antibodies than parenteral immunization. The basic study of local immunity started after Heremans et al. (5) characterized a new type of immunoglobulin (Ig), IgA. It was observed that IgA predominated in milk and other exocrine secretions (6,7). These findings led to the conception of a local humoral immune system mediated by IgA antibodies. The idea that local immunity was regulated relatively independent of systemic immunity was greatly strengthened when distinct migratory pathways of IgA precursor lymphoblasts were discovered. The IgA precursors were shown to have a strong predeliction to "home" or lodge in the lamina propria of small intestinal tissue (8-10).

In the intestine, the mucosa- (gut) associated lymphoid tissue (GALT) comprises Peyer's patches (PP), the so-called solitary lymphoid nodules, and the lymphocytes in the lamina propria and epithelium of the villi. Describing lymphoid aggregates in the lung that resemble PP (the bronchus-associated lymphoid tissue, BALT), Bienenstock et al. (11,12) proposed that the secretory immune system links all mucosal surfaces. The concept of the common mucosal immune system arose when it was demonstrated that PP and BALT are sources of precursors of IgA plasma cells in the gut and respiratory tract, and that exposure to antigen at one mucosal surface leads to dissemination of the response to other mucosal surfaces (13). This concept was extended by the demonstration of selective mechanisms that transport circulating dimeric IgA into external secretions (14-17). By means of the J-chain, dimeric IgA selectively binds to a receptor, the secretory component, on the membrane of epithelial cells and hepatocytes. The complex is

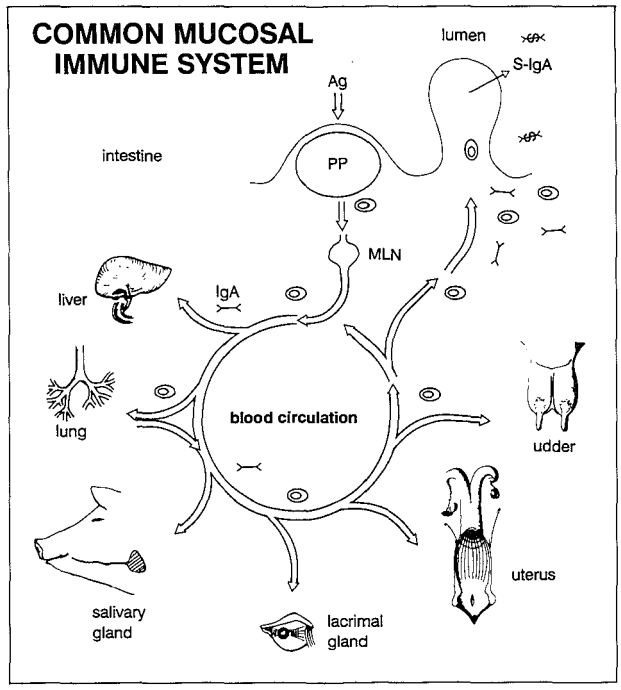

Figure 1. Schematic diagram of the common mucosal immune system.

then internalized, transported across the cell, and excreted as secretory-IgA (S-IgA) into the gut lumen directly or via the gall (20,25).

The common mucosal immune system has been studied extensively and its characteristics have been reviewed regularly (18-26). A schematic diagram of the mucosal immune system is shown in Figure 1. The following paragraphs describe 1) the antigen uptake via the intestine leading to the induction of Ig-secreting cells; 2) the regulation of isotype expression leading to the enhanced expression of IgA; and 3) the migration patterns of cells leading to their homing in the intestinal wall.

1.2. Antigen uptake

Antigenic macromolecules can cross the epithelial lining of the gut. Ratner and Gruehl concluded that antigenically intact proteins could enter the bloodstream directly from the intestinal lumen (27). Cornell et al. (28) demonstrated uptake and transport of horseradish peroxidase through columnar epithelial cells in the small intestine of rats. Further studies demonstrated the presence of orally administered horseradish peroxidase in both intestinal lymph and the portal bloodstream (29). Besides proteins, intestinal uptake has been demonstrated for intact polysac-

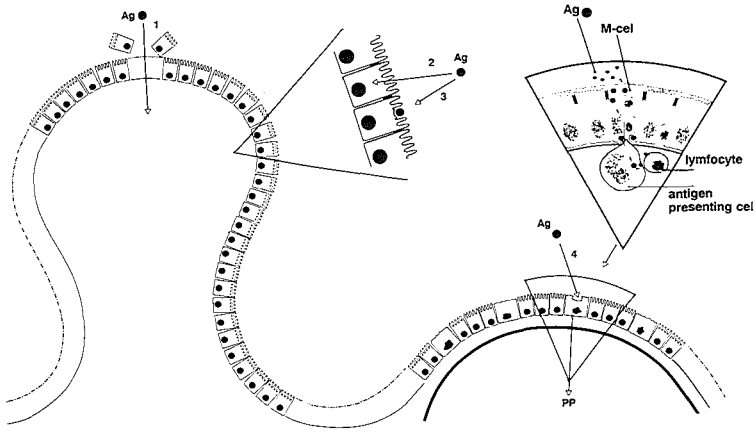

Figure 2. Ways of antigen uptake by the gut. 1) nonspecific uptake of antigen between epithelial cells; 2) nonspecific uptake via epithelial cells; 3) receptor-mediated uptake via epithelial cells; 4) specialised uptake via M-cells.

charides (30), inert particles (31,32), polypeptide fragments (33), and microorganisms (34). Several ways of antigen uptake have been described (24).

Figure 2 shows four different ways of antigen uptake: 1) nonspecific uptake of antigen between epithelial cells; 2) nonspecific uptake via epithelial cells; 3) specific uptake via epithelial cells; and 4) specialized uptake via M-cells.

Antigen can be taken up nonspecifically via broken tight junctions between absorptive epithelial cells especially in a damaged gut, for instance after a local hypersensitivity reaction (36). Antigens can also enter the body by passing the epithelial cells in the tip of the villi. The villi of many species maintain a cycle of contraction and relaxation which is associated with the continuous extrusion of cells from the villus tip. Ultrastructural examination of the villus tip has revealed gaps between the terminal epithelial cells, and villi have been shown to take up fluid via this cell extrusion zone during relaxation (37).

The small intestinal epithelial cell can engulf macromolecular antigens nonspecifically by an endocytotic process identical to the pinocytosis described for macrophages. Initially, large antigens within the intestinal lumen interact with components of the microvillous membrane of intestinal absorptive cells. When a sufficient concentration of molecules contacts the cell membrane, invagination occurs and small vesicles are formed. After invagination, antigens migrate within membrane-bound vesicles (phagosomes) to the supranuclear region of the cell where vesicles fuse with lysosomes to form large vacuoles (phagolysosomes). Within these structures intracellular antigens are digested. However, small quantities of absorbed molecules escape breakdown and migrate to the basal surface of the cell to be deposited in the interstitial space by a reversal of the pinocytotic process (38,39). Antigenically intact antigen has been detected in serum as soon as one hour after oral antigen presentation (40), although the antigen was converted

into a tolerogenic form by intestinal handling (41,42). It has long been known that oral antigen presentation easily induces a state of systemic tolerance specific for the presented antigen (43).

Next to nonspecific interaction of antigen with the microvillous membrane, specific binding to receptors is possible as demonstrated for adhesins from *Vibrio cholera* and *E. coli* or toxins like cholera toxin and *E. coli* heat labile toxin (44-46). Receptor-mediated antigen uptake can bypass lysosomal digestion and is therefore more effective in transporting luminal antigens to the underlying tissues (24).

Specialized epithelial cells exist within the gut to facilitate access of intraluminal antigens to intestinal lymphoid tissue. These epithelial cells, M-cells (membranous cells) show a poorly developed glycocalyx and a paucity of microvilli. Lysosomal organelles are absent (39). These features support the view that the M-cells are especially adapted for antigen uptake and transport. M-cells are found not only overlying PP, but can also be detected associated with microscopic lymphoid aggregates throughout the intestine (47). The combined area overlying these microscopic aggregates even exceeds that overlying the PP (48).

Histochemical studies have demonstrated a preferential uptake of horseradish peroxidase into M-cells after exposure of the gut to small quantities of this antigen (49). It has been demonstrated that particulate antigens can be transported by the M-cells as well (50). Antigen 'sampling' of luminal contents via the specialized epithelium overlying the PP appears to be quantitatively the most important route for intestinal antigen entry (51). After exposure to large amounts of antigen, uptake of antigen was noted not only in M-cells but in all epithelial cells, suggesting that the mode of antigen access to the underlying intestinal tissues may depend on the concentration of antigens in the intestinal lumen (39,49).

Transmission of maternal antibodies from mother to offspring can be seen as a special kind of antigen uptake by the gastrointestinal tract of newborn individuals. Antibodies in milk, among others induced by antigens present in the intestinal lumen of the mother, are passively transferred to their young. This proces, enabled by the common mucosal immune system, is referred to as the gut-mammary link (52). The gastrointestinal tract of newborn animals absorbs a variety of macromolecules and permits uptake of maternal antibodies via Ig-specific receptor-mediated endocytosis (53). Following exposure to colostrum, the intestinal columnar epithelial cells develop a vesicular and vacuolar apparatus that enables the neonatal gut to absorb antigens both specifically (receptor-mediated) and nonspecifically (54,55). The receptor-mediated uptake does not initiate a lysosomal response, whereas material that enters via nonspecific mechanisms is at risk for lysosomal degradation. The enhanced uptake of maternal antibodies and other macromolecules declines within a few days after birth and depends on the number of antigen molecules absorbed.

In young as well as in adult animals specific intestinal antibodies can inhibit the uptake of luminal antigens. In fact, the main function of intestinal antibodies is thought to be immune exclusion at the mucosal surface. There is considerable evidence that S-IgA antibodies block specific binding sites of the antigen and

thereby interfere with adherence of the antigen to the epithelial surfaces (56-59). Moreover, immune complexes stimulate mucus release from goblet cells, which also inhibit antigen uptake (60). Next to intestinal antibodies, leakage of systemic IgA and IgG antibodies can block antigen uptake as well (61,62). Inhibition of uptake of a particular antigen by intestinal or systemic antibodies coincides with an enhanced penetration of nonrelated antigens (61-63). This situation, for instance, does occur in allergic reactions (63), probably because immune complexes may induce gut damage and so facilitate the uptake of unrelated antigens (36).

1.3. Regulation of IgA expression

Virgin B-cells bearing both IgM and IgD on the membrane are commonly found in the gut mucosal follicles (64,65). Through stimulation by intestinal antigens, the IgM-IgD double-bearing cells undergo clonal expansion and differentiation (66). In the IgM-IgD-bearing cells, Ig heavy-chain genes are arranged in the VDJ-Cμ-Cδ-Cγ3-Cγ1-Cγ2b-Cγ2a-Cε-Cα order (67). The differentiation of IgM-IgD-cells to IgA-bearing cells (so-called IgA-switch) occurs vectorily via translocation of the VDJ gene to Cα gene (Figure 3). The intervening genes are deleted after translocation (68).

IgA commitment in PP may be achieved with the help of IgA-specific T-cells that bear an Fc receptor for IgA (69,70). A T-cell that causes the switch of IgM-bearing B-cells to IgA-bearing cells has been cloned from Concanavalin A-stimu-

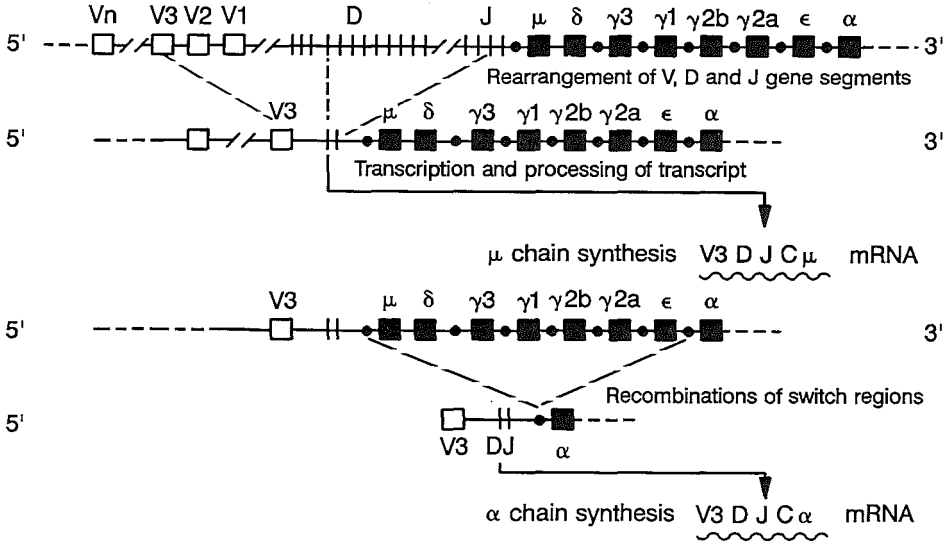

Figure 3. Schematic representation of the mouse Ig heavy chain gene segments and the processes leading to μ chain synthesis and the synthesis of α chains.

lated PP T-cell cultures (71-74). Isotype-specific T-cells have now been identified in various lymphoid organs (75). The switch-inducing cells seem to be antigen-specific (76,77), which was demonstrated elegantly with antigen-specific T-cell hybridomas (78). A second helper signal from another T-cell is necessary for terminal differentiation of switched cells to IgA secretion (79,80). The proposal that IgA-specific T-cells are responsible for the isotype switch to IgA expression is slightly weakened by studies that showed that bacterial lipopolysaccharides themselves can induce an increase of IgA-bearing cells in B-cell cultures (81,82).

Another regulation mechanism is suggested to be mediated by so-called contra-suppressor T-cells (83-86). Antigen presented via the mucosa is thought to induce antigen-specific T suppressor cells for each isotype. IgA-specific contrasuppressor cells abolish IgA suppression and so indirectly stimulate IgA-switch preferentially. In contrast, antigen presented systemically induces IgM- and IgG-specific contrasuppressor cells (87) and so indirectly stimulates IgM and IgG responses.

T-cells are thought to exert their effects through lymphokines produced by them. A T-cell derived lymphokine that enhances IgA production has been identified (78,88,89). In recent years, evidence has been presented that interleukin (IL) 4 and IL-5 may play a role in IgA switch and differentiation of B-cells. At first it was thought that IL-5 induced both the switch and differentiation of IgM-bearing cells to IgA-secreting cells (90,91). Now it is believed that IL-4 can induce switch to IgA expression, whereas IL-5 selectively stimulates IgA-bearing cells to mature (92-96). Yet the situation is not clear as IL-4 can induce, besides IgA-switch, switch of IgM-bearing cells to IgG1- or IgE-expression (97). IL-5 can also stimulate IL-4 induced IgE production (98). It is tempting to speculate that the factor derived from the cloned IgA-specific T cell hybridoma will show similarities with IL-4. The role of antigen in switch mechanisms is yet not clear (99). Antigen may define whether IL-4 induces B-cells to switch to IgG1, IgE or IgA expression. It already has been described that antigen can synergize with IL-5 to drive B-cell lymphoma proliferation and differentiation (100).

1.4. Selective migration of lymphoid cells

It has been convincingly established that PP are a major source of IgA plasma cell precursors. When PP-derived cells were transferred into irradiated, allogeneic rabbits, it was shown by the use of allotypic markers that donor cells repopulated the intestine and spleen of recipients mostly with IgA plasma cells (101,102).

Peripheral lymph node (PLN) cells repopulated the spleens and PLN mostly with IgG plasma cells. Tseng (103) reported that PP cells, adoptively transferred into sublethally irradiated congenic mice, were largely found in recipients' spleens up to 5 days after transfer. He suggested that PP-derived IgA-plasma cell precursors initially migrated to the spleen where they differentiated further before migrating to the intestine. It appeared that the spleen was an important but not obligatory site for further development and expansion of these precursors. IgA-plasma cell precursors can mature in mesenteric lymph nodes (MLN) as well. The

role of the MLN in the lodging of IgA-bearing cells into the small intestine was demonstrated by transfer of radiolabeled PP cells into normal syngeneic recipients, followed, 24 hours later, by a second transfer of the MLN of these primary recipients into secondary syngeneic recipients. Twenty-four hours after secondary transfer, radiolabeled IgA-containing cells could be identified in the lamina propria of the small intestine of the secondary recipients (104).

Other studies have now amply confirmed the role of PP as a source of IgA-plasma cells (105,106). Husband and Gowans (105) showed that, in rats, the intestinal antibody-containing cell response after immunization with cholera toxoid was abrogated if PP were surgically removed from the immunized segment of the intestine, whereas removal of MLN did not affect the response. In contrast, Keren et al. (47) and Hamilton et al. (48) demonstrated that an intestinal mucosal response could be induced in loops without PP, although the response was delayed and more than 10 times lower compared with the response induced by immunizing loops that contained a PP. In this regard it is important to notice that, although it is possible to remove all PP, it is impossible to remove all microscopic lymphoid aggregates of the intestine. Therefore, conclusions based on experiments in which PP were removed must be considered carefully.

It seems that not all PP are equally important in the generation of IgA-precursors. Reynolds and Kirk (107) suggested that two types of PP exist in sheep: jejunal PP that are more involved in generating intestinal immune responses and therefore are a rich source of IgA-plasma cells, and ileal PP that would function more, like the chicken Bursa, as a source of B-cell precursors.

Although PP are a major source of IgA-precursors, they are not the only ones. Lamina propria cells and BALT-derived cells can also repopulate the intestine of irradiated animals with IgAplasma cells (13,108,109). In rats, drainage of lymphocytes from the thoracic duct did not decrease the number of IgA-containing cells in the intestine below about 50% of the starting level (110). The remaining IgA-containing cells may arise within the lamina propria as a result of the interaction of absorbed antigen with sessile memory cells (105,111). Another possible explanation is that BALT and other mucosa associated lymphoid tissue supply the remaining IgA-containing cells. Under normal circumstances, however, BALT appears to be a poor source of IgA plasma cell precursors (112,113). Recently, it has been suggested (114) that Ly-1^+ peritoneal B-cells may be precursor cells for IgA-plasma cells in the intestine.

After switch in heavy chain expression, precursors for IgA plasma cells in PP bear surface IgA instead of surface IgM, and few contain cytoplasmic IgA (115-118). These cells traffic via the MLN and thoracic duct into the circulation and eventually return to the intestinal lamina propria. During this time, they further differentiate and acquire cytoplasmic IgA (119). In the mouse, 50 to 75% of the cells that display surface IgA in the MLN and thoracic duct also contain cytoplasmic IgA (10,115). The majority of IgA plasma cell precursors in lymph are lymphoblasts, indicating that they have been activated before or during migration.

The cell traffic outlined above is important because immunizing GALT results in a pool of circulating plasma cell precursors, mostly of the IgA class, which

return to the mucosal tissues with a preference for the organ where they were induced (111). Although migration studies largely concern IgA-committed cells it is well known that populations of GALT-derived T lymphoblasts can follow the same route (10,120). GALT-derived transferred B lymphoblasts return exclusively to the lamina propria, whereas T lymphoblasts can also be found in the epithelium as intraepithelial lymphocytes (10,120-123).

As for the GALT, BALT-derived IgA-precursors migrate in a similar route to mucosal sites in the respiratory tract (124). Moreover, both GALT and BALT nodules contain cells that can repopulate the lamina propria of irradiated animals with IgA plasma cells at either site (13,102,125), indicating the existence of a common mucosal immune system. This was substantiated by the findings that MLN cells can lodge selectively in lactating mammary gland (124,126), uterine cervix (124), salivary and lacrimal glands (127) as well as the intestine. Nevertheless, there is a certain preference to localisation. For instance, cells from the mediastinal nodes localize much better in the lungs than in the small intestine, while cells from the mesenteric lymph nodes prefer the small intestine (124). Selective localisation in the mammary gland and genital tract appears to be under hormonal influence (124,126,128). Hormonal influence has also been demonstrated for IgA levels in tears (129).

Lymphocytes must leave the blood circulation and migrate through the high endothelial venules (HEV) in order to traffic through mucosal lymhpoid structures. PP HEV bear glycoproteins that are distinct from those on the HEV in PLN. These glycoproteins, adressins, are utilized by small, recirculating lymphocytes in discriminating between the two sites (Figure 4; 130,131). Also in BALT HEV are important in lymphocyte migration (132). The lymphocytes themselves have specialized surface structures, homing receptors, that promote binding to distinct

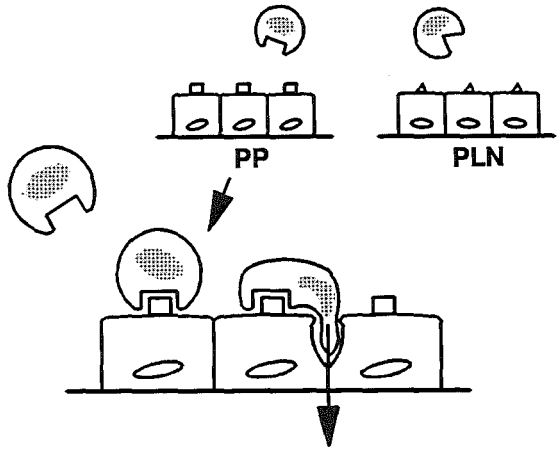

Figure 4. Recognition of distinct adressins on high endothelial venules by circulating lymphocytes leading to specific homing of lymphocytes to the underlying tissues.

HEV adressins after collision with the endothelium (133,134). For various species adressin-specific monoclonal antibodies have already been developed (135). Binding of lymphocytes to HEV can even be demonstrated in vitro (136). This enables detailed studies of the mechanisms of lymphocyte recognition (137). So, selective migration pathways can be explained for all tissues containing HEV. Knowledge about the structure and function of lymphocyte homing receptors and cell-adhesion mechanisms by HEV is cumulating rapidly and has recently been reviewed (135,138-140).

Lamina propria lacks HEV, although post-capillary venules with determinants equivalent to HEV have been reported (141). First it was proposed that, in the lamina propria, antigen is involved in trapping circulating lymphocytes bearing specific IgA antibodies (105,111,142). This was proven incorrect by experiments demonstrating homing to germ- and antigen-free intestinal mucosa (143). So, homing to the lamina propria is not antigen-dependent.

However, immediately after lodging into the tissue, cells are retained in the presence of antigen and induced to proliferate. The ultimate effect is that the response in the intestine depends on the presence of antigen (144). Recently, Nakache et al. (145) described a tissue-specific endothelial cell adhesion molecule for circulating lymphocytes in the lamina propria. This molecule was shown to be responsible for the selective binding of lymphocytes. It may be that recognition of this molecule is involved in selective migration of cells to the lamina propria.

1.5. Mucosal immunity and vaccination

The preceding paragraphs demonstrate that a lot is known about the mechanisms that lead to mucosal immune responses. Different aspects like antigen uptake, induction of immune responses in PP, isotype regulation, migration of activated cells to mucosal tissues, and excretion of S-IgA have been studied extensively. Live replicating antigens, for example bacteria and viruses, presented to the mucosa are known to evoke antigen-specific S-IgA responses by these mechanisms (35). In contrast, nonreplicating antigens, like food antigens, usually do not induce mucosal immune responses when presented mucosally. More often the opposite effect is achieved as the individual is easily rendered tolerant to ingested antigens (43). Unfortunately it is not clear yet what causes the difference of the type of response against replicating and non-replicating antigens (146,147).

It is an attractive thought to use the mucosal immune compartment in vaccination, because most pathogens enter the body via the mucosae. S-IgA is known to protect the mucosal sites from infections by its ability to inhibit the adherence of pathogens to mucosal epithelial surfaces (148,149), to inhibit antigen uptake (150), and to neutralize toxins (151,152). However, at present most vaccines are applicated by a non-mucosal route, which results in a systemic rather than a mucosal immune response. The problem we are faced with is, that only live antigen vaccines are known to induce good mucosal immune responses upon mucosal antigen presentation.

However, live mucosal vaccines are hardly available and, when available, are a considerable risk to the individual as they still can induce pathological symptoms and can reverse to the virulent type of pathogen. Mucosal vaccines containing non-replicating antigens do not induce mucosal immune responses. Therefore, various ways of antigen presentation are studied to alter or increase the immunogenicity of mucosally presented non-replicating antigens to overcome this problem.

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CHAPTER 2

INTRODUCTION TO THE EXPERIMENTAL WORK

Antigen uptake by the gut continuously stimulates the immune system of the individual. This stimulation is reflected in the number of immunoglobulin-secreting cells (Ig-SC) in the gut-associated lymphoid tissue of individuals (1). Studies in mice have shown that the number of Ig-SC in bone marrow, MLN and PP depends greatly on exogenous antigenic stimulation, whereas the number in the spleen does not (2-6). As antigen uptake mostly occurs via mucosal surfaces it is likely that in particular mucosal Ig production depends on exogenous antigenic stimulation. Immunohistological studies already demonstrated that many Ig-containing cells are present in the intestinal tissues and that the presence of these cells depends on the gut flora (7-12). As real quantitative data on the number of Ig-SC in the mucosal tissues are lacking, little is known about the various factors that influence the total Ig-production in the intestine, the contribution of the mucosal tissues to the total Ig production in the individual and consequently about the significance of the mucosal immune compartment for the defense of the individual to enteric pathogens.

The experiments described in this thesis focus on the intestinal B-cell compartment of mice. Next to antibodies, T-cells play, as effector T-cells, an important role in the protection against potential pathogens at mucosal surfaces. The T-cell compartment, however, is beyond the scope of this thesis.

The intestinal B-cell compartment can be studied best by quantitative analysis of the number of Ig-SC in the intestinal tissues. Therefore we developed (Chapter 3.1) a method to isolate functionally active lamina propria lymphocytes from the intestine. The functional activity was monitored by the capability of the isolated cells to secrete Ig. The number of Ig-SC was quantitated by the protein A plaque forming cell assay (13,14).

The ultimate expression of the Ig-SC responses at the mucosal surface was studied by measuring the Ig content of intestinal secretions. In Chapter 3.2 two sampling procedures for intestinal secretions were evaluated for their applicability to study intestinal Ig responses. The Ig content in intestinal secretions was measured by ELISA procedures.

In Chapter 4.1 the number of Ig-SC in the intestine was compared with the number of Ig-SC in other lymphoid organs. To that purpose, the number of Ig-SC in the small intestine and in various other lymphoid organs like spleen, bone marrow, MLN and PP was quantitated and the relative contribution of the small intestine to the total number of Ig-SC calculated.

The development of the intestinal B-cell compartment in the first few weeks of life and factors that influence this development are described in Chapter 4.2. The influence of the time of weaning itself was investigated by comparing the number of Ig-SC in early-weaned mice with that in non-weaned littermates. The influence of the presence and the type of gut-flora was studied by comparing the number of intestinal Ig-SC in conventionally housed mice with that in germfree mice and mice housed under specific pathogen free conditions.

The development of the intestinal B-cell compartment in elderly mice is described in Chapter 4.3. To ensure that the results were not due to unnoticed illnesses of the animals, we used old CBA/Rij mice from cohorts, that were housed under specific pathogen free conditions and were continuously screened for pathological disorders. These results were compared with those from conventionally housed C3H/He mice. The papers presented in Chapters 4.1 to 4.3 show that the small intestine is a dynamic lymphoid organ with high immunological activity.

To investigate whether it is possible to induce antigen-specific immune responses in this compartment, in Chapters 5.1 and 5.2 the induction of antigen-specific immune responses in the intestinal mucosa is described. In Chapter 5.1 we evaluated the sensitivity of quantitating antigen-specific antibody-secreting cells in the small intestine as a method to measure antigen-specific mucosal immune responses. The effect of a carrier-protein and an adjuvant on these responses was studied in Chapter 5.2.

The data obtained in the experimental studies are discussed in relation to the literature data in Chapter 6, the General Discussion.

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CHAPTER 3

ASSAYS FOR THE ISOLATION OF LYMPHOID CELLS AND SECRETIONS FROM THE INTESTINE

3.1 Improved procedure for the isolation of functionally active lymphoid cells from the murine intestine.

3.2 Comparison of two methods for collecting murine intestinal secretions to detect antigen-specific antibodies.

3.1 IMPROVED PROCEDURE FOR THE ISOLATION OF FUNCTIONALLY ACTIVE LYMPHOID CELLS FROM THE MURINE INTESTINE

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SUMMARY

An isolation procedure for functionally active lamina propria lymphoid cells (LPL) from the murine intestine is described. The procedure involved EDTA-dithiothreitol incubation of intestinal tissue to remove epithelial and intraepithelial cells, followed by collagenase digestion of the basement membrane to liberate part of the LPL. The LPL were suspended by squeezing the remaining tissue strips through a nylon gauze filter. Functional activity was tested by enumeration of the immunoglobulin-secreting cells in the cell suspensions obtained by an isotype-specific protein A plaque-forming cell assay. On average 1-2 x 10^8 LPL were isolated from the intestine of C3H/He mice. 11% of these cells actively secreted Ig. From these Ig-secreting cells 99% produced IgA. The isolation procedure described in this paper permitted a higher recovery of viable cells than has previously been obtained with other methods.

INTRODUCTION

The intestinal mucosa is the portal of entry for many infectious agents. Therefore, an adequate enteric mucosal immune system is important as a first line of defence (Befus and Bienenstock, 1982). To obtain insight into the mechanisms regulating the enteric mucosal immune response quantitation of the humoral mucosal immune response is of great importance.

The *in vivo* expression of the intestinal mucosal immune system has been studied by immunohistological techniques (Sminia et al., 1983; Pierce, 1984) and by measuring the levels of IgA in faeces (Elson et al.,1984; Van Zaane et al.,1986). Immunohistological studies on the localization of immunoglobulin-containing cells (Sminia et al., 1983; Pierce, 1984) give direct information about the *in vivo* expression of the mucosal immune system. However, quantitative data are difficult to obtain with this method and the number of immunoglobulin-containing cells does not necessarily reflect the number of cells that actually secrete antibody (Benner et al., 1982) and contribute to the intestinal IgA response. Similarly, the quantity of IgA, measured in faeces using an ELISA technique (Elson et al.,1984; Van Zaane et al.,1986), is not merely a reflection of intestinally produced and secreted IgA, since systemically produced IgA can also contribute to the IgA levels in faeces (Solari and Kraehenbuhl, 1985). Moreover, in bovine species, IgA can be demonstrated by ELISA in the faeces of young calves, but the amount of IgA found in faeces decreases below detectable levels during ageing. This is in contrast to immunohistological observations which have shown that IgA-containing cells are still present in the intestinal wall of older animals (Kimman et al., 1987). This decrepancy may be due to the degradation of IgA by proteolytic enzymes or to complexing of IgA with other components in faeces.

We believe that quantitative data on the enteric humoral immune response can best be obtained by isolation of lymphoid cells from the intestine and by subsequently testing the capacities of these cells to secrete immunoglobulins.

Several procedures for the isolation of intraepithelial and/or intestinal lamina propria lymphoid cells of rat (Lyscom and Brueton, 1982; Mayrhofer and Whately, 1983) and mouse (Cebra et al.,1977; Davies and Parrott, 1981; Tagliabue et al.,1982; Tseng, 1982; Leventon et al., 1983; Dillon and MacDonald, 1984; Lycke, 1986) have been described. However, most of these investigators do not deal with the functional activities of the isolated cells. Moreover, although the isolation procedures described may yield a representative intestinal cell population, they were not developed to give a quantitative estimation of the total number of lymphoid cells present in the intestine.

Based upon procedures described earlier we have developed an isolation procedure for murine lamina propria cells that gives a high yield of viable lymphoid cells which can be tested for their functional activities. Since the enteric humoral immune response is our first interest, the isolation procedure was optimized on the basis of a maximal yield of immunoglobulin-secreting cells (Ig-SC) as tested in an isotype-specific protein A plaque-forming cell (PFC) assay.

MATERIALS AND METHODS

Mice: Clean conventional female C3H/He mice were purchased from HARLAN-OLAC Ltd., Bicester, U.K. and used when aged 8-20 weeks. The mice were housed under conventional conditions.

Isolation of intestinal lymphoid cells: Mice were killed with carbon dioxide and immediately thereafter the intestine was isolated and thoroughly rinsed with physiological saline. All macroscopically visible Peyer's patches were removed. Subsequently the intestine was opened longitudinally and the resulting tissue strip was washed in Ca- and Mg-free balanced salt solution (BSS) and cut in 0.5-1 cm pieces.
These tissue pieces were first incubated during 10-15 minutes in 25 ml of Ca- and Mg-free BSS supplemented with DTT (0.145 mg/ml) and EDTA (0.37 mg/ml) in a shaking waterbath (110 strokes/min, 37°C). The supernatant, primarily containing epithelial cells, was collected by decantation. The cells in the supernatant were washed with RPMI 1640 (containing 5% FCS, 20 mM HEPES and 0.1 mg/ml DNAse (Sigma)) and subsequently squeezed through nylon gauze filters (100 μm followed by 50 μm) to obtain a single cell suspension (Fraction 1).

The debris remaining after decantation was rinsed once with RPMI 1640 (containing 5% FCS and 20 mM HEPES) and then incubated during 75-90 minutes in 25 ml of RPMI 1640 containing 5% FCS, 20 mM HEPES, 0.15 mg/ml collagenase (75 Mandle U/ml; Serva) and 0.1 mg/ml DNAse in a shaking waterbath (110 strokes/min, 37°C). The supernatant of this incubation mixture was collected by filtration through a stainless steel filter of 1 mm. The filtered cells in this supernatant were then washed once with RPMI 1640 (containing 5% FCS, 20 mM HEPES and 0.1 mg/ml DNAse) and squeezed through nylon gauze filters (100 and 50 μm) to obtain a second single cell suspension (fraction 2). The pieces of intestine remaining on the stainless steel filter were also squeezed through nylon gauze filters (200, 100 and 50 μm) to produce a third single cell suspension (Fraction 3). The cell suspensions 1, 2 and 3 were washed in RPMI 1640 (containing 5% FCS, 20 mM HEPES and 0.1 mg/ml DNAse), and counted in a haemocytometer. Viability of leukocytes was determined by nygrosine exclusion. To avoid bacterial growth, penicillin and streptomycin were added to all the RPMI 1640 solutions used during the isolation procedure.

Density gradient purification: Isolated lamina propria lymphoid cells were further purified by filtration through cotton wool, followed by discontinuous density gradient centrifugation. The gradient consisted of a bottom layer of 18.4% Nycodenz (Nyegaard, Denmark; density 1.098) and a top layer of 10.3% Nycodenz (density 1.056). After loading the cell suspension on the top of the gradient the tubes were spun for 20 min at 600xg and 4°C. The cells recovered at the interface between 1.056 and 1.098 density were washed in RPMI 1640 (containing 5% FCS, 20 mM HEPES and 0.1 mg/ml DNAse) and counted in a haemocytometer.

Isotype-specific protein A plaque forming cell (PFC) assay: The protein A PFC assay has been described previously (Van der Heijden et al., 1986). Rabbit-anti-mouse IgM, -IgG en -IgA were obtained commercially (Nordic, Tilburg, The Netherlands). Penicillin and streptomycin were added to the incubation mixture to avoid bacterial growth during the performance of the assay. Specificity control of the antisera by ELISA techniques with purified myeloma IgM, IgG1 or IgA (Litton Bionetics, U.S.A.) showed that the antisera used in the protein A PFC assay were specific for the different isotypes.

Immunofluorescence staining: Preparations of cell suspensions were fixed with cold ethanol-acetic acid. All cells with surface and/or cytoplasmic Ig were labelled directly with rabbit-anti-mouse IgA(Fc)/FITC, goat-anti-mouse IgM(Fc)/FITC or goat-anti-mouse IgG(Fc)/FITC conjugates (Nordic, Tilburg, The Netherlands).

T-cell markers were detected by incubation of the cell preparations, after fixation with cold acetone, with a rat-anti-Thy-1 monoclonal antibody (kind gift of Dr. W. van Ewijk, Erasmus University, Rotterdam, The Netherlands (Van Ewijk et al., 1981)), followed by staining with a rabbit-anti-rat Ig/FITC conjugate (Dako, Glostrup, Denmark). After incubation the preparations were washed and examined for fluorescence staining.

RESULTS

Recovery of viable mononuclear cells and the quantitation of Ig-SC in the different intestinal cell fractions.

The small intestines were removed from C3H/He mice of 8-20 weeks of age. Intestinal cell populations (Fractions 1, 2 and 3) were isolated as described in the materials and methods section and subsequently the number of viable cells in each fraction was determined. The functional activity of the isolated cells was assayed by testing the fractions for the presence of Ig-SC by means of the isotype-specific

Table 1. Number of Ig-SC in murine intestinal cell fractions obtained after enzymatic digestion.

Fraction	No. of viable cells	Epithelial cells (%)	No. of Ig-SC		Distribution of IgSC (%)
1	39×10^6	70	660×10^3	(266×10^3)[a]	5
2	65×10^6	10	6.600×10^3	(1500×10^3)	53
3	46×10^6	5	5.300×10^3	(699×10^3)	42
Total	150×10^6	25	12.600×10^3	(1600×10^3)	100

[a] Numbers are expressed as the mean values (n=5) with the SD shown in brackets.

Table 2. The percentages of Thy-1, IgM, IgG and IgA positive cells in the LPL suspension as determined by immunofluorescence staining.

	Thy-1	IgM	IgG	IgA
% positive cells	7 (3)[a]	<1	<1	12 (4)

[a] mean values (n=10) with the SD shown in brackets.

protein A PFC assay. Table I shows the results of the enumeration of viable cells and Ig-SC in each fraction.

Fraction 1 contained, as expected, the highest percentage of epithelial cells (70 %), while most of the viable cells in the Fractions 2 and 3 were non-epithelial mononuclear cells. Almost 10% of all viable isolated cells (Fractions 1, 2 and 3) produced Ig. It appeared that most Ig-SC occurred in Fractions 2 and 3 (53% and 42%, respectively). Therefore Fraction 1 was discarded in further experiments concerning the intestinal Ig-SC, while Fractions 2 and 3 were pooled to give one cell suspension containing almost all lamina propria lymphoid cells (LPL). Throughout the rest of the experiments pooled LPL suspensions were used.

Immunofluorescence staining of LPL with anti Thy-1, -IgM, -IgG and -IgA.
The presence of Thy-1 and Ig-positive cells in the LPL suspension (Fractions 2 and 3) was studied by immunofluorescence staining with anti-Thy-1, anti-IgM, -IgG or -IgA. Table II shows the results of this study. A clear distribution between cytoplasmic-Ig-only (c-Ig) or membrane-plus-cytoplasmic Ig (m/c-Ig) positive cells could not be made and, as the percentage membrane-Ig-only (m-Ig) positive cells was negligible, in Table II only the percentages of Ig-positive cells are given. Staining of the LPL with the anti-isotype specific antisera showed a strong predominance of the IgA-isotype among the Ig-positive cells.

Quantitation of Ig-SC in the intestine in adult mice.
The numbers of IgM, IgG and IgA-secreting cells in the intestine were determined in mice of 20 weeks of age using the isotype specific protein A PFC assay. The results are shown in Table III and expressed as the mean values obtained from ten mice. The isotype distribution revealed that the isolated Ig-SC contained almost exclusively IgA-producing cells. The isotype distribution and the percentage of Ig-SC found with the protein A PFC assay agree with the isotype distribution and the percentage of IgA-positive cells in the same suspension as detected by immunofluorescence.

Purification of Ig-SC from the LPL suspension.
Further purification of Ig-SC from the LPL suspension via filtration through cotton wool followed by density gradient centrifugation results in a less than two-fold increase of the percentage Ig-SC (Table IV). However, after this procedure only 24% of the total number of viable cells was recovered. The total number of

Table 3. Number of Ig-SC per intestine and per 10^6 viable intestinal lymphoid cells of mice of 20 weeks of age.

Isotype	No. of Ig-SC per intestine ($x10^{-3}$)	No. of Ig-SC per 10^6 LPL cells
IgM	16 (9)[a]	119 (70)
IgG	57 (44)	416 (325)
IgA	12,200 (5700)	88,000 (41,000)

[a] mean values (n=10) with the SD shown in brackets.

Table 4. Effect of cotton wool filtration followed by density gradient centrifugation on the percentages Ig-SC in the LPL suspension.

	Viable cells recovered	Ig-SC (%)
LPL before purification	1.5×10^8[a] (0.4×10^8)	11 (5)
LPL after purification	0.4×10^8 (0.2×10^8)	17 (5)

[a] Results are expressed as the mean values (n=10) with the SD shown in brackets.

Ig-SC recovered after the procedure was only about 40% of the number found in the unpurified LPL suspension.

Filtration through cotton wool followed by density gradient centrifugation did improve the quality of the LPL suspension. The percentage of epithelial cells in the suspension dropped from an average of 10% to 2% and the percentage dead cells declined from 55% to 35%. The relative increase in the percentage Ig-SC found after cotton wool filtration followed by density gradient centrifugation was also reflected in the percentages IgA and Thy-1 positive cells found after immunofluorescence staining of the purified cell suspensions.

The percentage Thy-1 positive cells increased on average from 7% to 11% and the percentage IgA positive cells from 12% to 18%.

DISCUSSION

The first quantitative data on murine intestinal antigen specific Ig-SC were obtained by André et al. (1978) after mechanical isolation of intestinal lymphocytes. Unfortunately, these authors did not give a detailed description of their isolation procedure. From our experience and from that of other investigators (Cebra et al., 1977; Davies and Parrott, 1981) it appears that appropriate single cell suspensions, necessary for quantitative, functional studies, can rarely be obtained using a mechanical isolation method.

Cebra et al. (1977) and Davies and Parrott (1981) demonstrated that, after some modifications, the EDTA-collagenase procedure (Bull and Bookman, 1977) is the method of choice for obtaining viable, functionally active single cell suspensions from the murine intestine. We modified the EDTA-collagenase isolation method described by Davies and Parrott (1981) for the isolation of murine intestinal Ig-SC and obtained average yields of 1.5×10^8 living LPL per adult C3H/He mouse. This is about ten times the number of viable cells isolated by Davies and Parrott (1981). As the various steps in the isolation procedure appeared to be critical with regard to the recovery of viable cells our isolation procedure requires more detailed discussion.

The EDTA-DTT incubation of the intestine is needed to remove mucus, epithelial cells and intraepithelial lymphocytes (Fraction 1), while the basement membrane remains intact. This was checked and optimized by histological examination. In our hands an incubation period of 10-15 minutes is sufficient to remove most of the epithelium and mucus and short enough to leave the basement membrane intact. Longer incubation times damaged the basement membrane after which many Ig-SC were liberated into the intraepithelial cell suspension. The relative absence of Ig-SC in the intraepithelial cell population obtained by us after EDTA-DTT incubation (Table 1) is in agreement with other studies (Tagliabue et al., 1982; Dillon and MacDonald, 1984; Ernst et al., 1985) in which it was shown that intestinal intraepithelial cell populations contain very few B-cells.

Subsequent incubation of the remaining intestinal tissue with collagenase breaks down the basement membrane after which villi and lamina propria cells are liberated and can be suspended. As batches of collagenase can differ in their enzymatic activity, the optimal concentration and time of incubation have to be determined for each batch separately. In our procedure 75-90 minutes was usually the optimal period of incubation. After longer incubation periods, up to 4 hours, the number of viable lymphocytes declined to 15%, while the number of functionally active Ig-SC declined to 50% (data not shown).

Moreover, the total number of Ig-SC obtained in suspension directly after collagenase treatment (Fraction 2) did not increase using incubation periods longer than 90 minutes. The Ig-SC in the tissue that was left after collagenase treatment could only be isolated by suspending the remaining tissue through a gauze filter (Fraction 3). This suspension still accounted for about 40% of all Ig-SC.

Recently an isolation method, also based upon the EDTA-collagenase procedure has been described by Lycke (1986). This author isolated $3.5 \pm 0.2 \times 10^6$ LPL per C57BL/6 mouse, while 0.5% appeared to be Ig-SC as tested in a reverse ELISA-spot assay. In contrast, we found 5-20% Ig-SC out of 1.5×10^8 LPL isolated per C3H/He mouse. Apart from the mouse strain used, these differences must also be explained by the method used. Lycke used a longer incubation period with EDTA (four times 15 minutes compared to our 10-15 minutes) and did not suspend the tissue pieces remaining after collagenase digestion. Another difference between our procedure and the one used by Lycke is the way of mixing. Lycke used a magnetic stirrer, with a magnet on the bottom of the flask containing the intestinal tissue strips. This way of stirring is injurious to cells in suspension.

We used a shaking waterbath to achieve good mixing and to avoid cell disruption.

In our studies the Ig-SC were quantitated using a protein A PFC assay directly after the EDTA-DTT-collagenase isolation procedure, while in most of the other studies (Cebra et al., 1977; Davies and Parrott, 1981; Tagliabue et al., 1982; Tseng, 1982) the LPL were characterized after an additional density gradient purification step. For direct comparison of our data with the data obtained by others we also applied density gradient centrifugation for further purification of the LPL suspension. Our results confirm the data presented by Cebra et al. (1977) and Tseng (1982) which indicate that 10^7 cells per mouse can be obtained after gradient purification of LP cells isolated with the EDTA-collagenase procedure.

Fluorescence studies demonstrated that gradient purified suspensions of LP cells contained on average 11% T-cells and 20% Ig-containing cells of which 95% were IgA-positive. These values are in agreement with published data (Tseng,1982) which indicated 9% Thy-1 positive cells and 22% Ig-containing cells of which more than 96% were IgA- -positive. Density gradient purification did improve the quality of the LPL suspension with regard to viability and the contamination with epithelial cells. A relative enrichment of Ig-SC, and of Thy-1 or IgA-positive cells was also achieved. However, more than half of the Ig-SC obtained after EDTA-DTT-collagenase digestion were lost during gradient purification. Therefore, it must be concluded that gradient purification is undesireable for a reliable quantitation of the Ig-SC in the intestine. In conclusion, the data presented in this paper suggest that our modified isolation procedure for intestinal Ig-SC is far superior to previously described methods with regard to recovery and viability as substantiated by the high number of Ig-SC detected in the isotype specific protein A PFC assay.

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3.2 COMPARISON OF TWO METHODS FOR COLLECTING MURINE INTESTINAL SECRETIONS TO DETECT ANTIGEN-SPECIFIC ANTIBODIES

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SUMMARY

Two methods frequently used to sample intestinal secretions are compared for evaluating intestinal immune responses. Intestinal samples were obtained by washing or scraping the intestine of mice immunized with ovalbumin (OA). An enzyme-linked immunosorbent assay was used to measure OA-specific responses in the washings and scrapings as well as in serum. Scrapings had the highest titres of OA-specific IgA.

OA-specific IgG responses were detected in scrapings and serum, but not in washings. Most of the OA-specific IgA detected in washings and in scrapings appeared to be locally produced. In contrast, most OA- specific IgG in scrapings originated from the blood. To confirm these findings, we immunized mice to induce a systemic but not a mucosal immune response and evaluated intestinal scrapings and washings again.

We conclude that scraping the intestine is the most practical method to study intestinal IgA responses. The method is more efficient in collecting IgA antibodies than washing. The occurrence of IgG antibodies in scrapings must be considered as an artefact.

INTRODUCTION

Secretory immunoglobulin A (S-IgA) is the predominant Ig isotype along mucosal surfaces and is thus the principal effector mechanism of the humoral immune system at the mucosal site (Bienenstock and Befus, 1980; Underdown and Schiff, 1986). The *in vivo* expression of the intestinal mucosal immune response can be studied by measuring S-IgA responses in intestinal secretions. Some of the methods used to sample intestinal secretions are to lavage the intestine *in vivo* by inducing a mild diarrhoea in the animal (Elson et al., 1984; Burr et al., 1987), to flush isolated loops of intestine *in vivo* or to flush intestinal sections *in vitro* (Keren et al., 1983; Nedrud et al., 1987; Keren et al., 1988, Senda et al., 1988), to scrape intestinal tissue (Falchuk et al., 1977; Anderson et al., 1985; de Aizpurua and Russell-Jones, 1988; Van Loveren et al., 1988), or to homogenise the entire intestine in a blender (Clements et al., 1986). Most sampling methods, except for the lavage procedures (Elson et al., 1984; Burr et al., 1987), have not been well standardized.

Some investigators have recovered only antigen-specific IgA antibodies from intestinal secretions (Yardley et al., 1978; Elson and Ealding, 1984; Burr et al., 1987), whereas others have recovered antigen-specific IgG antibodies as well (Keren et al., 1980; McKenzie and Halsey, 1984; Clements et al., 1986). These differences may be due to the sampling methods used.

The purpose of the present study was to determine whether the scraping technique contaminates intestinal Ig with Ig from the blood. In an earlier article (Van der Heijden et al., 1989) we described antigen-specific B-cell responses in the small intestine after an immunization regimen that induced both mucosal and systemic immune responses. Using the same immunization regimen as in the earlier study, we compared the gentle lavage technique (Elson and Ealding, 1984), with the rougher scraping technique for recovering intestinal Ig. The distribution of antigen-specific IgM, IgG and IgA in serum and intestinal secretions was compared to the distribution of total IgM, IgG and IgA in these fluids.

MATERIALS AND METHODS

Mice: Female C3H/He mice were purchased from Harlan-Olac Ltd., Bicester, Oxon, U.K., housed conventionally, and used at the age of 12-20 weeks.

Antigens and immunization: Ovalbumin (OA; Grade V, Sigma, U.S.A.) was polymerized according to the method of Holt et al. (1984). Briefly, glutaraldehyde (Merck, Darmstadt, FRG.) was added drop-by-drop to the protein in phosphate-buffered saline (PBS) to a final molar ratio of 25:1. During the next 4 hours the pH was repeatedly adjusted to 7.5. The reaction was stopped by adding excess glycine, and the resulting mixture dialysed and clarified by centrifugation. Mice were injected intraperitoneally (ip) with 0.1 ml of a water-in-oil emulsion (Bokhout et

al., 1981) containing 0.1 mg polymerized OA. Four weeks later the mice were given booster immunizations either orally with 80 mg monomeric OA in 0.5 ml 0.2 M $NaHCO_3$ or ip with 0.1 mg polymerized OA in 0.1 ml saline.

Intestinal secretions: Intestinal washings were obtained by lavage according to the method of Elson et al. (1984). After four gastric intubations of 0.5 ml lavage fluid (25 mM NaCl, 40 mM Na_2SO_4, 10 mM KCl, 20 mM $NaHCO_3$, 48.5 mM polyethylene glycol), light diarrhoea was induced by ip injection of 100 µl pilocarpine (1 mg/ml; Chibret, Haarlem, The Netherlands). Diarrhoea samples were collected and each was suspended in a 3-ml solution of 50 mM EDTA and 0.1 mg/ml trypsin inhibitor (Sigma, St. Louis, USA) and filled up to 6 ml with PBS. The suspensions were mixed vigorously and centrifuged (10 min, 650 x g).

To 4.5 ml of supernatant 45 µl phenylmethylsulfonylfluoride (PMSF; Sigma) was added and the solution was clarified by centrifugation (20 min, 27,000 x g). To the clarified supernatant 45 µl PMSF, 30 µl of 1% NaN_3, and 150 µl foetal calf serum were added and the samples were stored at -20 °C.

Intestinal scrapings were obtained in the following manner. Mice were killed with carbon dioxide and the small intestines were removed and flushed with PBS. The contents of the intestine were pushed out by squeezing the intestine between a petridish and the round tip of a pair of forceps. The squeezing was standardized, so that afterwards 34 ± 9% (w/w; wet weight) of the contents remained in the intestine and 66 ± 5% (w/w) was scraped out. The scrapings, about 1 ml per small intestine, were dissolved in 2 ml PBS containing 50 mM EDTA and 0.1 mg/ml trypsin inhibitor and mixed vigorously. The solution was clarified by centrifugation (10 min, 650 x g) and 10 µl of 1% NaN_3 was added before storage. Samples collected this way could be stored at -20 °C without detectable decrease in titre. The addition of PMSF did not enhance the recovery of Ig or the storage life of the samples.

Histologic examination: To determine the effect of the sampling method on the morphology of the intestinal tissue, we examined pieces of intestine histologically. "Swiss rolls" were prepared according to the method of Moolenbeek and Ruitenberg (1981) and stained.

Antigen- and isotype-specific detection of Ig: OA-specific antibodies in intestinal secretions and sera were assayed in an ELISA on microtitre plates coated with polymerized OA. Rabbit anti-mouse IgM- and IgG-peroxidase conjugates were obtained from Nordic, Tilburg, The Netherlands. Sheep anti-mouse IgA-peroxidase conjugate was obtained from Serotec Ltd., Bicester, England. Bound conjugate was made visible by adding 3,3,5,5-tetramethylbenzidin (Boehringer, Mannheim, FRG) and H_2O_2 as a colouring substrate. After incubation for 10 min, the reaction was stopped by adding 100 µl of 0.1 N H_2SO_4. Absorbance was measured at 450 nm.

Total Ig in intestinal secretions and sera were assayed in an ELISA on microtitre plates coated with rabbit anti-mouse-IgM, -IgG or -IgA (Nordic). The

ELISA was otherwise performed the same as the OA-specific ELISA described above. Total Ig levels were expressed as arbitrary Units/ml; the amount of Ig that gave an extinction of 0.300 at 450 nm was set on 0.1 Units/ml.

All antisera were selected on the basis of their specificity, which was determined on microtitre plates coated with purified myeloma IgM, IgA, or IgG subclasses (Litton Bionetics, Charleston, USA) as described earlier (Van der Heijden et al., 1987).

RESULTS

Evaluation of OA-specific and total Ig content of intestinal washings and scrapings Mice were primed ip with OA in a water-in-oil emulsion and were given booster immunizations orally four weeks later. Intestinal secretions were collected 7 and 8 days after booster immunization. Intestinal secretions were collected from one half of the mice by lavage, and from the other half by scraping. Serum samples were collected by heart puncture. Table 1 shows the isotype-specific anti-OA titres and total Ig Units/ml. In scrapings the antigen-specific IgA titre equalled the IgG titre, whereas in washings only low titres of antigen-specific IgA were measured. Serum samples had relatively high titres of antigen-specific IgG and low titres of IgA and IgM. The predominant total Ig isotype found in serum was IgG, whereas in scrapings and washings it was IgA. Only scant amounts of total IgM and IgG could be detected in washings. To determine whether IgA in

Table 1. Total and OA-specific Ig content in sera, scrapings and washings after oral booster immunization.

	Total IgM[1]	Spec. IgM[2]	Total IgG	Spec. IgG	Total IgA	Spec. IgA
Serum						
Day 7	566 (80)	13.1 (6.0)	2830 (400)	8000 (2800)	200 (0)	30.3 (24.2)
Day 8	492 (65)	8.7 (2.4)	4660 (640)	18400 (5500)	318 (50)	40.0 (40.0)
Scrapings						
Day 7	2.3 (0.8)	<2.0 (-)	21.1 (3.7)	52.8 (14.3)	420 (40)	69.6 (19.8)
Day 8	2.0 (0.7)	<2.0 (-)	22.6 (4.1)	56.6 (10.0)	450 (20)	28.3 (12.1)
Washings						
Day 7	<0.20 (-)	<2.0 (-)	0.67 (0.4)	<2.0 (-)	80 (0)	5.0 (1.0)
Day 8	0.35 (0.06)	<2.0 (-)	0.90 (0.6)	<2.0 (-)	40 (10)	4.4 (1.2)

Results are expressed as the mean (n=10 for sera, n=5 for scrapings and washings) with the SEM in brackets. 1) Total IgM, IgG and IgA are expressed as Units/ml. 2) OA-specific IgM, IgG and IgA are expressed as titres.

Table 2. Ratios of total Ig (Units/ml) in serum to those in scrapings and in washings.

Immunoglobulins	Serum/Scraping ratios		Serum/Washing ratios	
	Day 7	Day 8	Day 7	Day 8
IgM	250	250	>2,830	1,406
IgG	134	206	4,230	5,177
IgA	0.48	0.71	1.9	2.3

intestinal secretions was produced locally or had leaked from the blood, we first calculated the ratios of total IgM, IgG, and IgA Units/ml in sera to those in scrapings and the ratios of those in sera to those in washings (Table 2). The serum/scraping ratios for IgM and IgG (on average 250 and 170) and the serum/washing ratios for IgM and IgG (on average >2118 and 4703) were much higher than the serum/scraping and serum/washing ratios calculated for IgA (on average 0.59 for scraping and 2.1 for washing). The lower ratios for IgA already indicated that IgA had not leaked from the blood. Next, from Table 1, we calculated the ratios of total IgA Units to total IgG Units in sera, scrapings and washings.

The IgA/IgG ratio in washings (82:1) was more than 4 times as high as in scrapings (20:1) and more than 800 times as in serum (0.07:1). Thus the IgA in scrapings and washings was not derived from the blood and therefore must have been produced locally.

Theoretically, if IgG demonstrated in scrapings was derived from the blood, the ratio of OA-specific IgG titres to total IgG Units/ml should be the same in both fluids. From Table 1, we calculated that the ratio of OA-specific IgG to total IgG for serum (3.0) was almost the same as that for intestinal scrapings (2.5). Thus, IgG measured in scrapings probably originated from the blood.

To determine whether or not the IgG was derived from the blood, in another series of experiments we immunized mice to induce a systemic but not a mucosal immune response. Mice were primed ip with OA in a water-in-oil emulsion and given a booster immunization ip four weeks later. Serum and intestinal secretions were collected 7 days after booster immunization. Intestinal secretions were collected by lavage and by scraping. Table 3 shows the isotype-specific anti-OA titres and total Ig Units/ml in the intestinal secretions and sera. After parenteral booster immunization the OA-specific IgG titre in serum was more than two times as high as after oral booster immunization. In scrapings, the OA-specific IgG titre after parenteral booster was the same as after oral booster. In contrast, the IgA titre in scrapings after parenteral booster (Table 3) was five times less than the IgA titre after oral booster immunization (Table 1). OA-specific antibodies were not detected in washings. Total Ig Units/ml measured after parenteral booster in serum, scrapings, and washings (Table 3) were comparable to those measured after oral

Table 3. Total and OA-specific Ig content in sera, scrapings and washings at day 7 after parenteral booster immunization.

	Total IgM[1]	Spec. IgM[2]	Total IgG	Spec. IgG	Total IgA	Spec. IgA
Serum	700 (105)	4100 (1400)	4900 (750)	39450 (11250)	340 (40)	<10 (-)
Scrapings	4.6 (1.3)	6.1 (0.8)	6.9 (1)	56 (29)	220 (30)	9 (3)
Washings	<0.2 (-)	<2.0 (-)	<0.4 (-)	<2.5 (-)	37 (5)	<2.5 (-)

Results are expressed as the mean (n=10 for sera, n=5 for scrapings and washings) with the SEM in brackets. 1) Total IgM, IgG and IgA are expressed as Units/ml. 2) OA-specific IgM, IgG and IgA are expressed as titres.

booster (Table 1). After parenteral booster, the ratios of OA-specific IgG to total IgG for scrapings (8.1) was nearly the same as that for serum (8.0).

Histological observations of the intestine after lavage and scraping.
The structure of the small intestine of mice was studied after lavage and after scraping. After lavage, the small intestine appeared to be virtually intact, whereas after scraping it was severely damaged (Fig.1). Most villi had been stripped off up to the crypt region.

DISCUSSION

We compared two methods for sampling intestinal secretions, namely lavage and scraping. Total and OA-specific Ig content was measured by ELISA. Immune responses were induced by immunizing mice according to an appropriate immunization regimen (Van der Heijden et al., 1989).

Scrapings had antigen-specific IgA titres that were ten times those measured in washings (Table 1). Antigen-specific IgG titres were measured in scrapings but not in washings.

Compared to total IgA titres, IgG titres were relatively higher in scrapings than in washings indicating that IgG transudated into the lumen at a much lower rate than IgA is excreted. IgA is transported across the epithelium into the intestinal lumen after binding to the secretory component on the basolateral membrane of the epithelial cells (Solari and Kraehenbuhl, 1985). No specific transport mechanism exists, however, for IgG, although IgG can transudate into the intestine (Brown, 1978). Furthermore, luminal IgG is less stable than luminal IgA (Underdown and Dorrington, 1974; Lindh, 1975; Keren et al., 1980). This may explain why the amount of total IgG per ml in washings was lower than the amount in scrapings. Actually, antigen-specific IgG was below the detection level in washings.

The point of contention is whether or not antigen-specific IgG is inherently

present in the intestinal lumen or whether it originates from blood during sampling intestinal secretions. Some investigators report intestinal IgG responses and, when measuring intestinal IgA titres, they correct for supposed contamination with blood (Clements et al., 1986). Other authors report intestinal IgG responses, but do not correct their IgA titres for supposed contamination (Butler et al., 1983; Anderson et al., 1985). In some studies intestinal IgG responses could not be detected (de Aizpurua and Russell-Jones, 1988), while in other studies only IgA responses are reported without measuring IgG responses (Senda et al., 1988; Van Loveren et al., 1988).

To determine the origin of the antigen-specific IgA in scrapings and washings, we compared the ratios of total IgM, IgG and IgA Units/ml in serum to those in scrapings and to those in washings (Table 2). The serum/scraping and serum/washing ratios for IgA were much lower than the ratios for IgG and IgM. Therefore we conclude that, even if all IgG and IgM had originated from serum, IgA detected in scrapings and washings was mainly produced locally and transported actively to the intestinal lumen. This conclusion was supported by the ratios of total IgA titres to total IgG titres in the various fluids, that clearly showed that washings and scrapings contained relatively more total IgA than sera.

Antigen-specific IgG in scrapings may be locally produced or may be derived from blood, the latter as a consequence of the method used to collect the intestinal secretions. Histological examination of the small intestine after scraping revealed severe damage. The villi were ripped off down to the crypts or even deeper (Fig. 1B). So, the occurrence of serum components in scrapings is likely. In contrast, after lavage the intestine was virtually intact (Fig. 1A).

Whether or not serum components contaminate the intestinal secretions can be determined in various ways. One can try to detect serum components that are not normally present in the intestinal lumen, in the intestinal samples to evaluate possible contamination. However, because the proteolytic degradation of the components of each sample is unpredictable, the contamination is always underestimated.

Therefore, we preferred to calculate the ratios of antigen-specific IgG titres to total IgG titres in serum and in scrapings. It was assumed that proteolytic degradation of IgG would affect antigen-specific IgG titres and the total IgG titres equally and therefore would not alter the ratio between these two values. Indeed, the ratios of the antigen-specific IgG titres to the total IgG titres in both serum and scrapings were nearly the same (3.0 and 2.5, respectively).

We concluded, therefore, that most of the IgG detected in scrapings originated from blood. To confirm this hypothesis, we determined the antigen-specific antibody titres and total Ig titres after an immunization regimen that induced a systemic response only (Van der Heijden et al. 1989). Again, the ratios of OA-specific IgG to total IgG were equal in serum and scrapings (8.0 and 8.1, respectively).

In conclusion, intestinal secretions collected by the lavage technique reliably indicate the isotype distribution of luminal antigen-specific immunoglobulins. This isotype distribution agrees with the isotype distribution of the Ig secreting

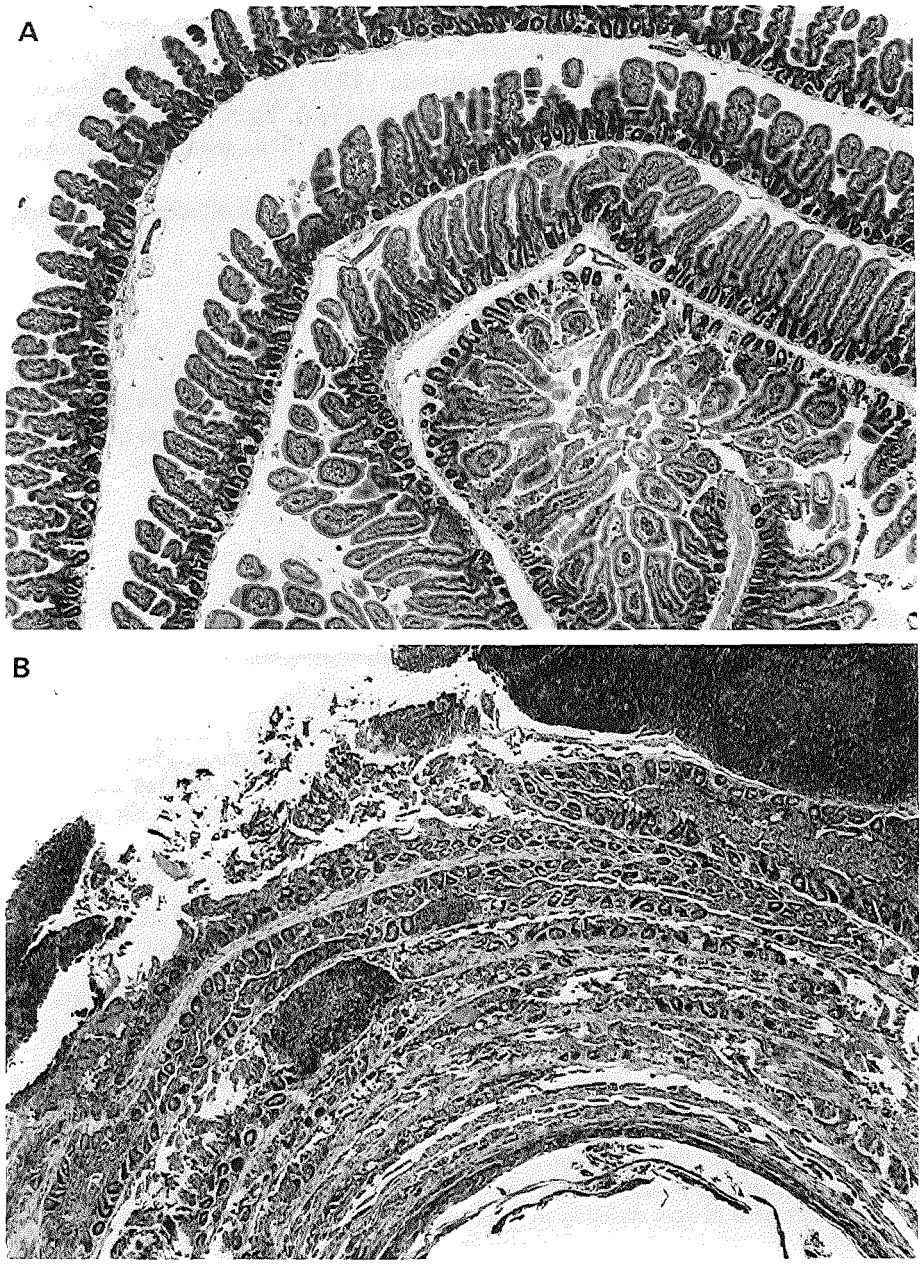

Figure 1. Sections of the small intestine after lavage (A) and scraping (B).

cells isolated from the small intestine (Van der Heijden et al., 1989). Moreover, although the lavage procedure is more laborious, it does not require killing the experimental animals, thus enabling longitudinal studies. Note, however, that the recovery of intestinal secretions from individual mice varies in the lavage technique, and corrections must be made for this variation (Elson et al., 1984). Moreover, only luminal IgA can be detected, whereas intestinal IgA tends to reside in the mucus layer (Magnusson and Stjernström, 1982). Scraping is less laborious and provides the possibility to detect mucus-bound IgA. Therefore scraping is the more sensitive technique for detecting intestinal IgA. This technique, however, necessitates killing the experimental animals.

Furthermore, most IgG detected in intestinal secretions collected by scraping is due to an artefact.

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CHAPTER 4

IMMUNOGLOBULIN-SECRETING CELLS IN THE INTESTINE

4.1 Contribution of immunoglobulin-secreting cells in the murine small intestine to the total "background" immunoglobulin production.

4.2 Background (spontaneous) immunoglobulin production in the murine small intestine before and after weaning.

4.3 Decrease of IgA-secreting cells in the intestine of mice during aging.

4.1 CONTRIBUTION OF IMMUNOGLOBULIN-SECRETING CELLS IN THE MURINE SMALL INTESTINE TO THE TOTAL "BACKGROUND" IMMUNOGLOBULIN PRODUCTION.

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SUMMARY

In this study we investigated the contribution of the small intestine of C3H/He mice to the spontaneous ("background") immunoglobulin (Ig) production in terms of the number of Ig-secreting cells (Ig-SC), and compared the results with the numbers of Ig-SC found in various lymphoid organs (spleen, bone marrow, mesenteric lymph nodes, Peyer's patches). The results show that in C3H/He mice of 20 weeks of age, on average 16 x 10^6 Ig-SC can be isolated from the small intestine.
Almost all of these Ig-SC produce IgA. Compared to the other lymphoid organs the small intestine contains more than 80% of all Ig-SC present in adult C3H/He mice. These results are in agreement with the need to maintain relatively high levels of Ig at the mucosal surfaces, especially of secretory IgA, for the prevention of penetration of these surfaces by microorganisms. In man and mouse most of these Ig are supposed to be produced locally in the underlying mucosal tissues and subsequently transported across the epithelium. Although the IgM and IgG levels in serum are predominantly maintained by non-mucosae associated lymphoid organs, the results of this study clearly indicate that the mucosal tissues are the major site of "background" Ig-production.

INTRODUCTION

Immunoglobulin (Ig) levels in serum and other body fluids of unintentionally immunized animals are formed and maintained by Ig-secreting cells (Ig-SC) in various lymphoid organs. To gain insight into the regulation of this spontaneous ("background") Ig production, it is important to know the distribution of the Ig-SC over the various lymphoid organs, the origin of these cells, their Ig class distribution and the stimuli that initiate their generation. The Ig class distribution per organ has been studied extensively in mice by means of cytoplasmic immunofluorescence (Benner et al., 1981b, 1982; Haaijman et al., 1975; Haaijman & Hijmans, 1978; Van Oudenaren, Haaijman & Benner, 1981a) and by the protein A plaque-forming cell assay (Benner et al., 1981a, 1982; Hooijkaas et al., 1984). In these studies it was shown that the total number of Ig-SC in the spleen is relatively constant during life, and less dependent on exogenous antigen stimulation. In contrast, the numbers of Ig-SC in bone marrow (BM), mesenteric lymph nodes (MLN) and Peyer's patches (PP) are greatly dependent on exogenous antigenic stimulation. Furthermore, the absolute numbers of IgG- and IgA-containing cells in all lymphoid organs are highly dependent on the presence of T cells (Benner et al., 1981b; Van Oudenaren et al., 1981a).

None of the studies mentioned above included the contribution of the mucosal lymphoid tissues, although high levels of secretory IgA (sIgA) in mucosal secretions have been reported (Tomasi & Bienenstock, 1968). These Ig molecules are considered to be produced locally in the mucosal tissue (Bienenstock & Befus, 1980; Cebra et al., 1977).
Moreover, in histological studies (Crabbe et al., 1968; Pierce, 1984; Sminia, Delamarre & Janse, 1983) it was shown that mucosal tissues, especially of the small intestine, contain many Ig-containing cells.

Previously (Van der Heijden & Stok, 1987) we described an isolation procedure for lamina propria lymphocytes (LPL) that enables us to quantify the number of Ig-secreting cells in the murine intestine. In this report we present the first quantitative data on the contribution of the intestine to the total number of "background" Ig-SC found in the most important lymphoid organs (spleen, BM, MLN and PP) and the class distribution of these Ig-SC. The biological significance of the Ig-SC in the intestine compared to the Ig-SC in the other organs is discussed.

MATERIALS AND METHODS

Mice: Female C3H/He mice (OLAC, Cat. 4+) of 6 weeks of age were purchased from H-OLAC Ltd., Bicester, Oxon, U.K., and housed under conventional conditions. The animals were fed standard laboratory animal feed (Hope farm, Woerden, The Netherlands). Mice were used at an age of 20 weeks.

Preparation of cell suspensions: Mice were killed with carbon dioxide. Immediately thereafter, spleens, MLN, PP, intestine and both femurs were removed.

Spleens were placed in a balanced salt solution (Hanks'), minced with scissors and squeezed through a nylon gauze filter (100 μm) to obtain a single cell suspension. MLN and PP were placed in RPMI-1640 medium containing 5% fetal calf serum (FCS), 20 mM HEPES and 0.1 mg/ml DNA-se (Sigma, St.Louis, MO.), minced with scissors and squeezed through nylon gauze filters (100 μm followed by 50 μm) to obtain single cell suspensions. The suspensions of PP and MLN cells were pooled per group of five mice. Femoral BM was used for preparing BM cell suspensions as described by Benner, Van Oudenaren & Koch (1981c). Viability of spleen and BM cell suspensions was always more than 90% and the viability of MLN and PP cell suspensions was about 70% as determined by nygrosin exclusion.

Lamina propria cell suspensions were prepared as described in detail before (Van der Heijden & Stok, 1987). Briefly, the procedure is as follows: the intestine is rinsed and the PP are excised from the intestine. Subsequently the intestine is cut longitudinally. The tissue strip is washed in Ca- and Mg-free balanced salt solution (CMF) and cut in small pieces of 0.5-1 cm. Thereafter the mucosal tissue strips are incubated for 10-15 min in 0.37 mg/ml EDTA and 0.145 mg/ml DTT containing CMF. After filtration the debris is further incubated for 75-90 min in RPMI-1640 containing 5% FCS, 20 mM HEPES, 0.1 mg/ml DNA-se (Sigma) and 0.15 mg/ml collagenase (0.8 U/mg, Serva, Heidelberg, FRG). The supernatant is collected and squeezed through nylon gauze filters (100 μm and 50 μm) to provide a single cell suspension. The tissue strips remaining after collagenase digestion are squeezed through nylon gauze filters (200 μm, 100 μm and 50 μm) to provide a second single cell suspension. Both suspensions were pooled and washed. The viability of the isolated cell suspension was about 60%.

In order to avoid bacterial growth, penicillin and streptomycin were added to all the solutions used during the isolation procedures of all cell suspensions.

Protein A plaque-forming cell (PFC) assay: In order to enumerate the total number of Ig-SC, the protein A PFC assay according to Gronowicz, Coutinho & Melchers (1976), and modified by Van Oudenaren,Hooijkaas & Benner (1981b), was used. Rabbit anti mouse-IgM and -IgA antisera (Nordic, Tilburg, The Netherlands) and rabbit anti mouse-IgG antiserum (Miles, Slough, Berks, UK) were obtained commercially. Guinea-pig complement (Behringwerke, Marburg-Lahn, FRG) was absorbed by passage over a Sepharose-bound protein A (Pharmacia, Uppsala, Sweden) column (Van Oudenaren et al., 1981b). Plaques were read after incubation of the slides for 4 hr at 37 °C followed by 18 hr at room temperature.

Specificity control of the rabbit anti-mouse antisera was done by ELISA techniques on microtiter plates coated with purified mouse myeloma IgM, IgG or IgA (Litton Bionetics, Kensington, MA). After incubation with the rabbit antisera, swine anti-rabbit-Ig peroxidase conjugate (Dakopatts, Copenhagen, Denmark) was used to test for bound immunoglobulins. The selected antisera used in the protein A PFC assay proved to be specific for the different isotypes.

In order to calculate the total number of Ig-SC in the bone marrow of the whole animal, we multiplied the number of Ig-SC found in the cell suspension obtained

from the two femurs with a factor of 7.9, since ^{59}Fe distribution studies did show that 12.6% of the total bone marrow is located in both femurs together (Benner et al., 1981c).

RESULTS

Distribution of Ig-SC along the murine intestine.
In order to get insight into the distribution of Ig-SC along the intestinal wall the complete intestine of a C3H/He mouse of 20 weeks of age was divided into five parts: duodenum, the first and second half of the jejunum (jejunum 1 and jejunum 2), ileum and colon with caecum. After isolation of LPL from each part of the intestine, the numbers of Ig-SC in each LPL suspension were determined using the protein A PFC assay. Since more than 99% of the Ig-SC isolated from the entire intestine of adult mice produce IgA (Van der Heijden & Stok, 1987), only the distribution of the IgA-SC was investigated.

The relative distribution of IgA-SC along the intestine is shown in Fig. 1. The percentages presented in Fig. 1 are the mean values calculated from three different experiments. The results demonstrated that more than 98% of the IgA-SC of the intestine were localized in the small intestine (SI) and that in the SI itself the number of IgA-SC declined from cranial to caudal. Therefore only the SI was investigated in the subsequent experiments concerning the contribution of the intestine to the total "background" Ig production.

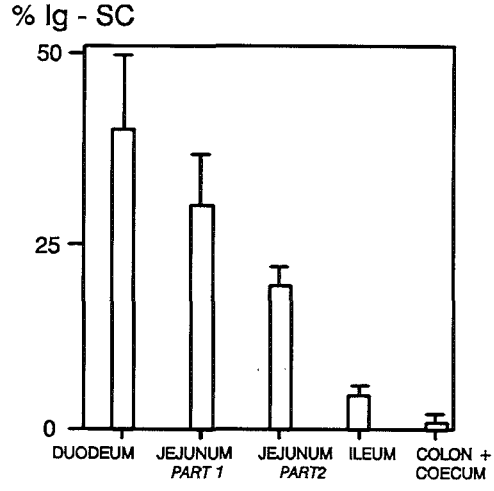

Figure 1. Relative distribution of IgA-SC along the murine intestine. Results are expressed as the mean values (with the SE) of each part of the intestine relative to the total number of IgA-SC of the intestine (n=3).

Quantification of Ig-SC in various organs in adult mice.
Spleens, MLN, PP, femora and SI were dissected from C3H/He mice of 20 weeks of age. After preparation of the cell suspensions of each organ the IgM-, IgG- and IgA-SC in each suspension were enumerated using the protein A PFC assay. The numbers of Ig-SC of the various isotypes per organ, expressed as the mean values of the results obtained from 15 mice, are shown in Fig. 2.

The results show that the IgM- and IgG-SC were predominant in the spleen, followed by the BM. Most (98%) of the IgA-SC were found in the SI. The contribution of the MLN and PP to the total number of Ig-SC is relatively low. From the absolute numbers shown in Fig. 2, it can be calculated that of all organs investigated, more than 80% of all Ig-SC were localized in the SI (Table 1). More than 99% of these cells secreted IgA (Fig.2).

The relative contribution of each isotype to the total number of Ig-SC in each organ is shown in Table 2. From these results it is clear that in the spleen IgM was

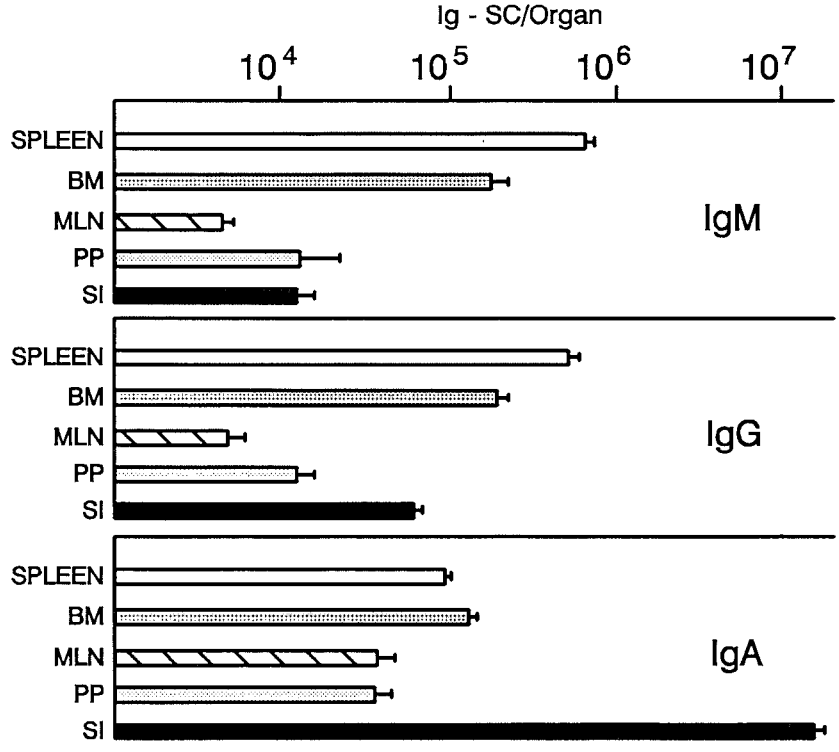

Figure 2. Numbers of IgM-, IgG- and IgA-SC in spleen, bone marrow (BM), mesenteric lymph nodes (MLN), Peyer's patches (PP) and small intestine (SI).
Results are expressed as the mean values with the SE (n=15 for spleen, BM and SI; n=3x5 for MLN and PP).

Table 1. Numbers of Ig-SC per organ and the relative contribution of each organ to the total number of Ig-SC found in C3H/He mice of 20 weeks of age.

Organ	Number of Ig-SC	% of total
Spleen	1,273,933	7.2
BM	515,988	2.9
MLN	49,066	0.3
PP	64,749	0.4
SI	15,775,290	89.2

Numbers of Ig-SC per organ were calculated as the sum of IgM-, IgG-, and IgA-SC. Results are expressed as the mean values (n=15 for spleen, BM and SI; n=3x5 for MLN and PP).

Table 2. Relative contribution of each isotype to the number of Ig-SC per organ.

Organ	% IgM	% IgG	% IgA
Spleen	51	41	8
BM	35	38	27
MLN	10	9	81
PP	20	20	60
SI	0.1	0.1	99

Results are expressed as the mean values (n=15 for spleen, BM and SI; n=3x5 for MLN and PP).

the predominant isotype. In the BM IgG-SC were predominant, while in MLN, PP and SI IgA was the most expressed isotype.

The number of isolated lymphoid cells differed per organ. Therefore, in Table 3 the frequencies of the Ig-SC per organ are shown. These frequencies are expressed as the number of Ig-SC per 10^6 cells. Comparison of the results of different organs show that the highest frequency of IgM- and IgG-SC was found in the spleen. The highest frequency of IgA-SC was found in the SI. About 10% of the isolated lamina propria cells from the SI actively secreted Ig. In contrast, only about 0.5% of the cells in the spleen secreted Ig.

DISCUSSION

In this paper we describe the quantification of background Ig-SC in the intestine of adult (20 weeks old), conventionally housed C3H/He mice and compared the results to the numbers of Ig-SC found in the spleen, BM, MLN and PP.

The distribution of IgA-SC along the murine intestine (Fig. 1) showed that the IgA-SC are mainly localized in the SI and decline from cranial to caudal. This is in

Table 3. Number of Ig-SC different isotypes per 10^6 lymphoid cells of various lymphoid organs of 20-week-old C3H/He mice.

	Ig-SC/10^6 cells						No. of living cells (x10^6)	
	IgM		IgG		IgA			
Spleen	2686	(261)*	2182	(236)	419	(35)	241	(7)
Bm	929	(116)	1019	(105)	711	(69)	194	(13)
MLN	142	(21)	139	(12)	1197	(279)	33	(5)
PP	584	(217)	577	(165)	1730	(268)	22	(6)
SI	88	(17)	435	(78)	109000	(13900)	144	(10)

* Results are expressed as the mean values (n=15 for spleen, BM and SI; n=3x5 for MLN and PP) with the SE shown in parentheses.

agreement with data obtained by immuno-fluorescence (Crabbé et al., 1968), which showed that the plasma cell population in the intestine almost exclusively belongs to the IgA class and declines from cranial to caudal. Therefore in the subsequent studies dealing with the relative contribution of the intestinal Ig-SC compartment to the total number of Ig-SC, we confined ourselves to the SI.

Compared to the lymphoid organs tested, the SI contained a high number of Ig-SC. More than 80% of all Ig-SC found in all lymphoid organs together were located in the SI. So, quantitatively, the SI is the most important site of residence of Ig-SC.

The numbers of Ig-SC found, and the isotype distribution of the Ig-SC in spleen, MLN, BM and PP are in agreement with the numbers described by Benner et al. (1981a, 1982), and Hooijkaas et al. (1984). As in their experiments, in mice of 20 weeks of age, which were used in this study, the spleen and BM accounted for the majority of the IgM- and IgG-SC. IgA-SC were found in significant numbers in the BM. However, our data clearly show that more than 98% of the IgA-SC were localized in the SI. So, the SI, as a representative of the mucosal tissues, contributes greatly to the total Ig production in adult mice.

The concentration of IgA in mouse-serum is low compared to the concentration of IgG or IgM (Lindmark, Thoren-Tolling & Sjoquist, 1983). In contrast, IgA has been demonstrated in significant quantities in faeces of mice (Elson, Ealding & Lefkowitz, 1984; Tomasi & Bienenstock, 1968). Therefore, it is most likely that the IgA secreted by the Ig-SC in the SI is excreted into the intestinal lumen.

This implies that the Ig-SC in the SI secrete dimeric IgA, as only dimeric and not monomeric IgA binds to secretory component exposed on the membrane of epithelial cells or of hepatocytes. These complexes are transported across the epithelial cells or the hepatocytes to the intestinal lumen or bile respectively (Brandtzaeg, 1981; Fisher et al., 1979; Hall, Gyure & Payne, 1980). Dimeric IgA

production has already been demonstrated for IgA immunocytes at various secretory sites in man (Brandtzaeg, 1985). At present we are unable to discriminate between monomeric and dimeric IgA production of murine Ig-SC to evaluate this hypothesis.

Secretory IgA protects the mucosal sites by its ability to inhibit the adherence of pathogens to mucosal epithelial surfaces (Porter, 1979; Williams & Gibbons, 1972), to inhibit antigen uptake (Walker et al., 1975) and to neutralize toxins (Kaur, Burrows & Furlong, 1971; Pierce, 1980). As there is no possibility of accumulation of Ig in enteric mucus due to the peristaltic movement of the intestine and enzymatic degradation, the turnover rate of sIgA in enteric mucus is high. In order to maintain the observed levels of sIgA in enteric mucus, many IgA-SC had to be present in the mucosal tissues.

Immunohistological studies already demonstrated the presence of many Ig-containing cells underneath the mucosal epithelium (Crabbe et al., 1968; Pierce, 1984; Sminia et al., 1983). The results presented in this paper show the first quantitative data of the high number of actual IgA-SC in the SI necessary to maintain the observed concentrations of sIgA in the mucus lining the epithelium. These findings are in agreement with the supposition that in man and mouse sIgA antibodies are produced locally in the mucosal tissues (Bienenstock & Befus, 1980; Husband, 1985; Tomasi & Bienenstock, 1968).

It has been demonstrated that the number of Ig-SC in the spleen is relatively constant during life, while the generation of Ig-SC in the BM is greatly dependent on exogeneic antigen exposure (Benner et al., 1981b; Van Oudenaren et al., 1981a). At the intestinal mucosal surface food antigens, the normal bacterial flora and infectious agents provide a constant stimulation of lymphoid cells, especially of those underlying the mucosal epithelium (Bienenstock & Befus, 1980; Husband, 1985). The high number of Ig-SC in the SI is very probably caused by the continuous occurrence of antigens at the intestinal mucosa. As other mucosal sites are under continuous antigenic exposure as well, a considerable number of Ig-SC might also occur in these tissues.

The regulation of the spontaneous Ig production in the SI by exogenous antigenic stimulation is subject to further experiments in which the development of the number of Ig-SC in the SI is investigated as a function of age and immune status of the mouse. This study already shows that the mucosal tissues contributes strongly to the total Ig production in adult mice.

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4.2 BACKGROUND (SPONTANEOUS) IMMUNOGLOBULIN PRODUCTION IN THE MURINE SMALL INTESTINE BEFORE AND AFTER WEANING

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SUMMARY

The ontogeny of the murine intestinal B-cell compartment before and after weaning was studied by quantitative analysis of immunoglobulin-secreting cells (Ig-SC) in the small intestine (SI). Before weaning, few Ig-SC were detected in the SI, whereas spleen and bone marrow already contained many Ig-SC. The number of Ig-SC in the SI started to increase immediately after weaning. Comparing early-weaned mice with non-weaned mice of the same age clearly demonstrated that weaning brought on the development of Ig-SC in the SI. The influence of a gut flora on the number of Ig-SC in the SI was examined by comparing the number of Ig-SC in the SI of conventionally housed, specific pathogen free (SPF) and germfree mice. A bacterial flora was apparently needed for the normal development of Ig-SC in the SI. Comparing mice containing an aerobic Gram-negative bacterial flora with mice containing only an anaerobic Gram-positive bacterial flora demonstrated that the type of bacterial flora is relatively unimportant. No evidence was found that circulating maternal antibodies suppressed the development of the "spontaneous" intestinal and systemic B-cell response. The results show that bacterial colonization of the intestine plays a pivotal role in the development of the Ig-SC compartment in the SI.

INTRODUCTION

Shortly after birth, the immune system of rodents is only partially developed (Silverstein, 1977; Porter, 1979). Maternal immunity, transferred via placenta or milk from mother to offspring, protects young animals from infectious diseases (Porter, 1979). However, maternal antibodies may suppress antigen-specific immune responses in young animals (Leiper and Solomon, 1976; Yamaguchi et al., 1983), which leads to a partially developed immune system at weaning. Therefore, many infectious diseases occur shortly after weaning of the young, when passive protection by maternal antibodies is no longer maintained.

The ontogeny of the systemic B-cell compartment has been studied in detail (Benner et al., 1981a; Osmond, 1985; Bos et al., 1987). In contrast, the ontogeny of the mucosal B-cell compartment, a major port of entry for infectious diseases, has been studied only partially. The small intestine (SI) of many newborn mammals contains Peyer's patches, which develop in the absence of antigenic stimulation (Ferguson and Parrott, 1972; Kantak et al., 1987; Reynolds and Morris, 1984). The newborn intestinal tract contains few plasma cells or intra-epithelial lymphocytes and the occurrence of those cells depends on antigenic stimulation (Crabbé et al., 1969; Milne et al., 1975). The effect of maternal antibodies on the development of the B-cell compartment in the SI has not been documented, although circulating maternal antibodies have been reported to suppress not only systemic immune responses, but also antigen-specific responses in the intestine of pigs before weaning (Watson et al., 1979).

In a previous paper, we described a method to quantitate Ig-secreting cells (Ig-SC) in the lamina propria of the murine intestine (van der Heijden and Stok, 1987). We used this method to study the effect of age on the background (spontaneous) immunoglobulin (Ig) secretion in the SI of mice (van der Heijden et al., 1988). In the present paper we describe the development of the murine B-cell compartment in the SI before and after weaning and the influence of the time of weaning on this development. In addition, the influence of type and presence of the gut flora on the number of intestinal Ig-SC is reported. The results show that immediately after weaning the intestinal immune system develops exponentionally. Moreover, the results indicate that the bacterial flora, and not food-antigens, is the major stimulus for the induction of this sudden increase in mucosal immune reactivity.

MATERIALS AND METHODS

Mice: Breeding pairs of C3H/He mice were purchased from Harlan-Olac Ltd., Bicester, Oxon, U.K. The offspring of these mice were used in the experiments and housed under conventional conditions at our institute. The mice were weaned at 3 weeks of age, unless stated otherwise.

Four additional groups of CBA/Rij mice were obtained from the Radiobiological Institute TNO, Rijswijk, The Netherlands, and used at 8-10 weeks of age.

These groups consisted of conventionally housed mice, specific pathogen free (SPF) mice, colonization resistance factor (CRF) mice and germ-free mice. CRF mice were the offspring of germ-free mice that had been contaminated with a strictly anaerobic microflora originally obtained from selectively decontaminated conventional mice. The microflora was named CRF because it provides resistance against newly colonizing microorganisms (van der Waaij et al., 1971). The CRF flora is largely composed of spore-forming Gram-positive rods (mainly different *Clostridium* spp; Wensinck and Ruseler-van Embden, 1971). Although CRF flora protects mice against low doses of contaminants, the CRF mice were housed in plastic film isolators to prevent undue contamination.

SPF mice were originally associated with the CRF flora. The flora of the SPF animals at the time of the experiments was defined as: CRF flora (non-pathogenic anaerobes), *Enterococcus faecalis, Escherichia coli* (present in low concentrations in a limited percentage (40-80%) of the animals), *Staphylococcus saprophyticus* and *Staphylococcus aureus* (both isolated from skin or nasal washings only).

Preparation of cell suspensions: Mice were killed with carbon dioxide. Cell suspensions of spleen, mesenteric lymph nodes (MLN), Peyer's Patches (PP) and lamina propria lymphocytes (LPL) were prepared as described previously (van der Heijden and Stok, 1987; van der Heijden et al., 1988). Cell suspensions of femoral bone marrow were prepared according to the method described by Benner et al. (1981b). In some experiments MLN, PP and bone marrow cell suspensions of each group of mice were pooled before testing for practical reasons.

Protein A plaque-forming cell (PFC) assay: A modified protein A PFC assay (van Oudenaren et al., 1981) was used to quantitate the number of Ig-SC in isolated cell suspensions. Slides were incubated for 4 h at 37°C and kept for 18 h at room temperature, after which plaques were read. Rabbit anti-mouse -IgM and -IgA antisera (Nordic, Tilburg, The Netherlands) and rabbit anti-mouse-IgG (ICN, Lisle, USA) were purchased. The specificity of the antisera was determined by an ELISA using microtitre plates coated with purified myeloma IgM, IgA, and all subclasses of IgG (Litton Bionetics, Charleston, SC), as described earlier (van der Heijden et al., 1987). The number of Ig-SC in the femur cell suspension was multiplied by a factor of 7.9 to calculate the number of Ig-SC in the total bone marrow (Benner et al., 1981b).

RESULTS

Development of Ig-SC before and after weaning: The Ig-SC in the SI, spleen, bone marrow, MLN, and PP of conventionally housed C3H/He mice were quantitated at 2, 3 (time of weaning), 3.5, and 4 weeks of age. The results showed that the number of Ig-SC in the SI increased immediately after weaning and that IgA was the predominant isotype (Fig. 1). In spleen and bone marrow the number of Ig-SC gradually increased during the testing period and IgM was the predominant isotype

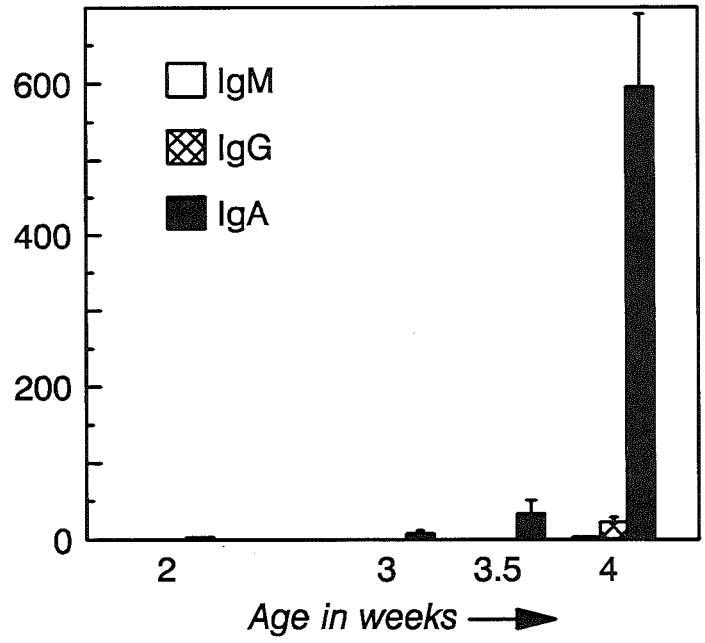

Figure 1. Number of Ig-SC (x 10^{-3}) in the small intestine (SI) of C3H/He mice before and after weaning. Mice were weaned at the age of 3 weeks. Results are expressed as the mean number of Ig-SC per organ (n=5) with the SEM.

Table 1. Number of isotype-specific Ig-SC in spleen, bone marrow (BM), mesenteric lymph nodes (MLN) and Peyer's patches (PP) before and after weaning. C3H/He mice were weaned at 3 weeks of age. Results for the spleen were expressed as the mean number per organ (n=5) with the SEM shown in parentheses. As suspensions were pooled, no SEM could be calculated for the BM, MLN and PP groups.

		Age of mice in weeks			
		2	3	3.5	4
Spleen	IgM	250 265 (26 400)	390 937 (20 900)	565 000 (31 200)	434 500 (41 000)
	IgG	82 140 (14 400)	205 625 (19 555)	307 187 (21 500)	276 500 (28 300)
	IgA	17 875 (1435)	32 750 (4508)	57 750 (5570)	44 600 (2150)
BM	IgM	15 800	32 588	56 090	140 225
	IgG	9875	18 763	30 810	78 200
	IgA	3655	8888	26 070	59 250
MLN	IgM	62	600	1975	390
	IgG	27	140	2725	1785
	IgA	42	240	1075	2875
PP	IgM	0	110	1450	9500
	IgG	0	20	700	18 500
	IgA	0	75	300	8400

(Table 1). In MLN and PP the number of Ig-SC increased simultaneously with those in the SI. In contrast to the other organs, the predominant isotype in PP at 4 weeks of age was IgG.

Influence of the time of weaning on the development of Ig-SC: Half of each litter of C3H/He mice was weaned when 19 days old, whilst the others were kept with their mothers until 29 days old. At 29 days, Ig-SC in SI, spleen, bone marrow, MLN and PP of all mice were quantitated (Fig. 2). The number of IgA-SC in the SI of early-weaned mice was 90% higher than that of their non-weaned littermates. In MLN and PP the number of IgG- and IgA-SC was approximately six times higher in early-weaned mice.

The number of Ig-SC in bone marrow differed little between early- and non-weaned mice, although slightly more IgG- and IgA-SC were detected in early-weaned mice. In contrast, the number of IgM- and IgG-SC in the spleen of non-weaned mice was 40% higher than that of their early-weaned littermates.

Influence of the gut-flora on the number of Ig-SC: The increase of the number of Ig-SC in the SI coincided with the change of diet and with the bacterial colonization of the SI after weaning. To evaluate the effect of bacterial colonization in the SI in more detail we quantitated the number of Ig-SC in the SI of 10 weeks-old CBA/Rij mice kept either under conventional, SPF or germ-free conditions. A group of CRF mice was also included. The number of Ig-SC in the SI depended greatly on the presence of a gut-flora (Fig. 3). The SI of conventionally reared mice contained three times as many IgA-SC than that of SPF mice. The SI of SPF mice again contained three times as many IgA-SC as the SI of germ-free mice.

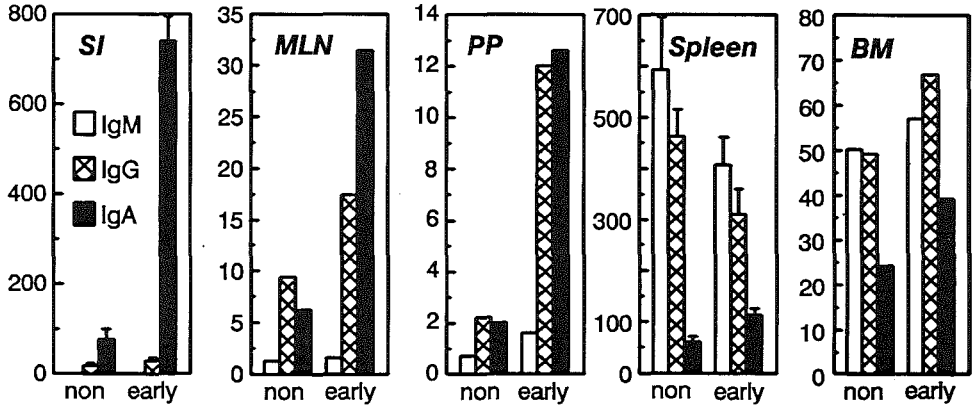

Figure 2. Number of Ig-SC (x 10^{-3}) in the small intestine (SI), mesenteric lymph nodes (MLN), Peyer's patches (PP), spleen and bone marrow (BM) in 29-days-old non- and early-weaned C3H/He mice. Results are expressed as the mean (n=8) with the SEM. As MLN, PP and BM suspensions were pooled no SE were calculated.

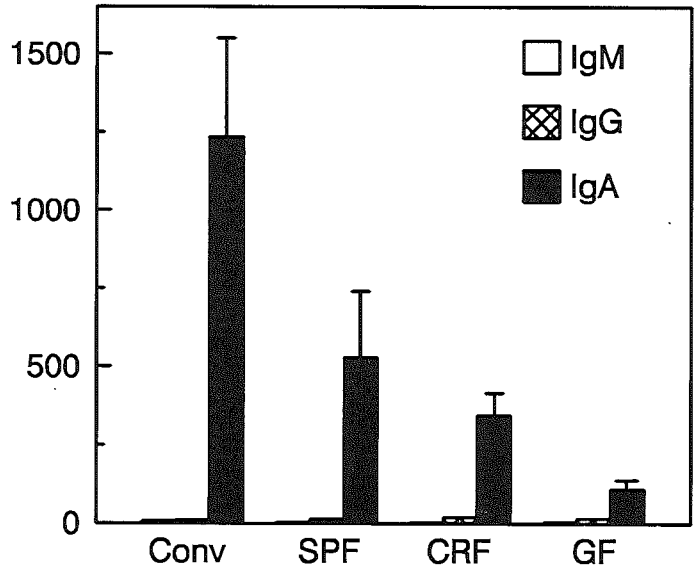

Figure 3. Number of Ig-SC (x 10^{-3}) in the small intestine (SI) of CBA/Rij mice of 8-10 weeks of age housed under conventionally, specific pathogen free (SPF), or germ-free (GF) conditions or under SPF conditions harbouring a colonization resistance factor (CRF) flora. Results are expressed as the mean (n=8) with the SEM.

Table 2. Number of IgA-SC per million living cells in the small intestine of conventionally, SPF, CRF or germfree reared CBA/Rij mice. Results are expressed as the mean (n = 10) with the SEM shown in brackets.

	IgA-SC/10^6		no. cells x 10^6	
Conv.	27,450	(8,544)	45	(16)
SPF	13,600	(3,780)	30	(7)
CRF	7,200	(1,417)	47	(18)
Germfree	1,600	(487)	50	(14)

CRF mice contained somewhat fewer IgA-SC in the SI than SPF mice. Table 2 shows the number of intestinal IgA-SC per million cells and the number of cells recovered from the various groups of CBA/Rij mice. Intestinal IgA-SC appeared two times more frequent in conventional mice than in SPF mice. The frequency of intestinal IgA-SC in SPF mice was two times higher than in CRF mice and eight times higher compared with the frequency in germ-free mice.

Table 3 shows the number of Ig-SC in spleen, bone marrow, MLN and PP of

Table 3. Number of Ig-SC in spleen, BM, MLN and PP of 8- to 10-week-old conventionally, SPF, CRF or germfree (GF) reared CBA/Rij mice. Results are expressed as the mean (n=8) with the SEM shown in parentheses.

		Conventional	SPF	CRF	GF
Spleen	IgM	296 200 (29 100)	256 200 (39 000)	185 300 (18 400)	358 400 (31 100)
	IgG	369 300 (161 300)	147 400 (12 100)	121 800 (8900)	214 100 (26 500)
	IgA	110 000 (21 300)	52 400 (7400)	31 800 (31 000)	43 700 (13 000)
BM	IgM	140 600 (25 600)	88 700 (17 300)	101 600 (20 500)	115 100 (12 300)
	IgG	164 500 (28 900)	123 900 (11 900)	114 400 (17 500)	114 000 (9600)
	IgA	420 700 (48 900)	238 600 (36 100)	159 500 (32 200)	41 600 (5100)
MLN	IgM	3000 (600)	1600 (400)	900 (80)	9700 (700)
	IgG	32 200 (19 600)	3900 (1400)	1400 (200)	9500 (1700)
	IgA	40 800 (10 700)	10 100 (600)	2300 (700)	2300 (50)
PP	IgM	3300 (700)	7800 (100)	1100 (100)	2100 (300)
	IgG	13 400 (3700)	23 200 (2700)	2600 (700)	3900 (200)
	IgA	32 800 (6200)	20 200 (100)	5400 (500)	3800 (50)

conventional, SPF, CRF and germ-free mice. Spleens of germ-free mice contained more IgM-SC than those of conventional and SPF mice. In bone marrow the differences in number of IgA-SC between conventional, SPF, CRF and germ-free mice showed the same pattern as in the SI (Fig.3).

DISCUSSION

This paper describes the development of background (spontaneous) Ig-SC in the murine SI before and after weaning. Before weaning, hardly any Ig-SC were detected in the SI (Fig. 1), whereas spleen and bone marrow already contained a relative high number of Ig-SC at this time (Table 1). Within the first week after weaning, the number of Ig-SC in the SI increased rapidly, whereas the number of Ig-SC in spleen and bone marrow increased gradually. The number of Ig-SC in MLN and PP (Table 1) increased in parallel with the number in the SI.

The number of Ig-SC and isotype distribution in spleen, bone marrow, MLN and PP agrees with previous reports (Benner et al., 1981a; Benner et al., 1982; Bjorklund, et al., 1985; Bos et al., 1987). Remarkably, in the SI, IgA appeared to be the dominant isotype from the moment that significant numbers of Ig-SC could be detected (Fig. 1). The number of IgM-SC in the SI, in contrast to the other organs tested, never exceeded that of IgG- or IgA-SC. This difference can be explained by selective migration of IgA-committed B cells to the mucosae through binding of receptors that are specific for high endothelial venules in the mucosal tissues (Butcher et al., 1982). Our finding that the IgA isotype consistently predominates during the development of Ig-SC in the SI agrees with histological examinations of Ig-containing cells that develop in antigen-free intestine (Milne et al., 1975).

To study the influence of the age of weaning on the development of Ig-SC, we quantitated the number in the SI and other organs of 29-day-old mice that were either weaned early (at 19 days of age) or not weaned (Fig. 2). The non-weaned mice presumably started consuming their mothers' diet at day 18 to 20, while continuing to suckle. The results clearly showed that weaning itself, and not the age, determines the number of Ig-SC in SI, MLN and PP. In contrast, the number of Ig-SC in spleen and bone marrow does not depend on weaning. The number of IgM- and IgG-SC in the spleen of non-weaned mice was even greater than in early-weaned mice. Bos et al. (1987) reported earlier that the number of Ig-SC in the spleen decreases after weaning. It may indicate that, after antigenic stimulation of the gut, Ig-SC from spleen migrate to the intestinal tissues.

The development of IgA-containing cells in the intestine depends on antigenic stimulation (Crabbé et al., 1969; Milne et al., 1975). Intestinal Ig-SC only begin to increase in number at weaning, despite preweaning exposure of the intestine to various antigens. This can be variously explained. Firstly, the intestinal immune system encounters antigen in greater amounts and variety after weaning (Moreau et al., 1982). Before weaning, maternal antibodies may block antigen uptake (Walker, 1979) or prevent bacterial colonization of the gut. Secondly, maternal antibodies can suppress the development of specific B-cell responses, both systemically (Leiper and Solomon, 1976; Yamaguchi et al., 1983) and at the mucosal site (Watson, et al., 1979). Thirdly, in vitro studies have demonstrated that in 1- to 3-week-old mice, temporarily high suppressor cell activity can restrain B-cell responses to T-dependent as well as T-independent antigens (DeKruyff et al., 1980). However, from Fig. 2 it appears that the onset of the increase of Ig-SC in the SI does not depend on age. The latter explanation is hence less likely.

To study the influence of the bacterial flora, we quantitated the Ig-SC in the SI of germ-free, SPF, CRF, and conventionally reared mice (Fig 3). The results clearly showed that the bacterial flora is crucial for the normal development of Ig-SC in the SI; the number of Ig-SC in the SI of germ-free mice is only about 10% of that of conventional mice. Frequency analysis of IgA-SC in the SI revealed that the differences in total number between conventional, SPF and germ-free mice did not stem from decreased numbers of cells isolated from the SI (Table 2). Our results agree with those of immunohistological studies that demonstrated that the intestines of germ-free mice had fewer IgA-containing cells than those of conventionally reared mice (Crabbé et al., 1968; Moreau et al., 1978). As the germ-free mice were fed the same diet as the SPF mice it appears that dietary antigens are not a major factor in inducing intestinal Ig-SC.

Lipopolysaccharide (LPS) is important in inducing oral tolerance after mucosal antigen presentation (Michalek et al., 1982). Furthermore, LPS can act as a polyclonal B-cell mitogen (Bjorklund et al., 1985). Moreover, when inoculated into germ-free mice, Gram-positive, LPS-negative bacteria induce only 20% of the number of IgA-containing cells in the villi as Gram-negative, LPS-positive bacteria (Moreau et al., 1978).

We examined the effect of a Gram-positive flora in CRF mice on the number of intestinal Ig-SC. The number of IgA-SC in the SI of CRF mice was only slightly

lower than the number in the SI of SPF mice containing a Gram-negative flora. This small difference may be due to the absence of LPS stimulation of the intestinal immune system in CRF mice. More likely, however, the difference is caused by the smaller variety in the bacterial flora in CRF mice compared with SPF mice and consequently the smaller variety in antigenic epitopes. Hence, in contrast to the histological results (Moreau et al., 1978), it is not LPS but the presence of a bacterial flora that induces the major part of the intestinal Ig-SC. Circulating maternal antibodies suppress the development of specific immune responses in offspring, presumably by masking the antigenic determinants (Auerbach and Clark, 1975), not only before weaning but also after weaning (Yamaguchi et al., 1983).

Circulating maternal antibodies do not notably affect the development of the "spontaneous" Ig-SC in the spleen or bone marrow. Moreover, the number of Ig-SC in the intestine increases immediately after weaning. We therefore conclude that circulating maternal antibodies also do not significantly suppress the development of "spontaneous" intestinal Ig-SC. This is supprising as it might be expected that at least some of the maternal antibodies would be directed against microbial and dietary antigens that are involved in the induction of Ig-SC in the young animal as well. Studies of antigen-specific responses in the SI will reveal whether antigen-specific maternal antibodies from the milk can suppress antigen-specific responses in the SI before and after weaning.

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4.3 DECREASE OF IgA-SECRETING CELLS IN THE LAMINA PROPRIA OF MICE DURING AGING

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SUMMARY

The intestinal B-cell compartment was studied in adult mice varying in age between 3 and 30 months. Immunoglobulin-secreting cells (Ig-SC) in lamina propria lymphocyte (LPL) suspensions were quantitated and the in situ localization of immunoglobulin-containing cells was examined in immunohistological sections of the intestine. The Ig-SC were quantitated in LPL suspensions of C3H/He and CBA mice. The number of LPL Ig-SC was compared with the number of Ig-SC of spleen, bone marrow (BM), and Peyer's patches (PP). The number of LPL Ig-SC increased upto 10^7 in CBA mice and upto 2.5×10^7 in C3H/He mice when the mice were about 12 months old. After an age of 12 months the number of Ig-SC in the small intestine decreased to 20% of its maximum values in conventionally housed C3H/He mice and to 60% in SPF housed CBA/Rij mice. The distribution of Ig-SC along the complete intestine did not change during aging. At all ages, the great majority of intestinal LPL Ig-SC was located in the small intestine and produced IgA. The "background" (spontaneous) Ig-SC response in the small intestine differed from the other organs tested in total number, isotype distribution and kinetics of the Ig-SC. Immunohistological examination of IgM-, IgG-, and IgA-containing cells in spleen and small intestine of CBA mice showed that the localization and isotype distribution of immunoglobulin containing cells was not affected by aging.

INTRODUCTION

It is generally accepted that immune responsiveness declines with age (1). This decline is often associated with health problems in elderly individuals. The decline of systemic B-cell responsiveness depends probably on changes of regulatory T-cells (2,3). It is still a matter of discussion whether aging is also accompanied by intrinsic changes in the B-cell compartment (4). In contrast to the decline of B-cell responsiveness upon immunization, no indication has been found that the overall B-cell activity in systemic lymphoid organs (background Ig production) decreases with age (5). To the contrary, Benner et al. (6,7) demonstrated that the number of immunoglobulin-secreting cells (Ig-SC) in mice increases in systemic lymphoid organs with age.

Most aging studies deal with the systemic immune system, while only a few aging studies are focussed on the mucosal immune system. Contradictious results have been found in studies that deal with immune responsiveness of gut-associated lymphoid tissue (GALT) on immunization during aging. Schmucker et al. (8) found that aging compromised the ability to induce specific B-cell responses in GALT. On the other hand, Wade and Szewczuk (4) found in their studies that mucosal B-cell responses persisted vigorous with age.

Quantitative data about changes with age of GALT background Ig-SC are only available for mesenteric lymph nodes (MLN) and Peyers patches (PP) (5-7). The number of Ig-SC of MLN and PP declines in mice already from an age of 14 weeks. There are only indirect data available about the changes of LPL Ig-SC with age. Some researchers studied the course of the total IgA level in the gut lumen with age. The results from these studies vary between a slight decrease (9) and a slight increase of luminal IgA with age (10,11).

We developed an isolation method of LPL that enables us to quantitate the number of Ig-SC in the intestinal lamina propria (12). This method showed to be a very sensitive way to study changes of the intestinal immune response quantitatively. Earlier, we studied the ontogeny of the murine intestinal B-cell compartment before and after weaning (13,14) and the influence of the gut flora on the number of Ig-SC in the intestine (14) by quantitative analysis of Ig-SC. We also demonstrated that the LPL is the major organ in terms of Ig-SC (15). Therefore, the quantitative study of intestinal Ig-SC during aging is of great interest.

In this study, we investigated the absolute number and the frequency of intestinal Ig-SC in adult C3H/He and CBA mice of various ages. We also looked for changes in the distribution of Ig-SC along the complete intestine. These data were compared with in situ observations of the distribution and frequency of Ig-containing cells in sections of spleen and small intestine.

MATERIALS AND METHODS

Mice: C3H/He mice were purchased from Harlan-Olac Ltd, Bicester, Oxon, U.K. in one shipment at an age of 6-8 weeks. The C3H/He mice were housed under

conventional conditions in the animal facilities of the Central Veterinary Institute, Lelystad, The Netherlands and were used in groups of 7 mice at the age of 3, 5, 12, 14, 17, and 20 months. CBA/Rij mice were bred and maintained in the mouse colonies of the REP institutes TNO in Rijswijk, The Netherlands. All CBA mice were from a pathogen-free and barrier-maintained colony and were used in groups of 6 mice at the age of 5, 12, 18, and 30 months.

Preparation of cell suspensions: Mice were killed by carbon dioxide exposition. Cell suspensions of spleen, Peyers patches (PP) and lamina propria lymphocytes (LPL) were prepared as described previously (13,14). Cell suspensions of femoral bone marrow (BM) were prepared according to the method described by Benner et al. (16). The PP cell suspensions of each group of mice were pooled before testing.

Protein A plaque-forming cell (PFC) assay: A modified protein A PFC assay (17) was used to quantitate the number of Ig-SC in isolated cell suspensions. The Cunningham chambers were incubated for 4 h at 37 °C and kept for 18 h at room temperature, after which plaques were counted. Rabbit anti-mouse-IgM and -IgA antisera (Nordic, Tilburg, The Netherlands) and rabbit anti-mouse-IgG (ICN, Lisle, USA) were purchased. The specificity of the antisera was determined by an ELISA using microtitre plates coated with purified myeloma IgM, IgA, and all subclasses of IgG (Litton Bionetics, Charleston, USA) as described earlier (13). The number of Ig-SC in the femur cell suspension was multiplied by a factor 7.9 to calculate the number of Ig-SC in the total BM (16).

Immunohistology: Small pieces of spleen and Swiss rolls of the small and large intestine of CBA/Rij mice were fixed in formalin-acetic acid-mercury chloride fixative. 3 μm thick sections were cut after dehydration and embedded in Paraplast. Single immunostaining was performed as described by Haaijman et al. (18) using the following antisera and conjugates: Sheep anti-mouse-IgM (Serotec Ltd, Blackthorn Bicester, U.K.), goat anti-mouse-IgG (Fc)7S and -IgA (Fc)7S (Nordic, Tilburg, The Netherlands) and peroxidase labelled rabbit anti-sheep Ig and -goat Ig (Dakopatts, Glostrup, Denmark). Total numbers of Ig-containing cells were counted in sections of spleen and the small and large intestine.

RESULTS

Effect of aging on the number of Ig-SC in the lamina propria of the small intestine of C3H/He mice.

C3H/He mice were purchased at an age of 4-6 weeks. This mouse strain was choosen because of our experience with this strain in earlier studies of in vivo regulation of the Ig-SC response in the lamina propria (12-15). The Ig-SC were quantitated in LPL suspensions of the small intestine, when the mice were 3, 5, 12, 14, 17, and 20 months old. Seven mice were used per age-group. The organs of the mice were checked macroscopically for pathological abnormalities. Mice with

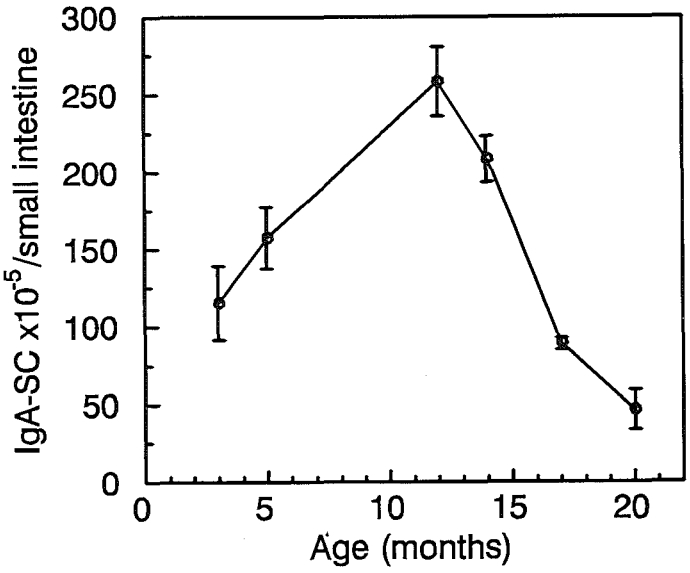

Figure 1. Effect of aging on the number of IgA-secreting cells in the small intestine of 3, 5, 12, 14, 17, and 20 month-old C3H/He mice. Each point represents the mean number of IgA-SC (n=7) with the SE shown in bars.

abnormalities were not included in the experiments. The number of LPL IgA-SC increased up to 12 months of age (Figure 1). A continuous decrease of IgA-SC was observed after 12 months of age. The number of LPL IgA-SC in 20 months old mice was 20% of the maximum number found in the small intestines of 12 months old mice.

Table 1. Frequency of IgA-secreting cells and number of nucleated cells in the small intestine of C3H/He mice at different age.

Age in months	Ig-SC x $10^{-3}/10^{6}$ nucleated cells		Number of nucleated cells x 10^{-6}	
3	84*	(17)	137	(21)
5	109	(14)	144	(10)
12	138	(12)	187	(19)
14	101	(7)	206	(14)
17	35	(1.6)	254	(15)
20	22	(6.8)	190	(20)

* Results are expressed as the mean values (n=7) with the SE shown in parentheses.

The number of mononuclear cells in the LPL cell suspensions of all age groups was determined and the frequencies of Ig-SC calculated (Table 1). In this way, we were able to determine whether the rise and fall of LPL IgA-SC with age was caused by a rise and fall of the frequency IgA-SC per 10^6 nucleated cells or by a rise and fall of the recovery of cells per organ. Comparison of table 1 and figure 1 shows that the kinetics of the frequency of LPL IgA-SC followed the kinetics of the number of LPL IgA-SC per organ. Both "graphs" displayed a clear maximum at 12 months of age. The recovery of cells at various ages displayed different kinetics with a slight maximum at 17 months of age.

The IgM- and IgG-SC were also quantitated in the LPL suspensions.These results are not shown. The total number of LPL IgM-SC and IgG-SC found in the C3H/He mice was always less than 1% of the LPL IgA-SC.

The distribution of IgA-SC along the intestine of C3H/He mice at different age.
It has been described for adult humans (19) that the highest frequency of intestinal Ig(A) containing cells is located in the large intestine. Therefore, we studied the distribution of LPL IgA-SC of 3, 14 and 20 months old C3H/He mice along the complete intestine. The intestine was divided in five parts : duodenum (duo), first half of the length of jejunum (jej1), second half of jejunum (jej2), ileum, and colon with caecum. LPL were isolated from each part of the intestine, and the IgA-SC in each LPL suspension were quantitated with the protein A PFC assay. The relative contribution of each part of the intestine was calculated by dividing the number of IgA-SC per part of intestine by the total number of LPL IgA-SC (Figure 2). We

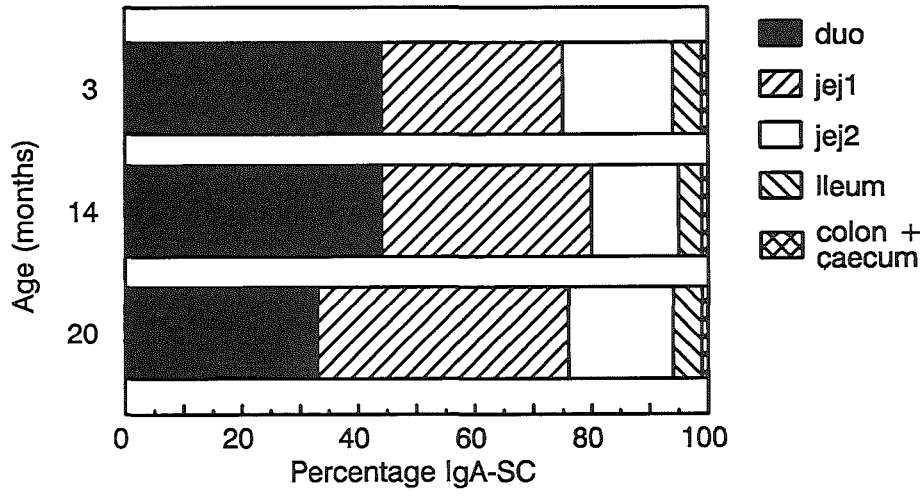

Figure 2. Relative distribution of IgA-secreting cells along the intestine of 3, 14, and 20 month-old C3H/He mice. Results are expressed as the mean values (n=7) of each part of intestine relative to the total number of IgA-secreting cells.

observed no significant shift in the distribution of IgA-SC with age. Ninetynine percent of all IgA-SC were located along the small intestine.

Moreover, at least 70 % of all intestinal IgA-SC were located in the first part of the small intestine (duodenum and jejunum 1) at all ages tested.

Effect of aging on the number of Ig-SC in the lamina propria of the small intestine of CBA/Rij mice.

The first experiments were done with C3H/He mice under conditions that were not optimal for aging studies. Therefore, we repeated the experiments with CBA/Rij mice that were obtained from pathogen-free aging colonies. Clinically healthy animals were used for this study only. The CBA strain is known as a long-lived strain that does not show specific immune pathology (5). Mice of 5, 12, 18, and 30 months of age were tested at the same time. The numbers of IgM-, IgG-, and IgA-SC were determined in LPL, spleen, BM, and PP suspensions (Figure 3). Spleen, BM and PP were included to compare our results to earlier studies in which Ig-SC were quantitated in spleen, BM, and PP of CBA mice at various ages (6,7). As in C3H/He mice, IgA was the dominant isotype amongst the LPL Ig-SC in CBA mice (Figure 3-IC). The percentage of IgM- and IgG-SC was small at all ages tested (Figure 3-IAB) but higher compared with percentage of IgM- and IgG-SC in LPL suspensions of C3H/He mice. The kinetics of the LPL IgA-SC in CBA mice (Figure 3-IC) were comparable to the kinetics of LPL IgA-SC in C3H/He mice (Figure 1). The highest number of LPL IgA-SC was also found in LPL suspensions of 12-month-old CBA mice. The number of LPL IgA-SC declined after 12 months. In 30-month-old mice the number of IgA-SC was 60 percent of that at the age of 12 months.

The number of splenic Ig-SC was already relatively high at the age of 5 months and increased slowly up to an age of 30 months. The isotype distribution shifted from IgM to IgG and IgA (Figure 3-II). The number of BM Ig-SC increased rapidly between an age of 5 and 12 months and reached a plateau at the age of 18 months (Figure 3-III). IgA was the dominant isotype among the BM Ig-SC at all ages tested. However, in contrast to the LPL suspensions, in which IgA was by far most the predominant isotype, IgG-SC contributed substantially to the total number of BM Ig-SC. The number of PP Ig-SC declined clearly in the period between the age of 5 and 30 months (Figure 3-IV). The decline of PP Ig-SC was mainly caused by a decline of IgM- and IgG-SC.

The frequencies of Ig-SC (number of Ig-SC per number of nucleated cells) were also calculated for all ages, organs and isotypes (data not shown). These data supported the above conclusions.

Distribution and frequency of Ig-containing cells in spleen and small intestine of CBA/Rij mice

Serial sections of spleen and small and large intestine of 5, 18, and 30 months old CBA/Rij mice were made and IgM-, IgG- ,and IgA-containing cells were visualized by immunohistological staining (Figures 4 and 5). The frequencies of IgM-, IgG-, and IgA-containing cells were determined in spleen and small intestine (Figure 6-A,B).

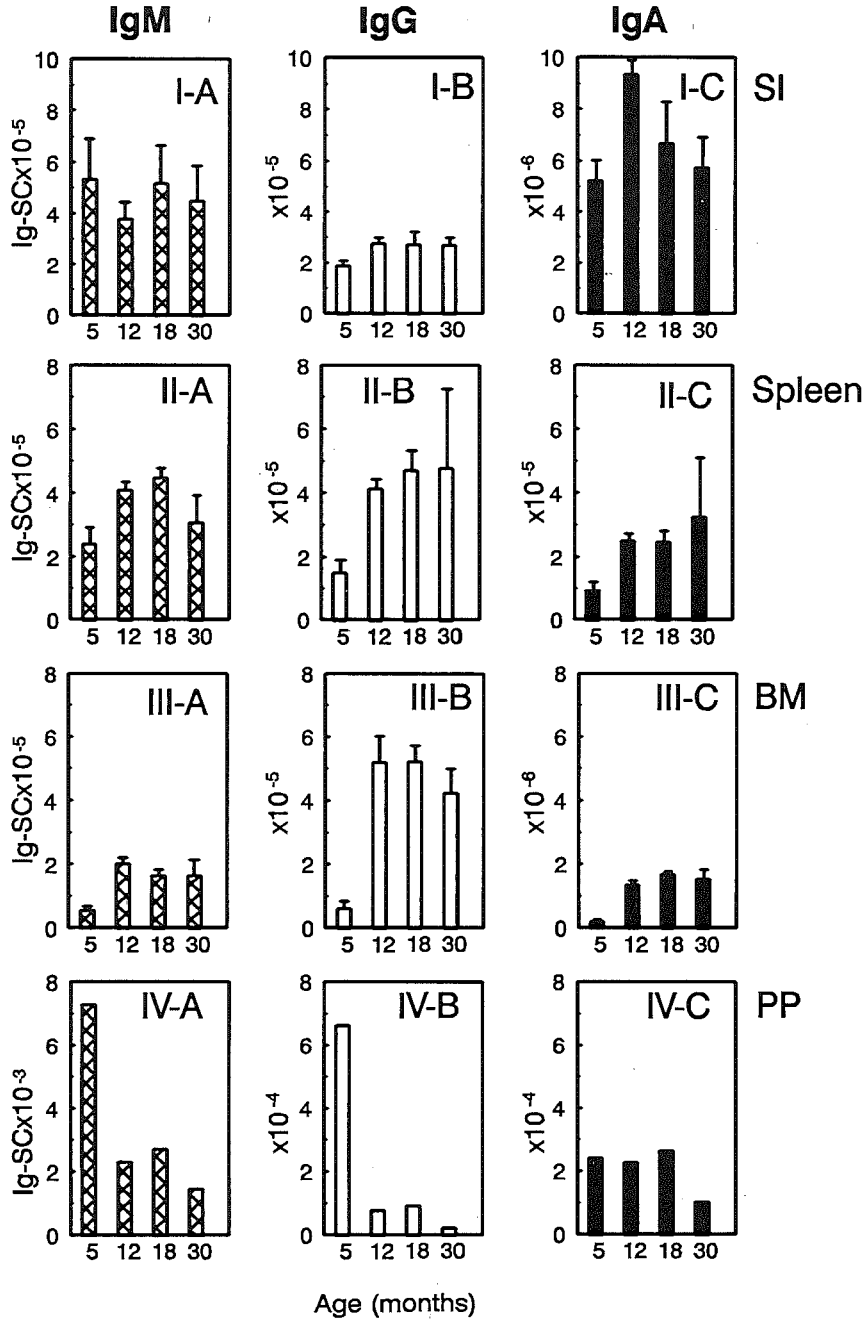

Figure 3. Effect of aging on the number of IgM-secreting cells (-SC), IgG-SC, and IgA-SC in small intestine (SI), spleen, bone marrow (BM), and Peyers patches (PP)# of CBA/Rij mice. Each bar represents the mean number of Ig-SC (n=6) with the SE. #Since PP suspensions were pooled, SE were not calculated.

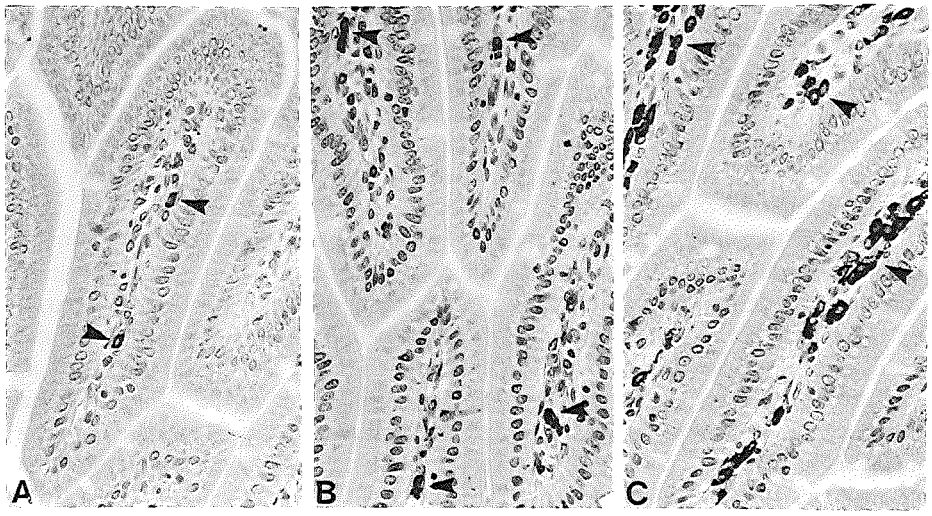

Figure 4. Sections of the same area of the small intestine of a 5 month-old CBA/Rij mouse stained for IgM (A), IgG (B), and IgA (C). Ig-containing cells (arrowheads) are located in the lamina propria in the villi.

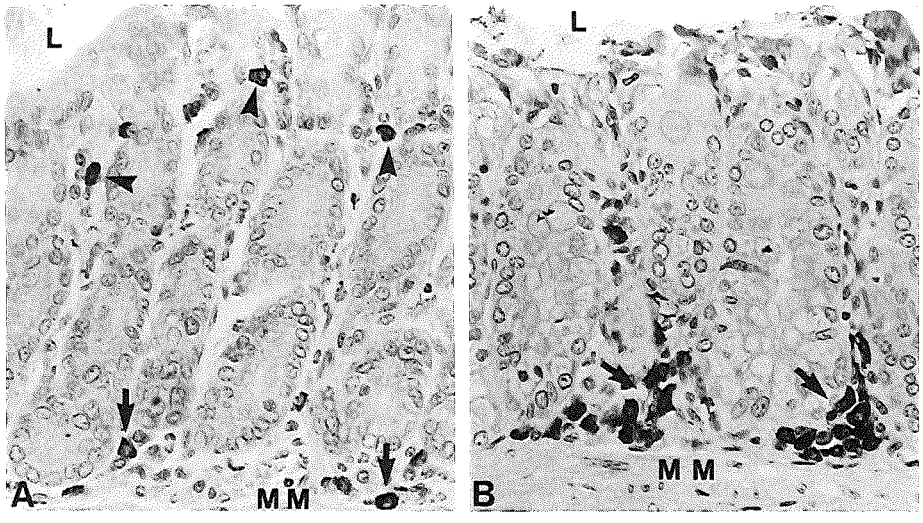

Figure 5. Sections of the large intestine of a 5 month-old (A) and a 30 month-old (B) CBA/Rij mouse stained for IgG. IgG-containing cells are located in the lamina propria around the crypts near the intestinal lumen (L) (Arrowheads) and near the muscularis mucosae (MM) (arrows).

The results demonstrate that the isotype frequencies of Ig-containing cells in the small intestines and spleens of different age groups were largely compatible with the frequencies of IgM-, IgG-, and IgA-SC in these organs (Figure 3-I,II and 6-A,B). The majority of the intestinal Ig-containing cells was also IgA positive, but the relative frequency of IgM- and IgG-containing cells compared with IgA-containing cells was about 4 times higher than the relative frequency of IgM- and IgG-SC compared with IgA-SC observed in the LPL suspensions.

Ig-containing cells in the small intestine were located almost exclusively in the lamina propria; in the villi and around the crypts (Figure 4). In the large intestine Ig-containing cells were found scattered throughout the lamina propria surrounding the crypts both in the proximity of the intestinal lumen as well as near the muscularis mucosae (Figure 5-A). We observed no major changes in the localization and in the isotype distribution of Ig-containing cells in the lamina propria during aging. However, for IgG-containing cells it was observed that the increase of such cells in old mice was found preferentially in the lamina propria of the large intestine near the muscularis mucosae (Figure 5-A,B). Aging caused a shift from IgM- to IgG- and IgA amongst the splenic Ig-containing cells. The decline of the frequency of IgM-containing cells was greater than the decline of IgM-SC.

DISCUSSION

The data presented in this study demonstrate that the absolute number and frequency of intestinal Ig-SC in lamina propria cell suspensions reached a maximum

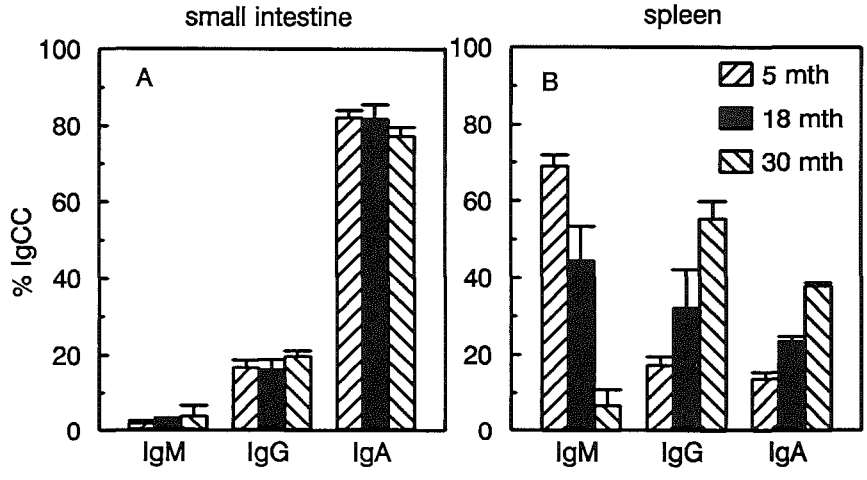

Figure 6. Effect of aging on the frequency of IgM-, IgG-, and IgA-containing cells in small intestine and spleen of 5, 18, and 30 month-old CBA/Rij mice. Each bar represents the mean percentage of Ig-containing cells (n=3) with SE.

in 12 months old C3H/He and CBA/Rij mice. From this age on, the "spontaneous" intestinal Ig-SC response decreased in both mouse strains. The strongest decrease of intestinal Ig-SC (80%) was found in C3H/He mice, probably because of the higher maximum of intestinal Ig-SC in this strain. In another study (unpublished observations), we have observed that differences of the number of intestinal Ig-SC can be partially due to the genetic background of the mice.

However, the difference between the maximum number of intestinal Ig-SC of C3H/He and CBA/Rij mice used in this study is most likely caused by different antigenic stimulation (14,19). The C3H/He mice were housed under conventional conditions, whereas the CBA/Rij mice were housed under specific pathogen-free conditions. Therefore, this study demonstrates that the onset of the decrease of intestinal Ig-SC did not depend upon the antigenic pressure during aging.

We also demonstrated that no significant change occurred in the relative distribution of the Ig-SC along the complete intestine during aging. The proximal part of the small intestine was the major site of intestinal Ig(A)-SC in mice at all ages. This is in contrast with the observations in adult humans where at least the same frequency of Ig-producing cells was found in large and small intestine (19). We also did not find a major change of the in situ localization of the Ig-containing cells along the intestine and of the distribution of the Ig-isotypes in the lamina propria. However, a small increase of IgG-containing cells was found in the lamina propria of the large intestine of old mice. This may be related to a different recirculation or homing pattern for such cells in old mice, possibly due to the antigenic history of these animals.

We observed some differences between the isotype distribution of the Ig-SC and the Ig-containing cells. These differences can be due to the assay systems used. Benner et al. (7) demonstrated that the protein A PFC assay always gives a higher number of Ig-producing cells as the immunofluorescence assay, the difference being 2-30 fold depending on Ig-class, age and immune status.

The splenic and BM Ig-SC responses of CBA/Rij mice increased till a maximum at the age of 12 months and persisted until the age of 30 months. The Ig-SC response of PP decreased rapidly from 3 months of age. These results confirm earlier observations by Haaijman et al. (5) and Benner et al.(6,7).

It is hard to imagine that the decrease of the Ig-SC response in PP of 3-month-old mice has a causal relation with the decrease of the intestinal Ig-SC response that starts 9 months later. However, it has been demonstrated by others that the PP are involved in induction as well as regulation of the Ig-SC response of the intestinal lamina propria (21,22). Kawanishi et al. (23-25) studied the regulatory T-cell circuit of PP during aging. They demonstrated by in vitro studies that the regulatory T-cell circuit deteriorated in old mice. This possibly explaines the decrease of the intestinal Ig-SC response by aging.

Other investigators have also studied the effect of aging on the enteric Ig response (8-11,23). They measured the concentration of luminal IgA. Different mechanisms can account for changes in the luminal IgA concentration. The IgA concentration of the intestinal lumen depends on (i) the number of Ig-SC in the lamina propria of the intestine, (ii) the efficiency of the transport of dimeric IgA

by the secretory component molecules of the epithelial cells, and (iii) the proteolytic degradation of IgA in the intestinal lumen (11). The results reported ranged from an increase (11) via no change of IgA concentration (8,23) to a slight decrease of the IgA concentration (9). These studies did not give insight into the site(s) of the IgA production and transport chain that were affected by aging.

Although our results do not give direct information about the effect of aging on the concentration of luminal IgA, the results clearly demonstrate that the number of lamina propria IgA-SC declines during aging.

Acknowledgements

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CHAPTER 5

ANTIBODY PRODUCTION IN THE INTESTINE

5.1 Quantification of antigen-specific antibody-secreting cells in the small intestine and other lymphoid organs of mice after oral booster immunization.

5.2 Manipulation of intestinal immune responses against ovalbumin by cholera toxin and its B subunit in mice.

5.1 QUANTIFICATION OF ANTIGEN-SPECIFIC ANTIBODY-SECRETING CELLS IN THE SMALL INTESTINE AND OTHER LYMPHOID ORGANS OF MICE AFTER ORAL BOOSTER IMMUNIZATION

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Immunology *66*, 404, 1989

SUMMARY

The intestinal immune response of mice against ovalbumin (OVA) was quantified by isolating lymphoid cells from the small intestine (SI) and testing them for antigen-specific immunoglobulin (Ig) secretion. The isolation procedure for functionally active lymphoid cells from the SI, originally developed to quantify the number of 'background' Ig-secreting cells in the SI, proved to be a useful method for evaluating antigen-specific intestinal immune responses quantitatively. The method was able to detect antigen-specific antibody-secreting cells (ASC) in the SI even when these cells occurred at a minimum frequency of only 0.006%. When mice were primed intraperitoneally (i.p.) with polymerized OVA and given an oral OVA booster immunization, OVA-specific ASC appeared in the SI from Day 3 after booster. After i.p. priming and an i.p. booster these cells could not be detected in the SI. The OVA-specific IgA-ASC responses in various organs after oral booster immunization were compared. From Day 5 after booster, when the response peaked, most OVA-specific IgA-ASC occurred in the SI. This suggested that these cells are mainly responsible for the OVA-specific antibodies demonstrated by ELISA in intestinal secretions from Day 6 after oral booster immunization. It is concluded that the quantitative method used in this study detects antigen-specific ASC in the SI with great sensitivity and could be used to evaluate immunization regimes aimed at inducing intestinal mucosal immune responses.

INTRODUCTION

Since most pathogens and toxins enter the body through the mucosae, vaccines and vaccination schedules should be able to induce effective mucosal immune responses. Secretory IgA (SIgA) is the predominating immunoglobulin along the mucosal surfaces, and therefore the principal humoral immunological effector mechanism at the mucosal site. The tissues adjacent to the mucosal surfaces are thought to synthesize IgA (Bienenstock & Befus, 1980; Underdown & Schiff, 1986).

Induction of antigen-specific SIgA responses in the mucosae depends on route, dose, and antigen-type (Fuhrmann & Cebra, 1981). Gastrointestinal or respiratory immunization generally stimulates SIgA responses better than parenteral immunization (Fuhrmann & Cebra, 1981; Koster & Pierce, 1983; Pierce & Koster, 1980). Vaccines containing 'life' antigens induce better immunity at mucosal surfaces than vaccines containing non-replicating antigens (Fuhrmann & Cebra, 1981; Lycke & Holmgren, 1986). An exception is cholera toxin (CT), a non-replicating antigen that induces a significant mucosal immune response (Elson & Ealding, 1984; Koster & Pierce, 1983; Pierce & Koster, 1980; Pierce, 1984). CT, in contrast to most non-replicating antigens, actively binds to the intestinal epithelium and stimulates adenylcyclase (Pierce, 1984).

Although 'live' antigen-containing vaccines are more immunogenic at mucosal surfaces than non-replicating antigen-containing vaccines, only a few safe mucosal vaccines have been developed. Therefore, inactive non-replicating vaccines, like subunit or peptide vaccines, are used to study immunization regimes aimed at inducing mucosal immune responses. Several methods to follow the induction of intestinal immune responses are employed. Antibodies in intestinal secretions can de detected by the enzyme-linked immunosorbent assay (ELISA) (Elson & Ealding, 1984; Elson, Ealding & Lefkowitz, 1984; Van Zaane, Ijzerman & de Leeuw, 1986), or antibody-containing cells can be detected immunohistologically (Fuhrmann & Cebra, 1981; Koster & Pierce, 1983; Pierce & Koster, 1980; Pierce, 1984; Sminia, Delamarre & Janse, 1983).

A very efficient method to evaluate and quantify intestinal immune responses is to isolate intestinal lymphoid cells and test them for IgA secretion (Lycke, 1986; Van der Heijden & Stok, 1987). Recently we described a method to isolate and quantify all Ig-secreting cells in the small intestine (SI; Van der Heijden & Stok, 1987; Van der Heijden, Stok & Bianchi, 1987). This procedure surpasses others because a greater number of functionally active lymphoid cells can be isolated (Davies & Parrott, 1981; Lycke, 1986). In this study we tested this procedure for its ability to isolate and quantify intestinal antigen-specific antibody-secreting cells (ASC) in C3H/He mice after intraperitoneal (i.p.) priming and oral booster immunization with ovalbumin (OVA). OVA-specific ASC in the SI were quantified by an OVA-specific plaque-forming cell (PFC) assay, and the number was compared with the number found in the spleen, bone marrow (BM), mesenteric lymph nodes (MLN), and Peyer's patches (PP). The kinetics of the OVA-specific ASC response was compared with the kinetics of the OVA-specific anti-

body response in intestinal secretions. The degree of sensitivity of the procedure was obtained from results of a dose-response relationship between oral booster and the number of OVA-specific ASC in the SI.

MATERIALS AND METHODS

Mice: Female C3H/He mice were purchased from Harlan-Olac Ltd., Bicester, Oxon, U.K., housed under conventional circumstances, and used at the age of 12-20 weeks.

Antigens and immunization: OVA (Grade V, Sigma, St Louis, MO) was polymerized as described elsewhere (Holt et al., 1984). In short, glutaraldehyde (Merck, Darmstadt, FRG) was added drop-by-drop to protein in phosphate-buffered saline to a final molar ratio of 25:1. During the next 4 hr the pH was repeatedly adjusted to 7.5. The reaction was terminated by adding excess glycine, and the resulting product dialysed and clarified. Mice were injected intraperitoneally (i.p.) with 0.1 ml of a water-in-oil emulsion (Bokhout, Van Gaalen & Van der Heijden, 1981), containing 0.1 mg polymerized OVA. Four weeks later the mice were given booster immunizations either orally with 80 mg monomeric OVA in 0.5 ml 0.2 M $NaHCO_3$ or i.p. with 0.1 mg polymerized OVA in 0.1 ml saline. In the first experiments a high dose of 80 mg OVA was administered orally, as it has been described (Challacombe & Tomasi, 1980; Hanson et al., 1979; Richman et al., 1981; Swarbrick, Stokes & Soothill, 1979) that doses up to 80 mg are needed for the induction of mucosal immune responses. In a later experiment the dose-response relationship was examined.

Assay for OVA-specific plaque-forming cells: Cell suspensions from the spleen, Peyer's patches (PP), mesenteric lymph nodes (MLN), and small intestine (SI) were prepared as described previously (Van der Heijden et al., 1987; Van der Heijden & Stok, 1987). Femoral bone marrow (BM) was used for preparing BM suspensions as described elsewhere (Benner, Van Oudenaren & Koch, 1981).

Sheep red blood cells (SRBC) were obtained from a single donor sheep (CVI, Lelystad), stored in Alsever's solution (CVI), and washed three times in saline. SRBC were conjugated with OVA by mixing three equal volumes of SRBC (33% suspension), OVA (0.33 mg/ml in saline) and $CrCl_3$ (3.8 mM in saline) for 10 minutes at room temperature. The anti-OVA PFC assay was performed as the anti-SRBC PFC assay described earlier (Van der Heijden et al., 1986). Rabbit anti-mouse IgG and IgA were obtained commercially (Nordic, Tilburg). Each cell suspension was assayed individually, with the exception of MLN and PP cell suspensions, which were pooled per group of mice (n=5) for practical reasons.

Detection of anti-OVA antibodies in intestinal secretions: Intestinal secretions were obtained by the lavage technique described by Elson et al., (1984). Anti-OVA antibodies were assayed by ELISA on microtitre plates coated with polym-

erized OVA. Rabbit anti-mouse IgM- and IgG-peroxidase conjugates were obtained commercially (Nordic). Sheep anti-mouse IgA-peroxidase conjugate was obtained from Serotec Ltd., Bicester, Oxon, U.K. Bound conjugate was made visible by adding 5-Amino-salicyl acid (Merck) and H_2O_2 as a colouring substrate. Absorbance at 450 nm of each well was measured after 1 h of incubation.

RESULTS

Detection of OVA-specific ASC after oral booster immunization.
Mice were primed i.p. with polymerized OVA and were boosted orally 4 weeks later with monomeric OVA. From Day 3 to 7 after booster, OVA-specific ASC were quantified in cell suspensions of SI, MLN, spleen, BM, and PP. Significant responses were found in spleen, MLN, BM, and SI; Figure 1a-d shows the mean number of ASC (n=5) in these tissues. PP contained few OVA-specific ASC (data not shown). OVA-specific ASC in spleen, MLN, and BM produced predominantly IgG, whereas in the SI these cells produced predominantly IgA.

OVA-specific IgA-ASC were detected in the SI as early as Day 3 after booster immunization (Fig. 1a). A maximum of 48,600 OVA-specific IgA-ASC occurred at Day 5. Since the total number of Ig-secreting cells in 20-week-old mice is about

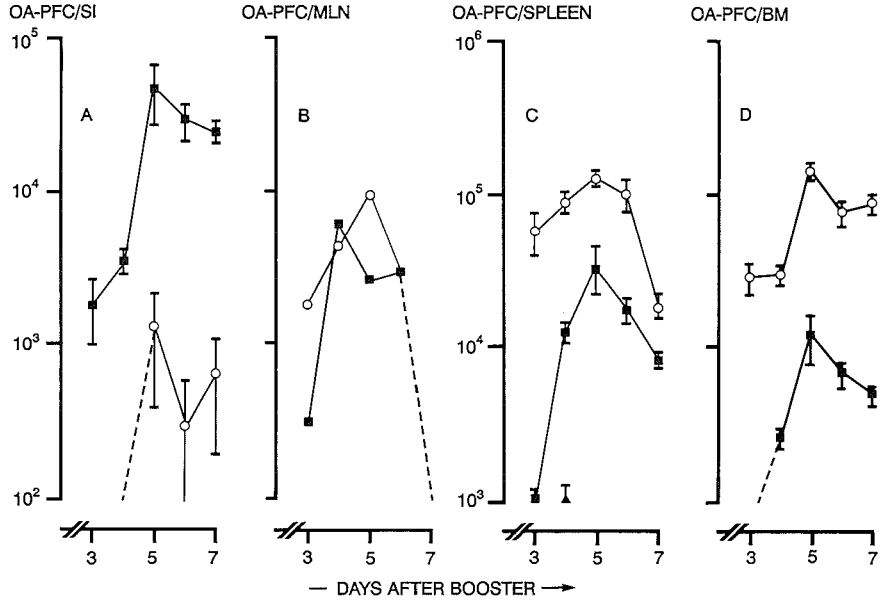

Figure 1. Number of OVA-specific ASC in small intestine (a), mesenteric lymph nodes (b), spleen (c) and bone marrow (d) after i.p. priming and oral booster immunization. Results are expressed as the mean (n=5) with the SE shown in bars.
Since mesenteric lymph node cell suspensions were pooled per group, no SE was calculated. Symbols represent IgM-ASC (▲), IgG-ASC (O), and IgA-ASC (■).

15 x 10^6 (Van der Heijden et al., 1987), we calculated the maximum frequency of OVA-specific IgA-ASC at 0.3%. After Day 5 the number of IgA-ASC in the SI decreased slowly, which indicated that the response persisted. At Days 5,6, and 7 after oral booster immunization only a few OVA-specific IgG-ASC could be detected in the SI among the majority of IgA-ASC.

The number of OVA-specific ASC in the MLN (Fig. 1b) peaked at Days 4 and 5. At Day 7 OVA-specific ASC were no longer detected. The number of OVA-specific ASC in the spleen peaked at Day 5 and thereafter decreased rapidly. OVA-specific ASC in the spleen produced predominantly IgG, but relatively high numbers of OVA-specific IgA-ASC were found as well. The mean IgG/IgA ratio from Day 4 to 7, calculated by averaging the daily number of IgG-ASC divided by the number of IgA-ASC, was 5.3:1.

Isotype distribution and peak response in the BM (Fig. 1d) were comparable to those found in spleen. The mean IgG/IgA ratio from Day 4 to 7 was 10.4:1. The slow decrease of the immune response in the BM, as in the SI, indicated that the response persisted. In none of the organs tested were OVA-specific IgM-ASC found at a detectable level.

Detection of OA-specific ASC after ip booster immunization.
To determine the significance of the oral route of the booster immunization to induce an intestinal anti-OVA response, i.p. primed mice were boosted i.p. with

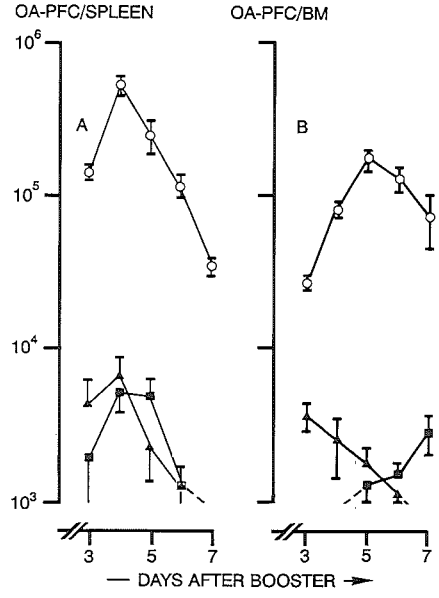

Figure 2. Number of OVA-specific ASC in spleen (a) and bone marrow (b) after i.p. priming and i.p. booster immunization. Results are expressed as the mean (n=5) with the SE shown in bars.
Symbols represent IgM-ASC (▲), IgG-ASC (O), and IgA-ASC (■).

polymerized OVA. Figure 2 shows the results of the OVA-specific PFC assay obtained with spleen and BM cell suspensions from i.p. boosted mice. The OVA-specific ASC response in the spleen (Fig. 2a) peaked 4 days after i.p. booster immunization; these cells produced predominantly IgG. The mean IgG/IgA ratio from Day 3 to 6 was 80:1. The OVA-specific ASC response in the BM (Fig. 2b) peaked at Day 5 after i.p. booster immunization. As in the spleen, OVA-specific ASC in BM produced predominantly IgG. The mean IgG/IgA ratio from Day 4 to 7 was 124:1. PP and MLN contained only a few OVA-specific IgG-ASC at Day 5 after i.p. booster immunization. In the SI no OVA-specific ASC were detected at all (results not shown).

Contribution of various organs to the total OVA-specific IgA-ASC response after oral booster immunization.

To determine the percentage of OVA-specific IgA-ASC contributed by each organ (spleen, MLN, BM, PP, and SI), we compared the number of OVA-specific IgA-ASC found in cell suspensions of each organ from Day 4 to 7. Table 1 shows the relative contribution of each organ. At Day 4 after oral booster, we recovered IgA-ASC primarily from the spleen and MLN. Thereafter, when the total OVA-specific IgA-ASC response peaked, the contribution of spleen and MLN decreased, while that of the SI increased. From Day 5 at least half of all OVA-specific ASC was recovered from the SI. In relation to the total number of OVA-specific IgA-ASC recovered, the percentage of these cells recovered from BM remained constant. PP contributed little to the total number of OVA-specific IgA-ASC recovered.

Table 1. Relative contribution of various organs expressed as percentages of the total OVA-specific IgA-ASC response after i.p. priming and oral booster immunization with OVA in C3H/He mice.
Results are expressed as the mean percentages (n=5). The total number represents the sum of all IgA-ASC in all organs tested. BM, bone marrow; MLN, mesenteric lymph nodes; PP, Peyer's patches; SI, small intestine.

	Days after booster			
	4	5	6	7
Spleen	51	35	31	22
MLN	24	3	5	0.5
PP	1	1	0.5	1
BM	10	13	12	14
SI	13	49	52	64
Total number	26,572	98,728	58,991	39,332

Anti-OVA antibody response in intestinal secretions after oral booster immunization.

In a separate experiment we determined whether i.p. priming followed by oral booster immunization with 80 mg OVA produced anti-OVA antibodies in intestinal secretions. Intestinal secretions obtained on Days 5-9 after oral booster were tested in an ELISA for the presence of IgM-, IgG-, or IgA-anti-OVA antibodies. IgM- and IgG-anti-OVA antibodies were not detected. Figure 3 shows the IgA-specific anti-OVA response as the mean absorbance at 450 nm (n=5) obtained with samples diluted five-fold. IgA anti-OVA antibodies were first detected at Day 6. At Day 8 after oral booster immunization the response peaked.

Intestinal secretions obtained after i.p. priming and i.p. booster immunization contained no detectable anti-OVA antibodies at any time after booster.

Determination of the dose-response relationship for oral booster immunization.

To evaluate the sensitivity of the procedure used to quantify OVA-specific ASC in the SI and to determine the relationship between the OVA dose used in the booster and the magnitude of the OVA-specific IgA-ASC response in the SI, i.p. primed mice were boosted orally with various doses of OVA ranging from 0.5 to 80 mg. Figure 4 shows the results of the OVA-specific PFC assay obtained with cell suspensions from the SI on Days 5,6, and 7 after booster immunization, when the response reaches its maximum level. The results show that already an oral booster immunization with 0.5 mg OVA produces a detectable immune response. From these results it can be calculated that the procedure is able to quantify antigen-

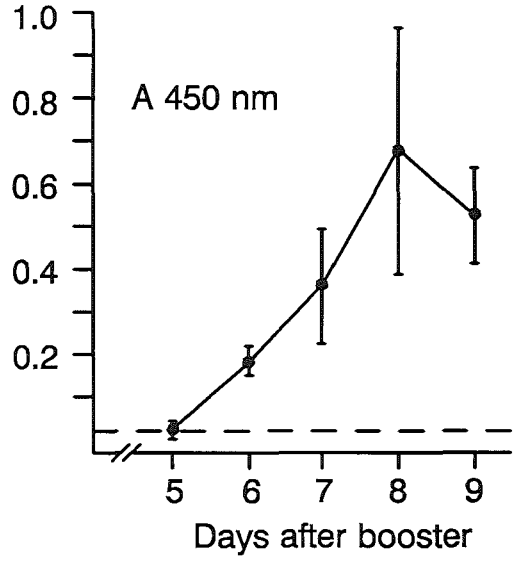

Figure 3. IgA anti-OVA response in intestinal secretions after i.p. priming and oral booster immunization. Results are expressed as the mean absorbance at 450 nm (n=5) with the SE shown in bars. Intestinal samples were tested in a five-fold dilution.

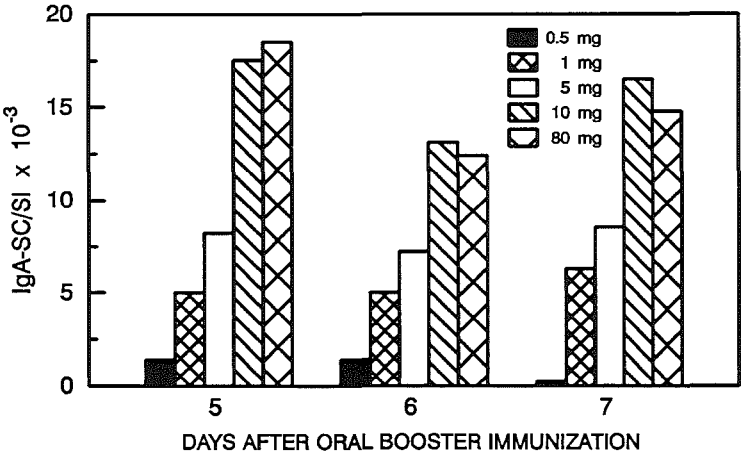

Figure 4. Number of OVA-specific IgA-ASC in the small intestine after i.p. priming and oral booster immunization with various doses OVA. Results are expressed as the mean (n=3).

specific ASC when these cells occur at a minimum frequency of 0.006% of all lymphoid cells in the SI. The response increases with a greater booster dose. An optimal response is produced with doses of 10 mg OVA or more.

Anti-OVA-specific antibodies in intestinal secretions were only detectable with booster doses of 10 mg OVA or higher (data not shown).

DISCUSSION

We described previously a procedure to isolate functionally active lymphoid cells (Van der Heijden & Stok, 1987). With this procedure we quantified the total number of Ig-secreting cells in the murine SI (Van der Heijden et al., 1987). In this report we show that this procedure can also be used to isolate and quantify antigen-specific ASC in the SI.

I.p. priming followed by oral booster immunization, an immunization procedure that normally induces intestinal immune responses (Challacombe & Tomasi, 1980; Pierce & Gowans, 1975; Pierce, 1984), also resulted in a clear response in the SI against the non-replicating, inactive antigen OVA (Fig. 1a). Results obtained from the spleen and BM (Fig. 1c, d) showed that this immunization procedure induces significant systemic immune response as well as an intestinal mucosal immune response. The simultaneous occurrence of these immune responses confirms results obtained by Elson & Ealding (1984). Moreover, our observations show that mucosal immune responses are not necessarily linked with systemic unresponsiveness and vice versa as suggested earlier (Challacombe & Tomasi, 1980; Hanson et al., 1979; Koster & Pierce, 1983).

OVA-specific ASC occurred in SI as early as Day 3 after oral booster immunization. OVA-specific antibodies, however, were detected in intestinal secretions only from Day 6 onwards. This observed delay has probably different causes. In part it may be caused by the relative insensitivity of the OVA-specific ELISA. Intestinal SIgA is degraded by proteolytic enzymes. Furthermore, specific coupling to the coated antigen used in the ELISA is inhibited, because intestinal SIgA binds to faecal components non-specifically. Moreover, before antibodies produced in the SI by ASC can be detected in intestinal secretions, they must be transported across the epithelial cells or the hepatocytes to the intestinal lumen or bile (Brandtzaeg, 1981; Hall, Guyre & Payne, 1980).

Apart from the delay of onset, the kinetics of the OVA-specific IgA antibody response in intestinal secretions matches that of the SI. These findings confirm suggestions (Bienenstock & Befus, 1980; Underdown & Schiff, 1986) that intestinal IgA is produced and secreted locally in the mucosal tissues.

In calculating the contribution of each lymphoid organ to the total OVA-specific IgA-ASC response (Table 1), we found that the contribution of each organ varied with the length of time after oral booster immunization. At Day 4 after booster immunization, when the first substantial IgA responses were measured, OVA-specific IgA-ASC occurred mostly in the spleen and MLN. At Day 5, when the response peaked, OVA-specific IgA-ASC occurred mostly in the SI. This change supports the proposition (Bienenstock & Befus, 1980) that, when stimulated by antigen in the PP, IgA-committed cells migrate via the MLN and thoracic duct into the blood circulation and spleen. Thereafter, they home selectively to intestinal and other mucosal tissues.

PP contributed little to the total number of OVA-specific IgA-ASC. This could indicate that PP have no significant function in inducing the OVA-specific IgA-ASC response after oral booster immunization.
Using the same immunization protocol, however, we clearly demonstrated by immunohistology OVA-specific antibody-containing cells in the PP (Bianchi, Zwart & Van der Heijden, 1989). These findings agree with earlier propositions that suggested that, after stimulation of lymphocytes by antigen in PP, activated lymphocytes may undergo heavy-chain class switching to IgA-bearing cells under influence of IgA-specific T cells (Kawanishi, Saltzman & Strober, 1983a). Terminal differentiation of switched IgA-bearing cells to IgA-producing cells only occurs after migration to other lymphoid tissues, i.e. the MLN, where appropriate maturation factors are available (Kawanishi, Saltzman & Strober, 1983b).

After i.p. booster immunization, OVA-specific ASC were not detected in the SI, whereas many were detected in spleen and BM (Fig. 2). Most of these cells secreted IgG. The mean IgG/IgA ratio in spleen was 80:1 and in BM 124:1. Comparison of these IgG/IgA ratios with the mean IgG/IgA ratios in spleen and BM after oral booster immunization (5.3:1 and 10.4:1) also shows that the isotype distribution in 'systemic' organs like the spleen and BM depends on the route of booster immunization. I.p. priming with OVA in a W/O emulsion apparently does not induce memory cells, which are committed to secrete IgA upon reactivation via a parenteral booster immunization. Thus the i.p. booster mainly produce IgG-

ASC. After antigen presentation via the PP, memory cells may undergo heavy chain class switching to IgA-bearing cells (Kawanishi et al., 1983a). Thus the oral booster produces IgA-ASC as well as IgG-ASC. Also in other studies intestinal antigen-specific ASC in mice have been quantified (Lycke, 1986; Lycke & Holmgren, 1986). In these experiments CT, known to be a potent inducer of mucosal immune responses, was used as antigen. The frequency of the CT-specific ASC – calculated as the percentage of antigen-specific ASC at the maximum of the response out of the total number of Ig-secreting cells in the suspension – was much higher than the frequency of OVA-specific ASC (7.8% compared with 0.3%). Although differences in isolation procedures can influence the frequency of antigen-specific ASC in the lamina propria cell suspensions, these results suggest that CT induces stronger immune responses than OVA. Remarkably, in our experiments, the maximum absolute number of ASC against OVA in the SI at Day 5 after oral booster (Fig. 1a) was more than 10 times higher than the maximum anti-CT responses reported by Lycke & Holmgren (1986). Responses, expressed as the absolute number of antigen-specific ASC per SI, were 4200 for CT and 48,600 for OVA. Because of the higher number of OVA-specific ASC per SI described in this paper, the method used by us to quantify antigen-specific ASC is much more sensitive.

Illustrative for the sensitivity of the method used by us is the study of the dose-response relationship (Fig. 4) between the OVA dose used in the oral booster immunization and the magnitude of the OVA-specific IgA-ASC response in the SI. The results showed that, although oral booster with 10 mg of OA or more is needed to produce an optimal response in the SI, a very weak response after oral booster immunization with as little as 0.5 mg of OA could be detected. The detection limit of the method used by us is estimated at 1000 antigen-specific ASC per SI or a frequency of 0.006%. In contrast, antibodies in intestinal secretions were only detected when mice were orally boosted with a dose of 10 mg OVA or more.

We conclude that isolating and testing lymphoid cells from the lamina propria for secreted antigen-specific antibodies is a very sensitive method for studying the intestinal immune response in mice quantitatively. After oral immunization with a non-replicating, inactive antigen, the SI contains more antigen-specific IgA-ASC than other lymphoid organs. Therefore, we recommend the above-described method for evaluating immunization regimes aimed at inducting mucosal immune responses.

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5.2 MANIPULATION OF INTESTINAL IMMUNE RESPONSES AGAINST OVALBUMIN BY CHOLERA TOXIN AND ITS B SUBUNIT IN MICE

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SUMMARY

We studied the effect of mucosal presentation of ovalbumin (OVA) conjugated to cholera toxin (CT) or cholera toxin B subunit (CTB) on the intestinal immune responses against OVA. Mice were primed intraperitoneally with OVA in a water-in-oil emulsion and boosted intraduodenally (i.d.) with OVA conjugated to CT or CTB in various molar ratios. Responses were evaluated by testing intestinal secretions for OVA-specific antibodies and by quantitating the OVA-specific antibody secreting cells (ASC) in the lamina propria of the small intestine. OVA-CT conjugates were tested in a molar ratio ranging from 1.8:1 to 4500:1. OVA-CTB conjugates were tested in a molar ratio ranging from 0.25:1 to 500:1. The optimum intestinal immune response was reached at a molar ratio of 1.8:1 for OVA-CT and 5:1 for OVA-CTB. The binding capacity of OVA-CTB, but not of OVA-CT, to GM1-ganglioside corresponded with the capacity to enhance the intestinal immune response. The effect of conjugating CTB or CT to OVA on the immune reponse against OVA was more striking when mice were not only boosted i.d., but also primed i.d. Both OVA-CT and OVA-CTB induced detectable immune responses, whereas free OVA did not. Therefore, the carrier effect of CT or CTB is essential to trigger a mucosal immune response against OVA when presented mucosally only. We conclude that enhancing antigen uptake greatly facilitates mucosal immune responses.

INTRODUCTION

Most dead, nonreplicating antigens do not induce mucosal immune responses after mucosal antigen presentation except cholera toxin (CT) and the heat-labile toxin of *E. coli* (LT), that induce significant mucosal immune responses upon oral immunization (Fuhrmann & Cebra, 1981; Klipstein, Engert & Clements, 1982). This is due to the fact that both CT and LT bind actively to the intestinal epithelium by their B subunits via the GM1-ganglioside receptor and stimulate adenylcyclase by their A subunits (Middlebrook & Dorland, 1984).

Further, CT and LT can provoke the induction of responses against non-related antigens administered orally together with CT or LT (Elson & Ealding, 1984; Nedrud et al., 1987; Clements, Hartzog & Lyon, 1988; Lycke & Holmgren, 1986). Although activation of adenylcyclase by the A subunit of CT or LT may be an important factor in the latter effect (Gilbert & Hoffman, 1985; Parker 1979), it is believed that enhanced antigen uptake through binding to the intestinal epithelium via the B subunit by itself will also lead to enhanced mucosal immune responses.

Therefore, the non-toxic B subunits from CT and LT (CTB and LTB) have been studied as possible carrier-proteins for mucosal antigen presentation. Unfortunately, the results obtained sofar are not conclusive. CTB has been found to stimulate mucosal responses when given simultaneously with the non-related antigen (Tamura et al., 1988; 1989). Others described that CTB must be conjugated with the antigen to be effective (McKenzie & Halsey, 1984; Czerkinsky et al., 1989). In contrast, Liang, Lamm & Nedrud (1988) and Lycke & Holmgren (1986) reported that CTB, either conjugated or not, does not stimulate mucosal responses. LTB has been found to be ineffective when given simultaneously with the antigen (Clements et al., 1988), but effective when presented as a fusion protein with the antigen (Schodel & Will, 1989).

In this paper we studied in detail the effect of conjugating CT and CTB to ovalbumin (OVA) upon the mucosal immune response against OVA in various immunization regimens. Furthermore we studied whether these effects correlated with the binding of the conjugates to GM1 ganglioside.

MATERIALS AND METHODS

Mice: Female C3H/He mice were purchased from Harlan-Olac Ltd., Bicester, U.K., housed under conventional circumstances, and used at the age of 12-20 weeks.

Antigens: Ovalbumin (OVA; Sigma, St. Louis, Mis., USA) was conjugated to CTB or CT (Sigma) by glutaraldehyde (Merck, Darmstadt, FRG) in various molar ratios. In short, OVA and CTB or OVA and CT were dissolved in 0.01 M phosphate-buffered saline (PBS; pH 8.0). Glutaraldehyde was slowly added to the mixtures untill a concentration of 15 mM was reached. After one hour of gently stirring, the reactions were terminated by adding excess glycine (60 mM). The

resulting mixtures, containing OVA-CTB and OVA-CT, were dialysed against PBS (pH 8.0). Polymerized OVA (pOVA) was prepared as described earlier (Van der Heijden et al., 1989).

GM1-ELISA: OVA-CT and OVA-CTB conjugates were tested for their ability to bind to ELISA plates coated with GM1-ganglioside (Sigma). Detection of bound CT and CTB was performed according to the method described by Svennerholm & Holmgren (1978). Rabbit anti CT-peroxidase conjugate (RaCT/PO; a kind gift from Dr. F.G. van Zijderveld, CVI, Lelystad, The Netherlands) was used to detect bound CT or CTB. Bound OVA was detected by adding mouse-anti-ovalbumin (MaOVA; CVI) followed by Sheep-anti-mouse Ig-peroxidase conjugate (ShaMIgGPO; Serotec, Kidlington, U.K.). Bound peroxidase conjugates were made visible by adding 3,3,5,5,-tetramethylbenzidin (Boehringer, Mannheim, F.R.G.) and H_2O_2 as a colouring substrate. After incubation for 10 min, the reaction was stopped by adding 100 µl of 0.1 N H_2SO_4. Absorbance was measured at 450 nm.

Immunizations: Mice were injected intraperitoneally (i.p.) with 0.1 ml of a water-in-oil (W/O) emulsion (Bokhout, Van Gaalen & Van der Heijden, 1981), containing 0.1 mg pOVA. Four weeks later the mice were given booster immunizations intraduodenally (i.d.) with 150 mg antigen in 0.5 ml 0.2 M $NaHCO_3$. To this end the mice were anaesthetised with Avertin (Aldrich, Brussels, Belgium; Koch et al., 1982), the abdominal cavity opened and the antigen injected in the duodenum approximately 1 cm after the stomach. The incision was closed in two layers. As antigens were used pOVA, pOVA with free CTB (150 µg) or free CT (10 µg), OVA-CTB, and OVA-CT.

In another series of experiments mice were primed i.d. with 150 µg of pOVA, OVA-CTB or OVA-CT. Four weeks later the mice were given booster immunizations i.d. with the same antigen preparations. In one separate experiment OVA and OVA-CTB immunized mice were boosted i.p. with 150 µg OVA one week after the second i.d. immunization.

Detection of anti-OVA antibodies in intestinal secretions: Intestinal secretions were obtained by scraping the isolated small intestine as described in detail previously (Van der Heijden, Dol & Bokhout, 1990). Briefly, small intestines were removed from the mice and flushed with PBS (pH 7.2). Mucus was squeezed out. The scrapings were dissolved in 2 ml PBS containing 50 mM EDTA and 0.1 mg/ml trypsin inhibitor and mixed vigorously. The solution was clarified by centrifugation (10 min, 650 x g) and 10 ml NaN_3 was added before storage at -20 °C. Anti-OVA antibodies were assayed by ELISA on microtitre plates coated with pOVA as described (Van der Heijden et al., 1989).

Detection of OVA-specific antibody secreting cells (ASC): Lymphocytes from the lamina propria of the small intestine were isolated as described (Van der Heijden & Stok, 1987). OVA-specific ASC were quantitated by an ELISA-spot assay (Bianchi et al., 1990).

RESULTS

Stimulation by CT and CTB: First it was studied whether CT and CTB could stimulate the intestinal IgA response against OVA in an i.d. booster after an i.p. priming of OVA in a W/O emulsion. Mice were boosted i.d. with pOVA, pOVA + free CT, OVA-CT (molar ratio 1.8:1), pOVA + free CTB, or OVA-CTB (molar ratio 0.25:1). Eight days after booster immunization intestinal secretions were collected and tested for anti-OVA IgA by ELISA. The results clearly demonstrate (Table 1) that CT stimulated the response to OVA regardless whether it was conjugated or not. CTB stimulated the response only when conjugated to the antigen.

Binding of OVA-CT and OVA-CTB to GM1: OVA was conjugated to CT in molar ratios varying from 1.8:1 to 4500:1 or conjugated to CTB in molar ratios varying from 0.25:1 to 500:1. The capacity to bind to GM1 ganglioside of the conjugated products was examined by ELISA on GM1 coated microtitre plates. Bound antigen was measured after addition of MaOVA followed by ShaMIgG/PO. The absorbance measured was optimal for OVA-CT at a molar ratio of 18:1 to 45:1 and for OVA-CTB at a molar ratio of 1:1 to 5:1 (Fig. 1).

When RaCT/PO was used as detecting agent, the conjugates with the highest CT or CTB input (molar ratio OVA:CT 1.8:1 and OVA:CTB 0.25:1) reached the highest absorbances (Fig. 1). The conjugates with the lowest molar ratio (4500:1 for OVA-CT and 500:1 for OVA-CTB) reached the lowest absorbances, which were, however, still above control level.

When free pOVA was used as antigen, no signal was observed in the assay with either RaCT or MaOVA as detecting antiserum (results not shown).

Effect of conjugating CT or CTB to OVA on the OVA-specific immune response in intestinal secretions: In two separate experiments mice were immunized i.p. with OVA in W/O emulsion and boosted i.d. with OVA-CT conjugates in various molar ratios or with OVA-CTB conjugates in various molar ratios. As a control

Table 1. Anti-OVA IgA titres in intestinal secretions of mice primed with OVA i.p. and boosted with various antigen preparations i.d.

Antigen in booster	IgA titre ± SE in intestinal secretions
pOVA	23 ± 11
pOVA + CT	208 ± 85
OVA-CT	256 ± 114
pOVA + CTB	20 ± 12
OVA-CTB	87 ± 22

Titres are expressed as the mean (n=5) with the S.E.

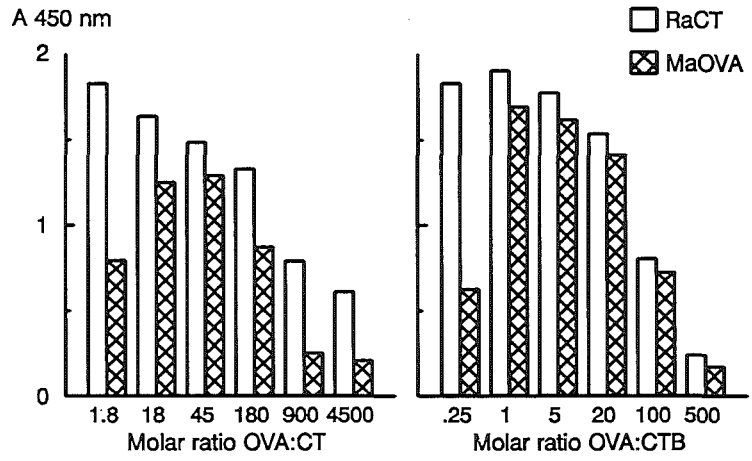

Figure 1. Binding of OVA-CT (left) and OVA-CTB (right) conjugates to GM1-coated ELISA plates detected by MaOVA/ShaMIgGPO. As a control bound CT and CTB was detected by RaCT. Results are expressed as the absorption at 450 nm at an antigen-carrier dilution of 0.02 mg/ml.

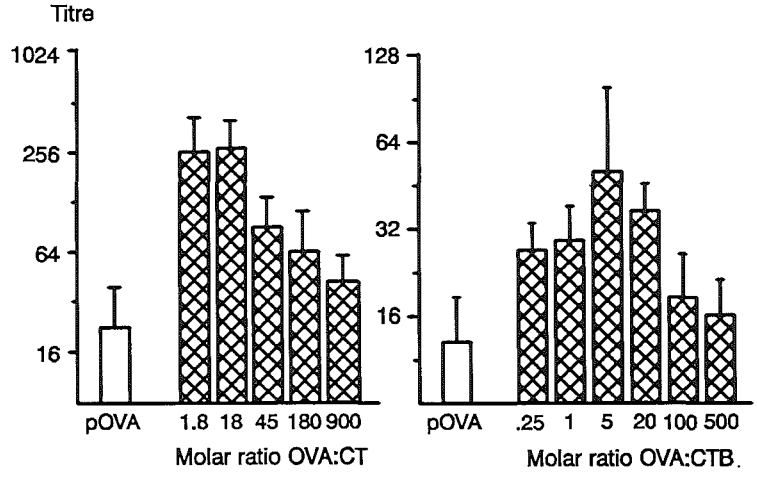

Figure 2. Effect of CT (left) and CTB (right) coupled in various molar ratios to OVA on the intestinal immune response measured by ELISA in intestinal scrapings 8 days after i.d. booster immunization. Results are expressed as the mean titre (n=10) with the S.E. shown in bars.

other mice were boosted i.d. with pOVA. The intestines of mice were removed and intestinal secretions collected by scraping eight days after booster immunization. The samples were assayed for the presence of OVA-specific antibodies by ELISA. OVA-CT stimulated the response best when conjugated in a molar ratio of 1.8:1 or 18:1 (Fig. 2). At higher molar ratios the stimulation decreased. OVA-CTB

stimulated the response best when conjugated in a molar ratio of 5:1. At higher molar ratios the stimulatory effect decreased gradually. The shape of the curve of intestinal responses after i.d. booster immunization with OVA-CTB conjugated in various molar ratios (Fig. 2 right) corresponded with the shape of the curve of the binding capacity of the OVA-CTB conjugates to GM1 detected by MaOVA (Fig. 1 right). This indicates that the binding capacity of OVA-CTB to GM1 correlated with the ability to stimulate the intestinal immune response.

Effect of conjugating CT or CTB to OVA on the number of intestinal OVA-specific ASC: We quantitated the number of OVA-specific ASC in the lamina propria of immunized mice to determine whether the enhanced mucosal immune responses were due to an enhanced secretion of SIgA per cell or to an enhanced number of IgA secreting cells in the lamina propria. Mice were immunized i.p. with OVA in W/O emulsion and boosted i.d. with OVA-CT (molar ratio 18:1) or OVA-CTB (molar ratio 5:1).

Lamina propria lymphocytes from the small intestines were isolated 5,6,7,8,9 and 10 days after booster immunization and the number of OVA-specific ASC measured by ELISA spot assay. The responses were compared with the response detected after booster immunization with free pOVA. Conjugating CTB stimu-

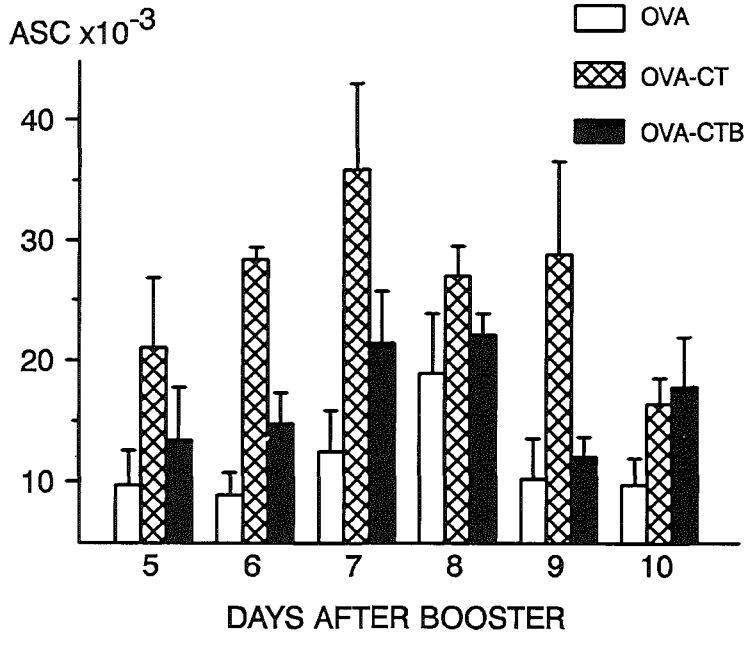

Figure 3. Number of OVA-specific IgA-ASC in the small intestine after i.d. booster immunization with OVA, OVA-CT or OVA-CTB. Results are expressed as the mean (n= 5 to 10) with the S.E. shown in bars. Molar ratios of the antigen-carrier conjugates were 2.5:1 for OVA-CTB and 18:1 for OVA-CT.

lated the response 1.5-2 times, whereas conjugating CT stimulated the response 2.5-3.5 times (Fig. 3). The kinetics of the responses after immunization with OVA-CT or OVA-CTB did not differ compared with the kinetics after immunization with free pOVA.

OVA-specific IgG-ASC were detected in the lamina propria cell suspensions in low numbers. Maximum responses were detected 6 days after i.d. booster immunization: pOVA immunized mice had 2220 IgG-ASC per small intestine, OVA-CT immunized mice 4320 and OVA-CTB immunized mice 2880. OVA-specific IgM-ASC were not detected above background level.

Effect of conjugating CT or CTB with OVA on the immune response after i.d. priming and booster immunization: In the experiments described above mice were primed i.p. with OVA in W/O emulsion. In another series of experiments mice were primed i.d. with pOVA, OVA-CT (molar ratio 18:1) or OVA-CTB (molar ratio 5:1) and boosted i.d. with the same antigen preparations to study whether OVA-CT and OVA-CTB could trigger detectable immune responses upon mucosal antigen presentation. OVA-specific IgA antibodies were determined in intestinal secretions and in serum 7 days after booster immunization. All pOVA immunized mice did not react with detectable responses in intestinal secretions or in sera (Table 2). In contrast, several OVA-CT and OVA-CTB immunized mice responded with IgA titres in scrapings and IgG titres in sera. Responder mice immunized with OVA-CT gave higher IgA titres in scrapings than responder mice immunized with OVA-CTB.

To determine whether the non-responder mice were primed for an anamnestic systemic immune response or were rendered tolerant to OVA, in a separate experiment mice were primed and boosted i.d. with pOVA or OVA-CTB. Two weeks after the second i.d. immunization the mice were injected i.p. with 150 μg OVA. One week after booster immunization blood was collected and serum tested for the presence of anti-OVA antibodies. Mice primed and boosted i.d. with OVA-CTB reacted to the i.p. injection (average titre 520 ± 250), whereas mice immunized with free pOVA did not respond (average titre ≤50).

Table 2. Anti-OVA antibody titres in scrapings and sera of mice i.d. primed and boosted with OVA, OVA-CT or OVA-CTB.
Titres are expressed as the mean of responder or non-responder mice.

Immunogen	responder/total no. of animals	IgA-titre scrapings resp.	IgA-titre scrapings non-resp.	IgG-titre sera resp.	IgG-titre sera non-resp.
OVA	0/10	-	<2	-	<50
OVA-CT	4/10	47.6	<2	4600	<50
OVA-CTB	3/10	20.0	<2	8800	<50

DISCUSSION

In this paper we studied the effect of conjugating CT or CTB to OVA on the i.d. induced intestinal immune response against OVA. First we demonstrated that CT and CTB stimulated the intestinal immune response after an i.p. priming and an i.d. booster immunization (Table 1). We confirmed earlier results (McKenzie & Halsey, 1984) that conjugating CTB to the antigen is a prerequisite to obtain the stimulatory effect, as free CTB did not stimulate the response above the level reached with pOVA alone. The stimulation by CT did not depend on conjugation.

It is thought that CTB can stimulate mucosal immune responses by facilitating the contact between the antigen and the intestinal epithelium. However, a direct correlation had not yet been demonstrated. We clearly demonstrated by ELISA that OVA-CTB binds to GM1-ganglioside (Fig. 1). The results obtained when MaOVA was used as detecting agent, showed that the efficiency to bind to GM1 depended on the molar ratio between OVA and CTB. An optimum was reached at a molar ratio of 1:1 to 5:1. Probably, at these molar ratios there is an optimal combination of available GM1-binding sites and antigenic determinants that can be detected by MaOVA. At a molar ratio of 0.25:1 antigenic determinants of OVA were probably partly blocked by excess CTB, which resulted in a lower signal. At molar ratios higher than 5:1 the binding capacity of the OVA-CTB conjugates decreased. The absorbance reached when RaCT was used as detecting agent continuously declined from a molar ratio of 0.25:1 to 500:1 as a result of decreasing concentrations of CTB.

As OVA-CTB conjugates bind to GM1-ganglioside in an ELISA system, they probably also adhere to GM1 on intestinal epithelial cells. We examined whether or not the capacity to adhere to GM1 in ELISA correlated with the stimulatory effect on an i.d. induced intestinal immune response. Indeed, the stimulation by CTB conjugated to OVA in various molar ratios (Fig.2) corresponded with the binding capacity of the OVA-CTB conjugates in the GM1 ELISA (Fig. 1). As Bland & Warren (1986) described that intestinal epithelial cells can function as antigen-presenting cells, active binding of antigen to these cells may result in enhanced immune responses.

In contrast to the effect observed using CTB, the stimulation by CT did not correlate with the binding of OVA-CT to GM1. The stimulation by CT showed a linear dose-response relationship that decreased from a molar ratio of 1.8:1 to 900:1, whereas binding to GM1 showed an optimum at molar ratios of 18:1 to 45:1. CT is known for its strong adjuvant proporties provided by its A subunit (Lycke et al., 1989; Wilson, Stokes & Bourne, 1989). Clearly, the adjuvant effect of the A subunit dominates over the carrier effect of optimal binding of the B subunit to the intestinal epithelium.

To study the stimulatory effect of conjugating CT or CTB to OVA at the cellular level we measured the numbers of OVA-specific IgA-ASC in the small intestine of mice that were boosted with pOVA, OVA-CT or OVA-CTB. CTB stimulated the response 1.5-2 times, whereas CT stimulated the response 2.5-3.5 times (Fig. 3). CT and CTB enhanced the number of OVA specific IgA-ASC in the

small intestine somewhat less than the level of OVA-specific IgA antibodies in intestinal secretions. This indicates that the stimulation by CT and CTB is not only caused by an increase in the number of antigen-specific ASC in the lamina propria but also by an enhanced transport of IgA over the epithelium and/or an increased secretion of IgA by individual plasma cells.

The stimulation by CT and especially by CTB observed in our experiments (Table 1, Fig. 2 and 3) is clear yet not very strong. This may be caused by the antigen used in our experiments, as Wilson et al. (1989) reported that CT stimulated mucosal immune responses against OVA less than the response against keyhole limpet haemocyanin. More likely, it is due to the immunization regimen chosen for the experiments, since free pOVA is quite able to induce a mucosal immune response by itself (Van der Heijden et al., 1989).

The effect of the carrier function of CT and CTB on the intestinal anti-OVA response was much more striking in an immunization regimen in which the antigen was presented to the mucosa twice via i.d. immunization (Table 2). Free OVA did not induce an immune response in this protocol. However, about half of the mice did show a detectable immune response upon immunization with OVA-CTB or OVA-CT. The percentage of responder mice can probably be increased by presenting the antigen to the mucosa more often, as André et al. (1973) and Wachsmann et al. (1985) stated that at least four oral immunizations are needed to induce mucosal immune responses. As we immunized the mice i.d. it was technically impossible to immunize the animals repeatedly on four occasions.

I.p. booster immunization with OVA revealed that mice i.d. immunized twice with OVA-CTB were primed for an systemic response, whereas mice i.d. immunized twice with free pOVA were not. It is known that oral presentation of protein antigens usually results in the induction of a state of tolerance (Tomasi, 1980; Strober, Richman & Elson, 1981). Our results indicate that conjugating CTB to OVA can prevent tolerance induction. These results confirm the conclusions reported by Elson & Ealding (1984), but are in contrast to the conclusions reported by Clements et al. (1988) and Lycke & Holmgren (1986). They suggested that the lack of tolerance induction by CT or CTB may be a function of binding to cell surface receptors, while the ability to influence the immunological response to a second antigen may reside with the A subunit of the toxin.

The results presented in this paper show that a carrier protein like CTB greatly facilitates a mucosal SIgA response and decreases the chance of tolerance induction by antigens that do not induce a mucosal immune response by themselves. The effect of a carrier protein is smaller in mice that are already primed for a mucosal immune response by another protocol (i.p. immunization of OVA in a W/O emulsion). In this situation whole CT still stimulates the mucosal immune response as a result of the activity of the A subunit. So, in agreement with Liang et al. (1989) we conclude that the carrier function of CTB enhances the immunogenicity of mucosally presented antigen, whereas the A subunit of CT provides adjuvant activity.

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CHAPTER 6

GENERAL DISCUSSION

The main physiological function of the intestine is the digestion and uptake of nutritious substances and the excretion of degraded products. Therefore, the intestine is in open contact with the outside world. This implies that potentially pathogenic microorganisms and substances can enter the gut lumen. So, next to mechanisms for the uptake of nutritious substances the individual is provided with specific as well as nonspecific defence mechanisms against pathogens.

Nonspecific defence mechanisms of the gut include among others mucus and fluid secretion, peristaltic movement, diarrhoea, enzymatic degradation etc. Specific defence mechanisms are provided by the mucosal immune system. Although it was clear that the intestine showed immunological activity, up till now little insight existed in the magnitude and development of this immunological compartment.

Consequently, the contribution of the gut to the total immunological activity in the individual had not been evaluated before. These items were subject to research in the experiments described in Chapter 4 of this thesis. To study the production of Ig by the intestine quantitatively we developed a method to isolate functionally active lymphocytes from the intestinal lamina propria (Chapter 3.1). The number of Ig-SC among these was determined by the Protein A plaque assay. For comparison of the immunological activity of different lymphoid organs this method is more appropriate than the estimation of the level of Ig in the intestinal lumen. The latter method, evaluated in Chapter 3.2, is more easy to perform but is less sensitive and shows greater variation in results. Therefore, we used the latter method only to screen specific intestinal immune responses after various immunization schedules, as illustrated in Chapter 5. The results in Chapter 4 clearly demonstrate that the intestine is a major immunological compartment as it contains more than two-third of all Ig-SC present in an adult mouse. The great majority of these intestinal Ig-SC secrete IgA. Moreover, the results showed that the small intestine is an unique lymphoid organ that differs in many characteristics from other lymphoid tissues like spleen and bone marrow. For instance, the development of the intestinal immunological compartment depends greatly on exogenous antigenic stimulation, whereas the development in the spleen does not. Further, the rate of development of Ig-SC in the intestine, when stimulated by antigen, is enormous,

whereas the rate of development is more gradually in spleen and especially in bone marrow. Also, the number of intestinal Ig-SC reaches a maximum at an age of about one year and decreases afterwards, whereas the number of Ig-SC remains relatively constant in spleen and continues to rise in bone marrow during life. Furthermore, and in contrast to other lymphoid organs, during ontogeny IgM is not a major isotype produced by the intestinal Ig-SC. The latter may apply for mice only as we have demonstrated that, for instance, in pigs IgM is the predominant secreted isotype by intestinal Ig-SC from early in ontogeny of the intestinal immune compartment untill weaning (1).

6.1. Development of the intestinal immune compartment

In man and in farm animals many diseases of the intestinal tract occur during infancy. Therefore, we investigated the development of the intestinal immune compartment during the first few weeks of life.
Relatively little is known about the development of the intestinal mucosal immune system. From our results and those from others it is clear that intestinal antigenic stimulation is a major stimulus for the induction of IgA-plasma cells in the intestinal lamina propria (2-8). In contrast to the presence of IgA plasma cells in the lamina propria, the development of the PP does not depend on antigenic stimulation, although the PP will involute prematurely when antigen-deprivation is continued (6,9,10). So, the mucosal immune system is already present before weaning. At that time the bacterial flora is not yet established in these young animals. Consequently, until than exogenous antigenic stimulation is minimal. Moreover, maternal antibodies will trap antigens in the intestinal lumen and therefore reduce stimulation of the mucosal immune system. It has been shown that the occurrence of germinal centres in PP as well as in MLN does depend on antigenic stimulation (6).

The influence of maternal immunity on the development of the mucosal immune compartment is an important aspect that is not fully understood yet. It has been shown in newborn pigs that circulating maternal antibodies suppress not only systemic immune responses, but also antigen-specific responses in the intestine (11). Paradoxically, maternal immunity is vital for the young animal to survive the first few weeks of life, while it simultaneously suppresses the development of active immunity by the young animal itself. So, if the maternal antibodies do not provide adequate protection due to a lack of specific antibodies or because the young are weaned very early as is common practice in modern farming, methods have to be developed to evade the suppressive activity of maternal antibodies. It should be possible to induce mucosal immune responses in the presence of maternal antibodies, as the potency of the PP and MLN to respond seems to be present already before weaning. To that purpose antigens must be applied locally without being trapped by maternal antibodies. This may be accomplished, for instance, by administering the antigens packed in microparticles that are selectively sampled by PP.

6.2. Comparison of the intestinal Ig production of C3H/He mice and other mouse strains

Most results in the experiments described in this thesis have been obtained by using C3H/He mice. To ensure that the data obtained were representative for mice in general, we quantitated the number of Ig-SC in the small intestines, MLN, PP, bone marrow and spleen of 12-week-old mice of various genetic background and calculated the contribution of the small intestine to the total Ig(A) production in each mouse strain. Table 1 summarizes these results. Although the small intestine of C3H/He contributes most to the total Ig(A) production, it is clear from Table 1 that these results apply for all mouse strain investigated. On average 85% of the IgA-SC and 65% of all Ig-SC are located in the small intestine.

Table 1. Contribution of the small intestine of various mouse strains to the total number of IgA-SC and Ig-SC. The results are expressed as the percentage contribution of the small intestine to the total number of Ig(A)-SC in spleen, bone marrow, PP, MLN and small intestine together.

Strain	H-2 type	% of IgA-SC	% of Ig-SC
C3H/He	H-2^k	98	90
C57BL/6	H-2^b	66	39
C57BL/Ka	H-2^b	80	66
CBA/Rij	H-2^q	93	75
BALB/c	H-2^d	91	55

The absolute number of IgA-SC isolated from the small intestines of mice of various genetic background differed (Table 2). The highest numbers of IgA-SC were found in C3H/He mice. This is probably caused by a higher recovery of cells from these mice, due to the fact that we developed and optimized the isolation procedure with C3H/He mice. The optimal conditions may differ slightly between different mouse strains. Nevertheless, in all mouse strains tested more than one million IgA-SC were detected in the isolates. There is not a clear correlation between the H-2 type and the number of IgA-SC isolated from the SI, although mice with a "black" background (C57BL/6, C57BL/10.ScCr and B10.BR) tend to harbour less IgA-SC in the intestinal lamina propria than the other strains investigated. A crossbred between C3H/He and C57BL/6 mice showed an intermediate response.

Table 2. Number of IgA-SC in the small intestine in various mouse strains.

Strain	H-2 type	Number of IgA-SC ($x10^{-6}$)	S.E. (n=10)
C3H/He	$H\text{-}2^k$	9.53	0.93
C3H/HeJ	$H\text{-}2^k$	6.50	1.22
CBA/J	$H\text{-}2^k$	8.62	1.42
B10.BR	$H\text{-}2^k$	1.87	0.28
BALB.K	$H\text{-}2^k$	4.18	0.93
BALB/c	$H\text{-}2^d$	8.62	0.91
(C57BL/6xC3H/He)F1	$H\text{-}2^{b/k}$	5.04	1.48
C57BL/6	$H\text{-}2^b$	1.21	0.39
C57BL/10.ScCr	$H\text{-}2^b$	2.32	0.26

6.3. Functional significance of intestinal IgA-SC

From histological examinations it was already clear that the intestine harboured many Ig-SC. Nevertheless, the numbers found in the experiments described in this thesis were supprisingly high. As pointed out in Chapter 4.1 many IgA-SC are needed to maintain the level of S-IgA in the enteric mucus as there is no possibility of accumulation of S-IgA due to peristaltic movement of the intestine and enzymatic degradation. To illustrate the dynamics of the intestinal immunological compartment, half of the Ig-SC in the intestine have a life span of only 3-4 days, while the other half have a life span of more than three weeks (12,13).

If the body invests more than two-third of the immunological activity in maintaining the S-IgA level in the gut, it must be vital for survival of the individual. However, it must be noted that about 1 out of 500 individuals is deficient for IgA, but normally lives a healthy life. In these individuals IgM usually replaces the function of IgA as mucosal Ig isotype (14). The high number of IgA-SC in the intestinal wall suggests that the body maintains a kind of permanent coating of the mucosal surfaces with S-IgA, directed against all antigenic determinants that it encounters, including food antigens. If all antigens are recognized by S-IgA present at the mucosal surfaces, under normal circumstances no antigen can reach the intestinal epithelium without complexing to S-IgA. When antigen, complexed with S-IgA, is taken up by the tissues, tolerance is mostly induced as it has been described that such complexes are potent inducers of a state of tolerance (15). This would explain why normally immune responses are not induced after mucosal antigen presentation. Induction of oral tolerance after feeding of protein antigens is a common phenomenon (16). Non-aggressive (dead) antigens that are not recognized by S-IgA, on the other hand, may induce immune responses, for example in young animals where the mucosal immune compartment is not yet fully devel-

oped or the antibodies present are still directed against only a limited number of antigenic determinants. In contrast to non-aggressive antigens, aggressive antigens, like toxins or live virusses and bacteria, may be able to evade trapping by S-IgA in adult animals and therefore induce mucosal immune responses. The presence of S-IgA antibodies directed against a greater diversity of antigenic determinants in older animals would explain that even the intestinal immune response against cholera toxin, known as one of the most potent inducers of mucosal immune responses after mucosal presentation, declines with age (17).

The above data suggests that the immunological compartment of the intestine does not discriminate between "good" and "bad" antigens. It may be that differences between immune responses against food-proteins ("good" antigens) and bacterium-derived proteins ("bad" antigens) after oral immunization are caused by the bacterial context in which the latter are presented (18,19).

6.4. Intraepithelial lymphocytes

T-cells play an important role in the regulation of immune responses.We demonstrated the role of T-cells in the development of the mucosal B-cell compartment by quantitating the IgA-SC in the small intestine of 16-week-old conventional and athymic nude BALB/c mice. In this study we found that the small intestine of nude

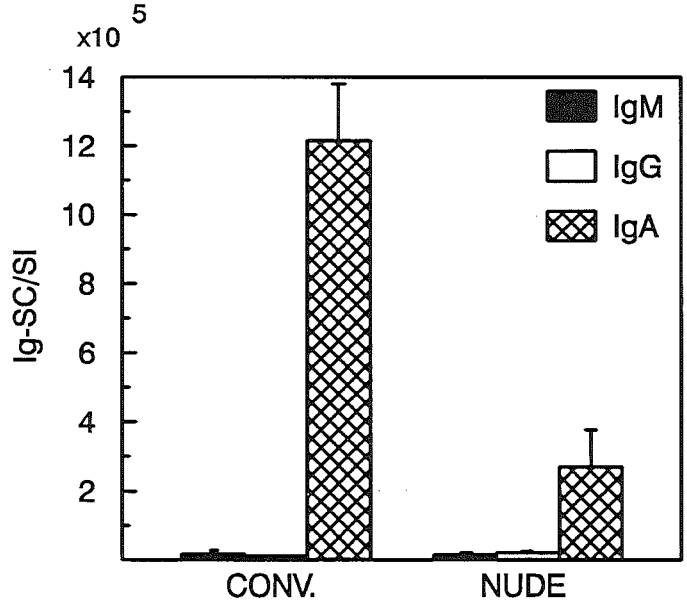

Figure 1. Numbers of Ig-SC in the small intestines (SI) of conventional euthymic and athymic (nude) BALB/c mice of 16 weeks of age. Results are expressed as the mean with the S.E. (n=10) shown in bars.

mice harbours far less IgA-SC than the small intestine of their conventional counterparts (Figure 1). This shows that the development of the intestinal B-cell compartment greatly depends on T-cells.

T-cells are present in the lamina propria and in between epithelial cells. The latter population, the intraepithelial lymphocytes (IEL), can be isolated by a procedure similar to the first part of the one described in Chapter 3.1 to isolate LPL. After incubation of rinsed pieces of intestine with DTT and EDTA, IEL can be separated from epithelial cells by density gradient centrifugation. It has been described that these T-cells express the Thy-1 marker in variable densities (20,21). Most of these cells also bear the CD8 marker.

Normally T-cells have a receptor consisting of a so-called alpha and beta chain. In a collaboration between De Geus et al. and our institute we showed, after analysis of mRNA from IEL by hybridization with cDNA probes, that most IEL have a receptor consisting of a gamma and a delta chain. Quantitative analysis showed that IEL are the richest source of these cells in the mouse (20). Further, De Geus et al. showed (22,23) that the presence of these cells did not depend on the presence of the thymus as the number isolated from athymic nude mice was as large as that from normal mice. These results have also been described by others (24-26).

The function of IEL is subject to discussion, although it is generally accepted that they can exert cytotoxicity or suppressor cell activity.
In rats (27) and guinea pigs (28) it was demonstrated that IEL exhibit both natural cytotoxicity and antibody-dependent cellular cytotoxicity (ADCC). In contrast, IEL from mice do not exhibit ADCC activity (29). It has been suggested that IEL may be precursors of mucosal mast cells. This possibility has been raised because of the histochemical and ultrastructural similarities between the granules in IEL and mucosal mast cells and because both cells contain histamine (30,31).

Studies in mice suggest, but do not prove, that IEL can differentiate into mucosal mast cells during nematode infestation (29). Unequivocal evidence for transformation of IEL into mucosal mast cells, however, is not available.

The T-cell population in the lamina propria consists of about equal numbers of alpha/beta receptor positive and gamma/delta receptor positive T-cells (20). This suggests that the T-cells in the lamina propria and the IEL are functionally different compartments. The characteristics and functional activities of IEL and T-cells from the lamina propria have been reviewed extensively (21).

6.5. Induction of mucosal immune responses

Numerous studies have shown that local antibody responses are best stimulated by local application of the antigen (38). The highest responses and best protection have been achieved with viable organisms (32-39). Probably the intestinal tract must be colonized with the immunizing microorganism in order to elicit a strong response since local antibody production is only seen in animals from which the specific organism can be cultured from faeces (40).

Non-replicating antigens usually do not induce mucosal immune responses upon oral vaccination, although particulate antigens sometimes induce significant mucosal immune responses. Mice, orally immunized with killed *Salmonella typhimurium* (41) or *E. coli* (42) and rats fed dead *Streptococcus mutans* (43), all showed a close relationship between the amount of antigen fed and the antibody response elicited. Maximum responses required large doses of antigen.

However, after oral immunization with inactivated whole organisms, even at the highest doses, the protection achieved was incomplete and, when compared, was inferior to natural infection and living oral vaccines. We demonstrated that oral immunization with inactivated *E. coli* K88ac even suppressed the intestinal immune response in mice on a subsequent oral application of live *E. coli* K88ac bacteria (44).

In Chapters 5.1 and 5.2 we demonstrated that mucosal responses against non-replicating soluble antigens can be induced by parenteral priming and oral booster immunization. These responses could be augmented by the use of carrier proteins like cholera toxin (CT) or cholera toxin B subunit (CTB) during the booster immunization. Moreover and more important, by using carrier proteins we were able to induce mucosal immune responses by immunization via the mucosal route. As the use of intact toxic CT has several disadvantages, many studies have focussed on the use of the non-toxic CTB (45-52). We clearly demonstrated (Chapter 5.2) that coupling of CTB to the antigen is a prerequisite for the stimulation of the mucosal response by CTB. As CTB is sufficient to trigger intestinal immune responses upon mucosal antigen presentation, some studies have focussed upon using synthetic peptides derived from this B subunit as a carrier. In these studies it was shown that synthetic peptides derived from CTB induced systemic (53,54) as well as mucosal responses (55) when given per os. Houghten et al. (56) used a peptide of the *E. coli* heat-labile toxin B subunit (LTB) to enhance responses against a peptide derived from the *E. coli* heat-stable toxin. We have used a synthetic peptide from CTB as a carrier with some success (results not shown), but further work needs to be done to evaluate this possibility.

Uptill now, chemical coupling procedures have been used to link carrier proteins like CTB or LTB to the antigen. These procedures are not very effective as it has been described that only 6 to 15% of the carrier is incorporated in an antigen-carrier conjugate (46,50). A new approach to prepare these conjugates is by making use of recombinant DNA techniques. Oligonucleotides that code for a specific antigen are inserted into plasmids in conjunction with the gene that codes for the CTB or LTB carrier protein (57). After cloning the vector in suitable bacteria, fusion-proteins can be isolated, that consist of the carrier protein combined with the antigen. As every antigen molecule is bound to a carrier, immunizations with these antigen-carrier fusion-proteins may induce antigen specific responses more efficiently than chemically prepared conjugates. Recently we produced fusion-proteins by recombinant DNA techniques that consisted of LTB and a viral epitope. These fusion-proteins adhere to GM1-ganglioside, the receptor of CTB and LTB on the epithelial cell membrane and are recognized by anti-epitope antibodies. Studies are in progress to evaluate the capacity of this product to induce

mucosal anti-epitope antibodies in an oral immunization regimen.

Another approach for the induction or stimulation of mucosal immune responses is the use of vector vaccines. It has already been stated that living organisms induce adequate mucosal responses when given orally. By recombinant DNA techniques it is possible to use a nonpathogenic vector for the expression and presentation of nonrelated antigenic determinants. A suitable vector is for instance an attenuated *Salmonella typhimurium* strain or an *E. coli* strain. Immunization with salmonellae expressing cloned streptococcal M protein induced protection against subsequent challenge (58). In another study *Salmonella* was used to express LTB (59). The outer membrane protein PhoE of *E. coli* has been used to express relevant antigenic determinants of foot-and-mouse disease virus (60,61).

In conclusion, the use of carrier molecules for the enhancement of mucosal immune responses is promising for future development of oral vaccines containing non-replicating antigens. Mucosal vaccines consisting of a non-pathogenic vector expressing relevant antigenic determinants, when available and considered safe enough, will especially be of great practical value.

6.6. Future research

As outlined in the introductory chapter of this thesis the induction of mucosal immunity is of great value as a first line of defence against invading pathogens. We showed that the intestine has enough immunological potential to provide this first line of defence. Further, we showed that specific immune responses in the intestine can be induced and that these responses can be enhanced by carrier proteins and suitable adjuvants. Future research must be aimed at further enhancing the intestinal immune response and at evaluating whether these responses indeed can protect the individual against infectious diseases. To this end a better understanding of the various ways of local antigen presentation will be necessary. In the near future carrier proteins will play an important role in this research, but also new technologies for the production of microparticles (62,63) that are selectively taken up by PP will contribute to the development of better mucosal vaccines. In the remote future, when more is known about the molecular biology of homing receptors and adressins on migrating lymphocytes and endothelial cells, these molecules may be used in guiding vaccine antigens to specialized lymphoid tissues after parenteral administration. This approach may lead to a new generation of vaccines in which mucosal immunization is achieved by systemic immunization.

6.7. References

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CHAPTER 7

SUMMARY

Most infectious agents enter the body via the mucosae. Therefore, expression of immunological activity at the mucosal surfaces is important as a first line of defence against invading pathogens. To that purpose each individual possesses a local, mucosal immune system, a special limb of the overall immune system. Although it has been known for many years that the mucosal tissues exert immunological activity, the magnitude and the developmental stage at various ages of this immunological compartment was not known. This resulted partly from a lack of suitable techniques to quantitate immunoglobulin-secreting cells (Ig-SC) and partly from the confusing data obtained with other techniques used to estimate the Ig production in mucosal tissues. Further, little was known about the induction of specific mucosal immune responses, especially with non-replicating (dead) antigens, and about ways to direct these responses.

The experiments described in this thesis were performed in a mouse model. The work started with the development of techniques to quantitate the Ig production in the intestine. In Chapter 3.1 a method is described that enables the quantitation of the number of Ig-SC in the intestine and in Chapter 3.2 methods are evaluated that enable the estimation of the Ig content of the intestinal lumen.

In Chapter 4.1 we demonstrate that the intestine is the major site of Ig production as more than two-third of all Ig-SC in the body, evaluated as the total number of Ig-SC in spleen, bone marrow, mesenteric lymph nodes, Peyer's patches and small intestine together, are located in the lamina propria of the small intestine. As more than 95% of the Ig-SC in the intestine secrete IgA, IgA is the predominant isotype produced in mice.

The development of the intestinal immune compartment is described in Chapters 4.2 and 4.3. In Chapter 4.2 the early development around the time of weaning was studied. In this chapter we demonstrate that the time of weaning and not the age determines the onset of development of the Ig-SC compartment in the intestine and that exogenous antigenic stimulation provided by the intestinal bacterial flora is the most important inducer. Gram-positive bacteria appeared as effective as Gram-negative bacteria for the induction of intestinal Ig-SC. After weaning at 4 weeks of age the number of intestinal Ig-SC rose exponentionally to reach a plateau value at the age of about 12 weeks. This plateau was maintained until the age of about one year. In Chapter 4.3 it is shown that in contrast to other lymphoid organs the number of Ig-SC in the intestine decreased significantly from maximum values at one year of age down to 20% of its maximum value at the age of two-and-a-half years. These results were confirmed by immunohistological examinations.

The induction of antigen-specific responses by immunization is the purpose of many vaccination schedules. In Chapter 5.1 we demonstrate that a soluble protein antigen as ovalbumin can induce an intestinal antibody response. Further we show that such a response can be quantitated by means of the techniques described in Chapters 3.1 and 3.2. In Chapter 5.2 we describe that intestinal responses can be stimulated by enhancing the antigen uptake via a carrier protein like the B subunit of cholera toxin and by adjuvant activity provided by the A subunit of the whole cholera toxin. To obtain the stimulatory effect by the B subunit it was found to be necessary to couple the B subunit covalently to the antigen, whereas the adjuvant activity of the A subunit did not depend on conjugation to the antigen.

The conclusions from the experiments described in this thesis are: (1) in mice the intestine is the major production site of Ig(A); (2) the intestinal immune compartment is fully active from weaning till the end of life, although maximum activity occurs at about one year and decreases afterwards; (3) it is possible to induce intestinal immune responses against a soluble protein antigen; and (4) intestinal responses can be enhanced by adjuvants like the A subunit of cholera toxin and by carrier proteins like the B subunit of cholera toxin. These conclusions indicate that it must be possible to use the immune compartment of the intestine to induce protective immunity at the mucosae by means of safe vaccines containing non-replicating antigens.

SAMENVATTING

De meeste infektieuze agentia dringen het lichaam binnen via de mucosae (slijmvliezen). Een goede immuniteit aan de mucosale oppervlakken is dan ook belangrijk als een eerstelijns bescherming tegen pathogenen. Het lichaam beschikt daarvoor over een lokaal funktionerend mucosaal immuunsysteem dat een speciaal onderdeel is van het totale immuunsysteem. Ofschoon het reeds lang bekend was dat mucosale weefsels immunologische activiteit vertonen, was het nog onbekend wat de betekenis is van dit immunologische compartiment, en hoe het zich ontwikkelde. Dit was mede een gevolg van het ontbreken van mogelijkheden om kwantitatief vast te stellen hoeveel immunoglobuline secernerende cellen (Ig-SC) zich in deze weefsels bevinden, en van tegenstrijdige resultaten m.b.t. het gehalte aan Ig in het darmlumen. Tevens was weinig bekend over het induceren van specifieke immuunresponsen aan de mucosae, vooral als dit moest gebeuren met dode, niet-replicerende antigenen. Ook over mogelijke methoden om de responsen aan de mucosae te manipuleren wist men nog weinig.

Het onderzoek dat in dit proefschrift wordt beschreven wil meer inzicht geven in deze aspekten. De experimenten werden alle uitgevoerd in een muizemodel. Allereerst werden technieken ontwikkeld om kwantitatief de Ig productie in de darm te meten. In hoofstuk 3.1 wordt een methode beschreven waarmee het aantal Ig-SC in de darmwand bepaald kan worden. In hoofstuk 3.2 worden twee technieken vergeleken waarmee het Ig-gehalte in het darmlumen bepaald kan worden.

In hoofdstuk 4.1 wordt aangetoond dat de darm twee-derde van alle Ig-SC in het lichaam bevat. Dit werd berekend door het aantal Ig-SC in de milt, het beenmerg, de mesenteriale lymfeklieren, de platen van Peyer en de dunne darm te bepalen. De darm is dus de belangrijkste produktieplaats van Ig. Daar bijna alle Ig-SC in de darm IgA secerneren betekent dit dat IgA het meest geproduceerde isotype is in de muis.

De ontwikkeling van het immunologische compartiment van de darm wordt beschreven in de hoodstukken 4.2 en 4.3. De ontwikkeling rond het moment van spenen wordt beschreven in hoofdstuk 4.2. Aangetoond werd dat het moment van spenen en niet de leeftijd de start van de ontwikkeling van het compartiment Ig-SC in de darm bepaalt en dat antigene belasting van buiten het lichaam, vooral die door de bacteriële darmflora, verantwoordelijk is voor die ontwikkeling. De aard van de bacterie flora was daarbij minder van belang. Na het spenen stijgt het aantal Ig-SC in de darm exponentieel tot een plateauwaarde wordt bereikt wanneer de dieren ongeveer 12 weken oud zijn. Dit plateau blijft gehandhaafd tot een leeftijd van 1 jaar. Daarna loopt het aantal terug tot 20% van zijn maximale waarde op een leeftijd van tweeenhalf jaar. Deze laatste resultaten werden bevestigd door immunohistologisch onderzoek. Zowel het moment waarop de ontwikkeling van het immuuncompartiment start als de snelheid waarmee dat gebeurt en

het verloop van de "achtergrond" Ig productie gedurende de leeftijd maakt dat de darm een uniek orgaan is. De andere lymfoïde organen vertonen heel andere ontwikkelingspatronen.

Vaccinaties zijn erop gericht beschermende antigeen-specifieke responsen te induceren. In hoofdstuk 5.1 wordt aangetoond dat in de darm ook tegen een oplosbaar, niet-replicerend antigeen als ei-albumine antilichaamvorming kan worden geinduceerd. Tevens bleek het mogelijk deze antilichaamvorming te kwantificeren m.b.v. de technieken die worden beschreven in de hoofdstukken 3.1 en 3.2. In hoofdstuk 5.2 wordt aangetoond dat deze responsen kunnen worden gestimuleerd door gebruik te maken van "drager-eiwitten" zoals de B subunit van het cholera toxine of van de adjuvans activiteit van de A subunit van het cholera toxine. Voor het stimulerende effect van de B subunit bleek het essentieel om het te koppelen aan het antigeen, terwijl de adjuvans aktiviteit van de A subunit niet afhing van koppeling aan het antigeen.

De conclusies van de experimenten die in dit proefschrift zijn beschreven zijn: (1) in de muis is de darm de belangrijkste produktieplaats van Ig(A) (2) het immuuncompartiment van de darm is vanaf het moment van spenen gedurende het gehele leven aktief, hoewel de aktiviteit afneemt vanaf de leeftijd van een jaar; (3) het is mogelijk immuunresponsen in de darm te induceren tegen een oplosbaar, inactief eiwit antigeen; en (4) deze immuunresponsen in de darm kunnen worden versterkt d.m.v. adjuvantia zoals de A subunit van het cholera toxine, en door drager-eiwitten zoals de B subunit van het cholera toxine. Deze conclusies tonen aan dat het in principe mogelijk moet zijn het immuuncompartiment van de darm te gebruiken om beschermende immuniteit aan de mucosae te induceren middels veilige vaccins die niet-replicerende antigenen bevatten.

ABBREVIATIONS

ASC	Antibody-secreting cell
BALT	Bronchus-associated lymphoid tissue
B-cell	Bone marrow derived lymphocyte
BM	Bone marrow
BSS	Balanced salt solution
CMF	Calcium- and magnesium-free balanced salt solution
Con A	Concanavalin A
CRF	Colonization resistance factor
CT	Cholera toxin
CTB	Cholera toxin B subunit
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ELISA	Enzyme linked immunosorbent assay
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
GALT	Gut-associated lypmphoid tissue
GF	Germ-free
HEV	High endothelial venule
H2-complex	Major histocompatibility complex of the mouse
i.d.	Intraduodenal
IEL	Intraepithelial lymphocyte
Ig	Immunoglobulin
Ig-SC	Immunoglobulin-secreting cell
IL	Interleukin
i.p.	Intraperitoneal
i.v.	Intravenous
LTB	*E. Coli* heat-labile toxin B subunit
LPL	Lamina propria lymphocyte
LPS	Lipopolysaccharide
M-cell	Membraneous cell
MLN	Mesenteric lymph node
O(V)A	Ovalbumin
PBS	Phosphate buffered saline
PFC	Plaque forming cell
PLN	Peripheral lymph node
PMSF	Phenylmethylsulfonylfluoride
pOVA	Polymerized Ovalbumin
PP	Peyer's Patches
RNA	Ribonucleic acid
SI	Small intestine
S-IgA	Secretory immunoglobulin A
SPF	Specific pathogen free
SRBC	Sheep red blood cell
T-cell	Thymus derived lymphocyte

DANKWOORD

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CURRICULUM VITAE

De schrijver van dit proefschrift is op 15 september 1953 te Zutphen geboren. Na het behalen van het diploma HBS-B in 1970 studeerde hij scheikunde aan de Rijksuniversiteit te Groningen. In januari 1979 behaalde hij het doctoraal examen met biochemie als hoofdvak bij Prof. Dr. M. Gruber (doctoraal onderzoek naar immuunglobulines op het membraan van konijne lymphocyten onder leiding van Dr. H.G. Seyen) en allergologie als bijvak (onderzoek naar karakterisering van antigenen van *Aspergillus fumigatus* en de ontwikkeling van een IgE-ELISA onder leiding van Dr. H.F. Kauffman). Tevens werd een onderwijsaantekening Scheikunde verkregen. Sinds april 1979 is hij verbonden aan de afdeling Immunologie van het Centraal Diergeneeskundig Instituut te Lelystad.

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