SERUM IMMUNOGLOBULIN LEVELS AND IMMUNOGLOBULIN HETEROGENEITY IN THE MOUSE

CONTROLLING FACTORS, WITH EMPHASIS ON THE INFLUENCE OF THE THYMUS

PROEFSCHRIFT

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CONTENTS

LIST OF ABBREVIATIONS

A5A	idiotype of antistreptococcal antibodies
Ars	azophenylarsonate
ATS	anti thymocyte serum
ATx	adult thymectomy
BGG	bovine gamma globulin
C	complement
C_H	constant region of the heavy chain
C-Ig cell	cytoplasmic immunoglobulin-containing cell
C_L	constant region of the light chain
ConA	concanavalin A
CRID	cross reactive idiotype of anti-azophenyl-arsonate antibodies
DNP	dinitrophenyl
Fab	antigen binding fragment of immunoglobulin molecule
Fc	crystalizable fragment of immunoglobulin molecule
GAT	linear copolymer of the L-amino acids L-glutamic acid, L-alanine and L-tyrosine
GF	germ free
GMuLV	gross murine leukemia virus
GT	linear copolymer of the L-amino acids L-glutamic acid and L-tyrosine
H-chain	heavy chain
HGG	human gamma globulin
H-Ig	homogeneous immunoglobulin
HRBC	horse red blood cells
Ig	immunoglobulin
IP	idiopathic paraproteinaemia
J-chain	joining chain
K cell	killer cell
KLH	keyhole limpet hemocyanine

L-chain	light chain
LPS	lipopolysaccharide
MHC	major histocompatibility complex
MLC	mixed lymphocyte culture
MOPC	mineral oil-induced plasmacytoma
NMS	normal mouse serum
NTx	neonatal thymectomy
PC	phosphorylcholine
PEG	polyethylene glycol1
SC	secretory component
SCID	severe combined immunodeficiency
SPF	specific pathogen free
SRBC	sheep red blood cells
SSSIII	pneumococcal polysaccharide type III
STx	sham thymectomy
T-15	major idiotype of anti-phosphorylcholine antibodies in BALB/c mice
TEPC	tetramethylpentadecane-evoked plasmacytoma
(T,G)AL	branched copolymer of the L-amino acids L-tyrosine, L-glutamine, L-alanine and L-lysine
TNP	trinitrophenyl
TRF	T cell replacing factor
V	variable region
V_H	variable region of the heavy chain
V_L	variable region of the light chain

1. *INTRODUCTION*

1.1. General remarks

Vertebrates can mount specific and nonspecific reactions to potentially pathogenic agents such as viruses, bacteria and fungi. Phagocytic cells can move to the site of infiltration to engulf and destroy such foreign invaders in a nonspecific way. In addition to this mechanism, vertebrates have a more specifically operating protective system, the immune system. Immune protection is provided by a dual system consisting of two basic defense mechanisms: the cellular and humoral immune systems. The cellular immune response is particularly involved in reactions against fungi, parasites, intracellular infections and foreign tissue (transplant rejection). The humoral immune response is primarily effective in the extracellular phases of infections with bacteria and viruses. Further, phenomena such as immediate hypersensitivity (e.g., hay fever, asthma) and Arthus reactions are based on this defense mechanism. Humoral immune responses are mediated by antibodies which are released into the blood by plasma cells found within the bone marrow and the lymphoid organs. Cellular immune reactions are directly mediated by cells of the lymphoid system and are transferable only by cells.

The dichotomy of the immune system is based upon two major subpopulations of cells which are morphologically indistinguishable: T and B lymphocytes. T cells mature in the thymus and mediate cellular immune responses. B cells differentiate mostly in the bone marrow. Their progeny produce the antibodies. Both T and B cells can recognize foreign entities of molecules that are not normal constituents of the organism itself. Such entities are called antigens. Antigen-activated T cells can directly eliminate antigenic cells in a cytolytic reaction, enhance engulfment of the antigen by macrophages or help antigen reactive B cells to mature into antibody-secreting plasma cells. These antibodies can combine with the antigen and this facilitates the clearance of the antigen from the body by cytolysis and/or phagocytosis. The process of antigen clearance via humoral immunity largely depends on the efficiency of the antibody in recognizing the antigen and extent to which other specific or nonspecific defense mechanisms can be recruited (see Chapter 2, section 2.4.).

Besides its specificity, the immune system has another important property which is known as immunological memory. This phenomenon is the ability of T and B cells to recall a pre-

vious antigenic experience. After the first antigenic stimulation, the immune system produces effector cells which are directly involved in the elimination of the antigen. This is called the primary immune response. However, the progeny of the activated cells include not only effector cells but also so-called memory cells. These memory cells retain the capacity to produce both effector and memory cells upon restimulation by the original antigen. After a renewed antigen contact, the specific immune response is faster and of greater magnitude than the primary response. Hence, the antigen will be removed more rapidly and more efficiently, which reduces the chance of harmful consequences of this contact. Such responses are called secondary or anamnestic immune responses.

A state of humoral immunity to a certain antigen is characterized by the presence of sufficient antibodies in the blood to eliminate the antigen. This antibody activity is mediated by globular proteins in the serum. Since these proteins provide immunity against the antigen, they are called immunoglobulins (Ig's).

The majority of the Ig molecules produced are released into the blood stream. Therefore, it is generally assumed that the Ig levels in the serum reflect the overall activity of the humoral immune system. Under normal conditions, the total activity of all B cell clones together will yield a heterogeneous spectrum of serum Ig molecules. In disease, however, such a heterogeneous serum Ig pattern can change. Some disorders of the immune system can lead to imbalanced activity of the B cell compartment and this can lead to excessively high (hyperglobulinaemic) or low (hypoglobulinaemic) serum Ig levels. Imbalanced B cell activity can also lead to a restriction in the heterogeneity of serum Ig's and the appearance of homogeneous Ig components or paraproteins. The occurrence of Ig components in the serum can be temporary or permanent, depending on the degree and nature of the disorder in the Ig synthesizing apparatus. Excessive production of such components is often the result of malignant B cell transformation.

1.2. Purpose of the investigation

The experiments described in this thesis were performed in attempts to obtain quantitative and qualitative data on the overall activity of the humoral immune system of mice, as reflected by the concentration and heterogeneity of the various Ig classes and subclasses in the blood. For most antigens, the

humoral immune response is regulated by the thymic dependent limb of the immune system. Therefore, special attention was paid to the influence of the thymus on the concentration and heterogeneity of serum Ig's.

2. *STRUCTURE AND FUNCTION OF MURINE IMMUNOGLOBULINS*

2.1. Humoral immunity and antigen elimination

The association between antibody activity and serum globulins was made by Breinl and Haurowitz in 1930 by showing that the bulk of a precipitate formed by horse hemoglobin and specific rabbit antibodies consisted of a protein similar to normal serum globulin (Breinl and Haurowitz, 1930). Subsequent analysis of serum globulins by electrophoresis revealed that antibodies belong to a particular group of globulins. Of the three principle separable globulins of vertebrate sera (α-, β- and γ-globulins), the γ-globulins were observed to be present in increased amounts in the sera of hyperimmunized animals (Tiselius and Kabat, 1939). With the development of the immunoelectrophoresis technique, it became clear that at least some of the β-globulins can also exhibit antibody activity and are antigenically similar to γ-globulins. Hence, it was postulated that all proteins that can behave as antibodies or that have antigenic determinants in common with antibody molecules constitute a single family of proteins, which are now called immunoglobulins (Ig's) (Heremans, 1959).

Although most antibody activity can be detected in the serum Ig fraction, it was found that serum Ig levels are not necessarily a measure for the capacity to produce specific antibodies upon immunization. Thus, mice with comparable serum Ig levels can show considerable differences in the capacity to produce specific antibodies after immunization (Amsbaugh et al., 1974). It is also known that, in addition to the formation of specific antibody, immunization can lead to synthesis of Ig's lacking the capacity to bind the specific antigen (Urbain-Van Santen, 1970; De Vos-Cloentens et al., 1971; Rosenberg and Chiller, 1979). Since both specific and nonspecific B cell stimulating factors have been described (see Chapter 4, section 4.4.), it is quite possible that both types of factors are released by specifically activated T cells. The release of nonspecific factors stimulating the entire repertoire of B cells can explain why the increase in the serum Ig levels after immunization is usually greater than the amount of specific antibodies produced (Moticka, 1974). Alternatively, nonspecific Ig production might be due to auto-anti-antibodies, since each antibody molecule is itself a potential immunogen (Najjar, 1963). Such auto-anti-antibody formation has been recently observed in several mouse strains reared under low pathogenic conditions (Van Snick and Masson, 1980).

Molecular and cellular antigen-antibody complexes have been used in studying biological activity of Ig's. Aggregated Ig's have also been used for such studies, since such complexes possess many of the properties of antigen-antibody complexes. Antigen-antibody complexes as well as aggregated Ig complexes can activate the complement enzymes which are normal constituents of the globulins in the serum. Via a cascade of proteolytic cleavage and protein-binding reactions, activated complement components can cause cell lysis (Mayer, 1973). The *in vitro* utilization of complement enzymes in antigen elimination is termed complement fixation. Ig's can also play an important role in the removal of antigens by phagocytic cells. The ingestion of antigenic particles by phagocytes increases when these particles are coated with antibodies. This process of preparing foreign particles for phagocytic ingestion is called opsonization and the antibodies mediating this process are called opsonins (Unanue, 1972). Besides elimination by complement and/or phagocytes, antigenic cells can be attacked by several antibody-dependent cellular cytotoxic mechanisms. A detailed review of the biological activities of Ig's will be given in section 2.4., since the various mechanisms by which antibody molecules eliminate antigens are closely related to their structure.

2.2. Structure and classification of immunoglobulins

Five classes of serum Ig's have been recognized on the basis of their physicochemical and antigenic properties: IgG, IgM, IgA, IgD and IgE. In most normal and hyperimmune individuals, the Ig fraction in the serum consists mainly of IgG. Accordingly, IgG has been studied most extensively. One of the first approaches to unravel the antibody structure of human IgG was to determine whether the molecules could be separated into subunits or fragments that still had the capacity to bind antigen. Two methods are most frequently used for this purpose, namely, proteolytic cleavage and cleavage by reduction of the interchain disulfide bonds.

Application of proteolytic enzymes such as papain and pepsin achieved a limited cleavage of the IgG molecules into fragments. Papain digestion revealed three fragments (Porter, 1959). Two of these were still capable of binding antigen and they were therefore designated as Fab ("antigen binding fragment"). The third fragment showed no binding capacity. In contrast to intact antibody molecules, this fragment was crystalizable. Hence it was called Fc ("crystalizable fragment"). Of the total digest

from papain, about 2/3 is Fab and 1/3 Fc. Comparison of the molecular weights of the respective fragments with the native antibody molecule has led to the conclusion that an intact divalent IgG antibody molecule consists of two univalent Fab fragments joined together by one Fc fragment. Pepsin has been used in a similar manner (Nisonoff et al., 1960). This proteolytic enzyme cleaves the antibody in such a way that the Fc part is broken down into several smaller fragments. The other part can still interact with antigen in a divalent way. Therefore, this fragment is designated as $F(ab')_2$.

The second approach to unravel the antibody structure is based upon cleavage of Ig molecules into subunits of polypeptide chains by reduction of the interchain disulfide bonds in a dissociating solvent (Edelman, 1959). This approach revealed that an IgG antibody molecule consists of two different chains of polypeptides, namely, heavy (H; relatively high molecular weight) and light (L; relatively low molecular weight) chains (Edelman and Poulik, 1961). Correlation of the data of the two approaches has led to the conclusion that IgG is a symmetrical four-chain antibody molecule (Fleischman et al., 1962). It was proposed that the basic unit of each antibody molecule consists of two identical H-chains and two identical L-chains which are linked together by interchain disulfide bonds and noncovalent bonds. The Fab fragment is composed of one L-chain and the amino-terminal-half of the H-chain (the latter is called the Fd fragment). The Fc fragment consists of the carboxy-terminal-halves of the H-chains. The $F(ab')_2$ fragment consists of two L-chains and two Fd fragments (Edelman and Gally, 1964).

In this model, the antigen-binding sites are localized on the amino-terminal portions of the H- and L-chains. Structural analysis of individual antibody molecules revealed that the amino-terminal half of the L-chains as well as a comparable portion of the amino-terminal part of the H-chains substantially differ in amino acid sequence even when the antibodies belong to the same Ig class or subclass. Therefore, these regions are called variable (V) regions. A comparative amino acid sequence analysis of V regions of both the H- and L-chains (V_H and V_L, respectively) of different antibodies revealed a further distinction into hypervariable (Wu and Kabat, 1970) and constant (framework) regions (Poljak et al., 1973). The hypervariable regions of the antibody molecules are particularly involved in antigen recognition (Amzel et al., 1974).

The marked heterogeneity of the amino-terminal portions of H- and L-chains contrasts with the relative invariability of the

amino acid sequence of the carboxyl-terminal parts of both chains (Cunningham et al., 1971). Consequently, these relatively constant parts of the H- and L-chains have been termed C_H and C_L, respectively. According to the model of Fleischman and coworkers (1962), the V_H region has approximately the same length as the V_L region. The C_H region of IgG is about 3 times as long as the C_L part of the Ig molecule.

Data from amino acid sequence analyses have shown that arrangements of intrachain disulfide bonds in homologous regions of both H- and L-chains contribute to certain relatively independent domains in the Ig molecule (Edelman and Gall, 1969; Edelman, 1973). L-chains can be folded in two domains, one in the V_L region and one in the C_L region. H-chains have one domain in their V_H part and, depending upon the Ig class, 3 or 4 domains in the C_H part, namely, C_H1, C_H2 and C_H3 for γ and α chains and an additional domain, C_H4, for the μ chain (Nisonoff et al., 1975; Cathou, 1978). A simplified scheme of the composition of an Ig molecule is given in Figure 1.

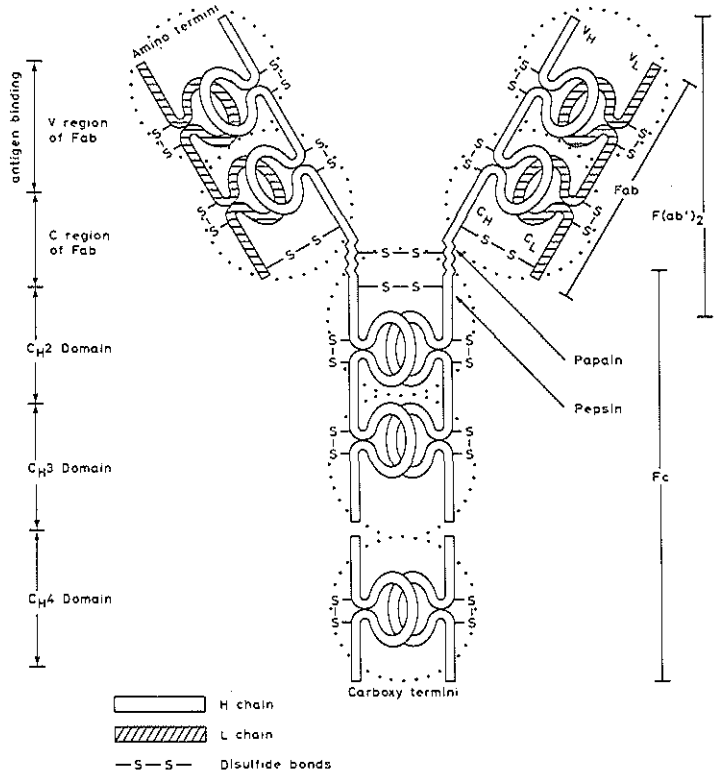

Figure 1. Scheme of the composition of an immunoglobulin molecule. The C_H4 domain only occurs in IgM molecules. The sites of cleavage by the proteolytic enzymes papain and pepsin as well as the corresponding fragments (Fab, Fc and $F(ab')_2$, respectively) are indicated (modified from Winkelhake, 1978).

For the structural analysis and classification of the various Ig's of the mouse, advantage has been taken from the occurrence of Ig-producing tumors, since their generally homogeneous products ("myeloma" proteins or paraproteins) can be easily isolated in large quantities from serum or other body fluids (Potter, 1972). The most widely used tumors for this purpose are plasmacytomas, which can be readily induced by intraperitoneal mineral oil injection into BALB/c and NZB mice.

Serological characterization of the paraproteins as well as their size, electrophoretic mobility, proteolytic peptide maps, occurrence and content of carbohydrates and amino acid sequences have led to a subdivision of Ig's. On the basis of structural analysis of Bence Jones proteins, a first division of human L-chains into two groups was made in 1956 by Korngold and Lipari and by Burtin and coworkers. According to the nomenclature of the World Health Organization (1972) L-chains are now divided into two groups: λ and κ. Characterization of the C_L regions of various murine myeloma proteins enabled for mice a subdivision into κ L-chains (Gray et al., 1967) and two variants of λ L-chains, namely, λ_1 (Appella, 1971) and λ_2 (Schulenburg et al., 1971). Serological examinations of normal mouse Ig show that 97% contain κ-chains (McIntire and Rouse, 1970). κ-chains show an extensive variation in their amino acid sequence (Gray et al., 1967; Hood et al., 1970; McKean et al., 1973). When compared with κ and λ_2, λ_1 polypeptides have a conservative amino acid sequence (Weigert et al., 1970).

Based on physicochemical and antigenic properties of the C_H part, murine Ig's have been divided into the above-mentioned 5 classes: IgM, IgA, IgG, IgD and IgE. In accordance with the nomenclature of the World Health Organization (1972), the H-chain of each Ig class is designated by the corresponding Greek letter, i.e., μ, α, γ, δ and ε, respectively. These are called H-chain *isotypes*. Characteristic differences in the Fc fragment are the basis for a further division of murine IgG into the IgG1, IgG2a, IgG2b, IgG3 subclasses (Fahey et al., 1964; Grey et al., 1971; Prouvost-Danon et al., 1972; Melcher et al., 1974; Abney and Parkhouse, 1974).

Native serum IgM molecules are polymers composed of 5 identical subunits which are cyclicly arranged around a central core (Parkhouse et al., 1970). The subunits are held together by disulfide bridges between the Fc regions and a polypeptide called the joining piece or J-chain (Cathou, 1978). Each subunit has a four-chain structure similar to that of IgG.

Monomeric IgM has been detected as a major constituent of the B lymphocyte membrane (Vitetta et al., 1971; Marchalonis et al., 1972). This IgM consists of two H- and two L-chians (Abney and Parkhouse, 1974; Melcher and Uhr, 1976). Aside from its monomeric form, membrane-associated IgM differs from its serum counterpart by having an extra hydrophobic piece at the carboxyl-terminal end of the molecule (Vitetta and Uhr, 1977).

A distinctive feature of IgA is its occurrence in different molecular (polymeric) forms. As for pentameric IgM, polymeric forms of murine IgA have been demonstrated to contain a J-chain (Rosenstein and Jackson, 1973). In the mouse, most of the serum IgA is dimeric (Vaerman, 1973). In contrast to serum, the external secretions yield a more heterogeneous spectrum of IgA molecules, because, despite a predominance of dimeric IgA, proportionally more IgA occurs in higher polymeric forms (Nash et al., 1970). In addition, another polypeptide chain (unrelated to J-chain) called secretory component (SC) is bound to secretory IgA via noncovalent and disulfide bonds. By binding both a J-chain and a unit of SC, the IgA molecule undergoes characteristic changes in electrophoretic mobility, molecular weight and antigenicity (Vaerman, 1973). With respect to the overall structure, the monomeric unit of IgA closely resembles that of IgG. For instance, electron micrographs indicate that monomeric IgG and IgA have the same shape and measurements of Fab and Fc fragments revealed comparable sizes for both isotypes (Munn et al., 1971). Additionally, crystallographic analyses revealed a close similarity between the tertiary and quaternary structures of Fab fragments of murine IgA and human IgG molecules (Segal et al., 1974). No serologically defined subclass specificities have been described for murine IgA. However, some differences in covalent and noncovalent bonds between H- and L-chains have been noted in the basic structure of IgA myeloma proteins from BALB/c and NZB mice, which resemble the two IgA subclasses (IgA1 and IgA2, respectively) in man (Abel and Grey, 1968; Potter, 1972; Heremans, 1974). Recently, more evidence for possible subclasses of murine IgA (IgA1) has been presented (Mushinski et al., 1976; Robinson et al., 1977).

IgG remains exclusively in the monomeric form. Of the four subclasses of IgG (IgG1, IgG2a, IgG2b and IgG3), IgG2a and IgG2b are most closely related serologically (Fahey et al., 1964). Comparison of the primary structure of IgG1, IgG2a and IgG2b myeloma proteins also showed many homologies between IgG2a and IgG2b (De Preval et al., 1970). In this respect, IgG1 was found to be far less similar to each of the two IgG2 subclasses (Svasti and Milstein, 1970). A detailed comparison of the four

IgG subclasses, however, has not yet been described. Complete amino acid sequences are available only from the γ1 and the γ2a chains of myeloma proteins (Beale and Feinstein, 1976; Fougereau et al., 1976; Adetugbo et al., 1977).

Murine IgD was first discovered as a constituent of the B lymphocyte membrane. It was described as an Ig molecule consisting of two disulfide linked H- and L-chains which can be precipitated from lysates of B lymphocytes with anti-κ-chain serum but not with anti-μ, anti-γ or anti-α sera (Melcher et al., 1974; Abney and Parkhouse, 1974; Melcher and Uhr, 1976). Only recently have minute amounts of IgD been detected in murine serum (Finkelman et al., 1979; Bargelessi et al., 1979). No myelomas of the IgD class are known in the mouse (Goding, 1979), although homogeneous Ig's of the IgD class have been found in sera of aged animals of the C57BL strain (Radl et al, 1980a). From estimations of the molecular weights of the L- and H-chains, a total molecular weight of membrane-associated or serum IgD molecules can be calculated which is intermediate between that of IgM (monomer) and IgG (Melcher and Uhr, 1976; Vitetta and Uhr, 1976; Radl et al., 1980a).

Murine IgE has been immunochemically identified by Prouvost-Danon (1972). Since the IgE concentration in the serum is too small (even in heavily immunized animals) to isolate enough material for structural analysis and murine IgE myelomas have not been observed (Potter, 1972), no information is available on the structure of murine IgE. However, a hybridoma cell line producing monoclonal mouse IgE has been recently described (Böttcher, 1978); this will enable a structural analysis of this Ig.

The only structural data on rodent IgE presently available are derived from IgE myeloma proteins found in rats (Bazin and Beckers, 1976). Proteolytic cleavage studies suggest that the Fd region of rat IgE contains 3 domains, whereas the Fc region is composed of two domains. The molecular weight of native IgE is comparable with that of IgG. Therefore, it is likely that IgE in rats is monomeric and that the molecules consist of two H- and two L-chains (Ellerson et al., 1978).

Isotypes, allotypes and idiotypes

Aside from the structural differences within the C_H- and C_L-parts of Ig molecules associated with class, subclass and L-chain type, a further distinction can be made according to serologically defined markers which are specified by structu-

ral genes. The genes coding for the constant parts of the H- and L-chains have been shown to occur in various allelic forms. Each allelic alternative is designated as an *allotype* (Potter and Lieberman, 1967; Herzenberg et al., 1968). For describing the polymorphism of murine allotypes, two nomenclature systems which were proposed by Herzenberg and by Potter and Liebermann, respectively, are used. Only recently have these two systems been incorporated into a generally accepted third proposal which satisfactorily describes the Ig allotypes of mice and the allelic forms of the genes coding for them (Greene, 1979). In the mouse, most allotypic specificities have been associated with the C_H2 and C_H3 domains, although some allotypes are defined by the C_H1 domain (Spring and Nisonoff, 1974; Lieberman, 1978). So far, 3 alleles for the C_H genes of the IgM locus, 12 alleles for IgG2a, 6 alleles for IgG2b, 2 alleles for IgG1, 5 alleles for IgA and 2 alleles for IgD have been found (Lieberman, 1978). Up to now, no different allotypes have been described for IgG3 and IgE (Goding, 1979).

As far as the L-chains are concerned, no allotypic determinants have been found on κ-chains. On λ_1 L-chains, one allotypic marker has been identified on the constant part which is common for most, but not for all, inbred mouse strains (Weigert and Potter, 1977).

Isotype and allotype heterogeneity do not contribute to the repertoire of antibody specificities of an individual. In fact, the heterogeneity of antibody specificities is the consequence of the repertoire of V regions that can be expressed. In other words, the unique amino acid sequence of the V region of each antibody molecule contains antigenic determinants which are defined by structural genes coding for V_H and V_L regions. These antigenic determinants have been called *idiotypes* (Oudin, 1966). At present, idiotypic markers are used for defining groups of Ig molecules with a related V-region specificity. Comparison of idiotypes of various antigen binding murine myeloma proteins revealed subsets of *v*-genes whose products are closely related in amino acid sequences (Hood et al., 1976). For instance, the phosphorylcholine (PC) binding properties of various BALB/c myeloma proteins usually correlate with one particular amino acid sequence of the V_H region. However, according to some characteristic differences in primary sequences, their V_L regions (all of κ type) fall into 3 subsets (Potter, 1972; Hood et al., 1976). In this way, several closely related V regions have been described for certain Ig families (i.e., Ig molecules all binding one particular antigen) in the mouse.

2.3. *Immunoglobulin class distribution of antibody responses*

The distribution of antibody activity over the various Ig classes and subclasses in adult mice depends upon the distribution of the antigen over the body, i.e., the route of administration determines whether the response will follow a systemic or a localized (mucosal) pattern. Systemic antibody production occurs when the antigen enters the body parenterally (e.g., subcutaneously, intravenously or intraperitoneally). In such a case, the antigen is transported through the body via the blood or lymph. Primary parenteral immunization initially induces the synthesis of IgM, followed later by IgG and eventually IgA antibody production (Uhr and Finkelstein, 1967; Andersson and Dresser, 1972). The secondary or anamnestic response consists primarily of IgG antibodies. These antibodies cause a higher avidity in antigen binding (Sarvas and Mäkelä, 1970).

The situation is different when the immunization occurs via a mucosal surface, particularly via the digestive tract. The antibody appearing in the serum is then largely composed of IgA and to a lesser extent IgM and IgG (Crabbé et al., 1969; Nash et al., 1969; Heremans and Bazin, 1971). After renewed enteric immunization with the same antigen, the kinetics is different from that seen after anamnestic systemic immunization. Primary enteric IgA antibody responses are relatively short lasting and are followed by a long refractory period. Furthermore, the kinetics of the secondary local IgA response do not differ essentially from the primary response (André et al., 1973). Ebersole (1979b) has determined the differential response of rats to systemic and local immunization with the antigen dinitrophenyl-bovine gammaglobulin (DNP-BGG). A single injection of this antigen via the hind footpads induced the production of serum antibodies consisting mainly of IgM, IgG and to a small extent IgA, whereas only low levels of IgG antibodies could be detected in the saliva. On the other hand, local immunization with the same antigen in the salivary gland vicinity elicited mainly IgA antibodies in the saliva.

Besides the route of antigen administration and time after immunization, the relative contribution of each class and subclass to the entire pool of circulating antibody is dependent upon the type and form of the antigen (Nossal et al., 1964; Torrigiani and Roitt, 1965; Kuhara et al., 1978), whether or not an adjuvant is used (White et al., 1963), the type of adjuvant (Torrigiani, 1971), the genetic background of the responder animal (Barth et al., 1965; Minga et al., 1975) and age of the responder (Makinodan and Peterson, 1966; Makinodan et al.,1976).

2.4. Effector functions of immunoglobulins

Apart from antigen binding, virtually all biological effector functions of Ig's reside in the constant parts of the H- and L-chains. The activation of the complement system and the cellular binding of Ig's (Ig cytotropism) are reviewed below.

2.4.1. Activation of the complement system

One of the most important effector functions of Ig's is the activation of the complement system, since complement represents an effective mechanism to eliminate foreign cells by cytolysis. Antibody plays an important role in this mechanism by identifying the foreign cells and activating and fixing the complement on the surface of the target cell.

The complement system consists of 9 components which cause cytolysis via a cascade reaction of proteolytic cleavages. The reaction must go to completion before lysis occurs. In addition to lysis, some components are also involved in other important activities of the inflammatory response (c.f. figure 2). The split products of the C3 and C5 components (C3a and C5a, respectively) evoke the release of vasoactive amines such as histamine. This results in local blood vessel dilatation and increased permeability of the blood capillaries. C3b is particularly involved in immune adherence and can enhance phagocytosis by promoting opsonization (see 2.4.2.). Furthermore, the fragment C5a and complexes of C5b, C6 and C7 have chemotactic properties for leukocytes (Mayer, 1973; Osler, 1976).

The lytic action of the complement system can occur via two pathways: the classical and the alternative (properdin). The classical pathway includes all 9 components, while only C3 and C5-C9 are involved in the alternative pathway (c.f. figure 2). It has been proposed that the alternative pathway may be used for antigen elimination when sufficient quantities of specific antibody are not available for activation of the classical pathway (Mayer, 1973). Activation of the classical pathway involves binding of the C1 component to antigen-antibody complexes or cross-linked Ig's (Augener et al., 1971).

C1 fixation by murine Ig's has been reported by Grey et al. (1971) for IgG2. IgG1, IgG3 and IgA were ineffective. In addition, IgM and IgG2a have been found to have the capacity of activating the classical pathway (Spiegelberg, 1974).

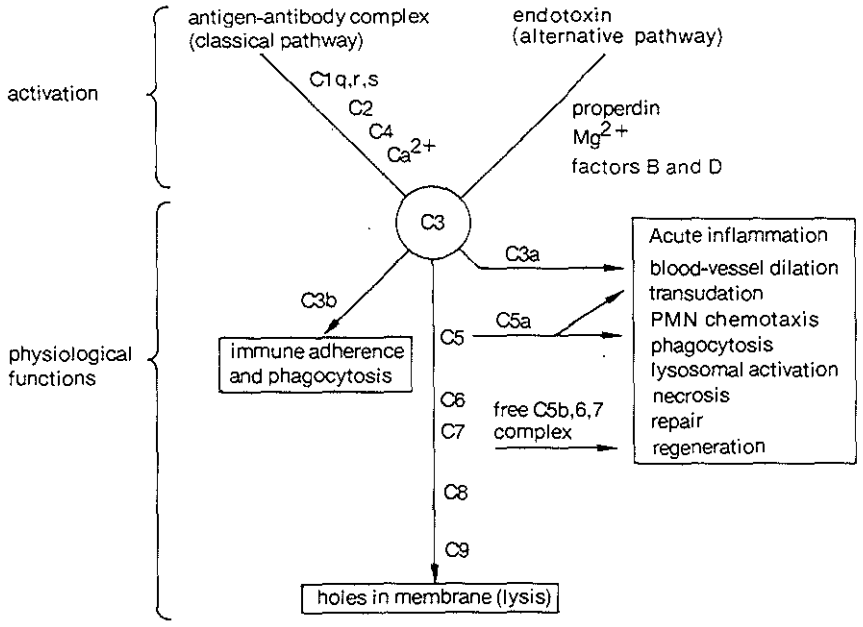

Figure 2. Activation pathways and physiological functions of complement components (From Hood et al., 1978).

Activation of the alternative pathway has been reported for IgG1, IgG2 and IgA (Lambert et al., 1973; Klaus, 1979). The latter report also discusses that autologous complement can not be activated by murine IgM hybridoma antibodies. In contrast, in the presence of heterologous (guinea pig) complement, these hybridoma antibodies do induce lysis of antigen-coated erythrocytes (Klaus, 1979). For IgM and IgG2a, the molecular structures responsible for C1 binding have been shown to reside in the C_H2 (Kehou and Fougereau, 1969) and the C_H4 (Spiegelberg, 1974) domain.

With respect to the humoral immune response, there are indications that the C3 component is required for selective trapping of antigen-antibody complexes in the follicles of the various lymphoid organs (Papamichail et al., 1975; Embling et al., 1978). Follicle-trapped immune complexes can have a prolonged half-life (Tew and Mandel, 1979). The occurrence of these complexes in the follicles is correlated with the generation of an effective secondary humoral immune response (Klaus and Humprey, 1977). It has been proposed that the C3-antigen-antibody complexes form an effective antigen specific B cell trap (Ponzio et al., 1977). Their efficiency depends on the Ig

class of the antibody which has been complexed (Klaus, 1979). It appears that antibodies of the IgG1, IgG2 and IgA classes are active in the process of B cell recruitment, IgG2 being the most effective. IgM immune complexes were shown not to localize the lymphoid follicles (Klaus, 1979).

2.4.2. Immunoglobulin cytotropism

2.4.2.1. Cytophilic antibody binding to phagocytes

Ig's can interfere with other elimination systems by virtue of their being recognized by Fc receptors on cells. In this way, Ig's can serve as mediators for antigen elimination by phagocytes (macrophages, polymorphonuclear leukocytes).

As far as phagocytes are concerned, two mechanisms of opsonization are possible. One concerns the classical nature of cytophilic Ig, in that it is simply mediated through a receptor for the Fc portion of the Ig involved. The other mechanism is complement dependent and operates through a receptor for the C3b factor. When soluble Ig or immune complexes are bound to phagocytes, this binding stimulates the selective release of lysosomal enzymes (Cardella et al., 1974), the expression of cell-mediated cytotoxic effects (Haskill et al., 1976) and the ingestion of particulate materials (Michl et al., 1976; Silverstein et al., 1977).

With respect to the binding of IgG to macrophages or macrophage-like cell lines, there is evidence for the occurrence of more than one type of Fc receptor. These receptors are more or less Ig subclass specific (Haeffner-Cavaillon et al., 1979). On mouse macrophages, there are at least two of such Fc receptors: one which can bind IgG2a and a second which can bind IgG2b, and probably also IgG1 (Walker, 1976; Heusser et al., 1977; Unkeless, 1977; Diamond et al., 1978).

Investigation of the binding site of mouse IgG1, IgG2a and IgG2b myeloma proteins to homologous macrophages revealed that the C_H3 domain accounts for the cytophylic properties of these IgG subclasses (Dissanayake and Hay, 1975). However, in other studies using IgG2b myeloma proteins with H-chain deletions, it has been shown that the mouse macrophage Fc receptor for IgG2b is located mainly in the C_H2 domain of the IgG2b molecule (Diamond et al., 1979), although it could not be excluded that the IgG2b Fc receptor recognizes a region between the C_H2 and C_H3 domains. There is an analogous discrepancy in the heterologous system. It has been found that human IgG proteins mainly bind via their C_H3 domain to Fc receptors

of normal murine macrophages (Yasmeen et al., 1976). However, in a transformed murine macrophage cell line, it has been described that human IgG binds via a site which is formed by both the C_H2 and C_H3 domains (Haeffner-Cavaillon et al., 1979).

The functions of the two Fc receptors on macrophages are a matter of controversy. A macrophage-dependent cellular cytotoxic reaction against syngeneic murine adenocarcinoma cells has been reported to be mediated by antibody of the IgG2a class (Haskill and Fett, 1976). On the other hand, it has been found that the IgG2a Fc receptors on a macrophage-like cell line can mediate phagocytosis after IgG2a binding, while the IgG2b receptor was found to be responsible for extracellular cytolysis of antibody-coated heterologous erythrocytes (Walker, 1977).

Macrophage Fc receptors may also play a regulating role in the humoral immune response. In mice, macrophages which specifically bind heterologous erythrocytes sensitized by autologous 7S antibody have been described. Such macrophages caused a feedback inhibition of *in vitro* antibody formation against these erythrocytes only if the Fc portion of the opsonizing 7S antibodies was present (Abrahams et al., 1973).

No Fc receptors have been found for IgM, IgG3 and IgA (Unkeless and Eissen, 1975; Heusser et al., 1977). However, immune complexes formed by IgM can induce phagocytic activity in the presence of serum complement (Silverstein et al., 1977). The mechanism of attachment is likely to be of the second category, i.e., IgM complexes induce phagocytosis through the C3 receptor of the macrophage. This explanation has been disputed by Walker (1977), who claimed that cytophylic IgM can bind to a trypsin sensitive site on macrophages without complement.

For murine IgE, no data are available on the involvement of cytophylic IgE in phagocytosis. In the rat, it has been reported that IgE antibodies can mediate the immune adherence of parasitic helminths to normal macrophages (Capron et al., 1975).

2.4.2.2. Homocytotropic antibodies

Antibodies that are cytophylic for basophils or mast cells have been called *homocytotropic* if their binding is species specific. Antigen complexed to homocytotropic antibodies can activate both types of cells when the antibody part is bound to the Fc receptor. When this binding occurs, the cells secrete vasoactive amines and chemotactic factors. These result in increased vascular permeability, bronchial smooth muscle con-

traction and eosinophilic influx. All of these events are characteristic for an anaphylactic reaction.

In the mouse, IgE and IgG1 are found to be real homocytotropic antibodies, in the sense that they can sensitize homologous basophils and mast cells (Barth and Fahey, 1965; Prouvost-Danon et al., 1966), while IgG2a is involved only in a heterologous anaphylaxis (*hetero*cytotropic antibody) reaction (Ovary et al., 1965). The mechanism of sensitization seems to be different for IgE and IgG1. Antibodies of the IgE class are firmly fixed to the membrane of mast cells and sensitization with IgE cannot be reversed by washing. In contrast, IgG1 sensitization can be easily abolished by a single washing of mouse mast cells. It appears, therefore, that the anaphylactic reaction produced by IgG is mediated by soluble complexes of IgG1 and antigen which act on the cell membrane (Prouvost-Danon and Binaghi, 1970). The mechanism of IgE sensitization probably involves a simple bridging of membrane bound IgE. It has been demonstrated that chemical cross linking of 2 or 3 rat IgE myeloma molecules on the surface of peritoneal murine mast cells can cause degranulation of these cells (Segal et al., 1977).

2.4.2.3. Cytophylic antibody binding to lymphocytes

The passive binding of exogenous cytophylic antibody to lymphocytes (in contrast to the membrane association of monoclonal Ig that has been synthesized in a given cell) has received much attention because there are indications that extracellular Ig's play a role in the interactions of immunocompetent cells during an immune response. Lymphocytes appear to bind monomeric Ig as well as artificially aggregated Ig complexes and antigen-antibody complexes on the lymphocyte membrane (Dickler, 1976). Most evidence indicates that the binding of Ig is mediated by sites on the lymphocyte membrane that specifically recognize the Fc portion of the Ig molecule. Hence, they are called Fc receptors. The vast majority of B lymphocytes, a substantial minority of T cells and many weakly defined lymphocyte-like cells appear to have such Fc receptors.

The B cells that bind Ig include the precursors of antibody-forming cells (Basten et al., 1972a; Cline et al., 1972; Paraskevas et al., 1972), whereas studies of plasmacytomas have indicated that the plasma cells probably do not bind Ig (Basten et al., 1972b; Cline et al., 1972; Ramasamy, 1974). There is general agreement that antibodies of the IgG class bind to B lymphocytes, but the results concerning the relative avidity

of binding of the various subclasses are controversial. Most reports indicate that B cells most effectively bind IgG2a and IgG2b (Andersson and Grey, 1974; Soteriades-Vlachos et al., 1974; Gyöngyossy et al., 1975). However, equal binding of IgG1, IgG2a and IgG2b has been found by Cline et al. (1972), while Basten et al. (1972) observed that IgG1 was most readily bound to B cells. The reports concerning the cytophilic properties of IgM and IgA are also conflicting. Binding of IgM to murine B cells has been reported by Basten et al. (1972b) and Lamon et al. (1976). Other laboratories have not been able to confirm binding of IgM to the Fc receptor of B cells (Cline et al., 1972; Soteriades-Vlachos et al., 1974; Gyöngyossy et al., 1975; Revillard et al., 1975). With respect to IgA binding, no cytophylic IgA has been reported until recently. It is now known that IgA-coated heterologous erythrocytes can specifically bind to a subpopulation of surface Ig positive spleen cells (Strober et al., 1978).

Fc receptors have also been described for T cells. Some antigen-activated T cells from spleen, lymph nodes and peritoneal exudate can bind Ig, while those of the thoracic duct do not (Basten et al., 1975; Krammer et al., 1975). Evidence is also accumulating that Fc receptors are present on nonactivated T cells in thymus (Andersson and Grey, 1974; Stout and Herzenberg, 1975) and spleen (Soteriades-Vlachos et al., 1974; Stout and Herzenberg, 1975) as well as on T cell lymphomas (Harris et al., 1973; Warner et al., 1975; Krammer et al., 1976). In all studies on the cytophylic activity of the various murine Ig H-chain isotypes, it was found that IgG can bind to T cells (Grey et al., 1972; Yoshida and Andersson, 1972; Ramasamy and Munro, 1974; Fridman and Goldstein, 1974). Studies using mouse myeloma proteins suggest that the Fc receptor of murine activated T cells binds mainly (Stout and Herzenberg, 1975) or solely (Krammer et al., 1975) IgG2b molecules. T cell binding activities have also been described for IgG1 and IgG2a, although their relative binding avidities are highly controversial (Dickler, 1976). Some T cells from the spleen were demonstrated to bear Fc receptors specific for IgA (Strober et al., 1978). No Fc receptors binding IgM have been demonstrated on murine T lymphocytes up to now. No studies have been performed in the mouse on possible cytophylic activities of IgG3, IgD and IgE against T or B cells.

The controversial results concerning the cytophylic properties of the various Ig isotypes might be due to different sensitivities of the methods employed (Dickler, 1976). However, some contradictions might be attributed to differences in size of

commonly used aggregates of highly purified myeloma proteins in the various bindings assays. Individual myeloma proteins appear to vary in the extent to which they will aggregate and the avidity of Ig complex binding to Fc receptors depends on the size of the complex (Andersson and Grey, 1974). In addition, it is important to know to what extent monomeric Ig's can interfere with binding of Ig aggregates, since they are known to weaken oligomeric binding and increase the exchange rate of bound Ig complexes from cells (Segal and Hurwitz, 1977). Therefore, if aggregated Ig's are used, a critical evaluation of negative results has to include study of the degree of heterogeneity of the aggregates used, their relative binding constants and the degree of interference by monomeric Ig's.

Data on the domains of the Fc fragments involved in the Ig binding to lymphocytes are scant. Based on rosette inhibition of mouse lymph node cells by isologous IgG1 myeloma proteins lacking almost the entire C_H1 or C_H3 homologous regions, it has been reported that an intact C_H3 region is essential for IgG1 binding to Fc receptors (Ramasamy et al., 1975). Heterologous binding of human IgG subclasses to the Fc receptor of activated murine T cells required mainly the C_H3 domain and, to a lesser extent, that of the C_H2 (Klein et al., 1977).

2.4.2.4. The role of the lymphocyte Fc receptor in the humoral immune response

Antigen-antibody complexes are known to be efficient regulators of the immune response (Uhr and Möller, 1968; Fitch, 1975); they can stimulate as well as suppress. At the B cell level, Fc receptors are suggested to play a functional role in feedback inhibition of B cell activity. Although definite proof is lacking, there are some indications suggestive for such activity. For instance, it has been shown that the *in vitro* proliferative response of B cells to a polyclonal activator such as *Escherichia coli* lipopolysaccharide (LPS) is effectively abrogated only if these cells are treated with antimouse Ig's with an intact Fc part (Sidman and Unanue, 1976). Sinclair and coworkers (1971; 1976) proposed that cross-linking of antigen and Fc receptors of B cells can effectively block their reactivity. Such a direct blocking of B cell activity has been recently shown *in vitro* for the induction phase of a primary immune response against heterologous erythrocytes (Oberbarnscheidt and Kölsch, 1978). By adding immune complexes consisting of these erythrocytes and autologous specific antibodies, effective inhibition of the IgM antibody production could be achieved. This suppression was highly effective if the Fc portion of the antibodies

in the immune complexes was intact. Complexes with $F(ab')_2$ fractions of these antibodies were only partly effective.

In vivo, it has been found that pretreatment of T cell-deprived mice with covalent hapten-antibody complexes causes suppression of the T-independent response to that hapten and this *in vivo* depression is also dependent on intact Fc fragments of Ig molecules in the immune complexes. Although it is likely that the B cell was the immediate target for this suppression, an influence via macrophages was not excluded (Tite and Taylor, 1979).

On the other hand, single binding of antibody to Fc receptors might provide a stimulating signal for B cell activity. This hypothesis is supported by *in vitro* experiments which showed that polyclonal stimulation of both the proliferative response and antibody production by murine B cells can be achieved by adding heat-modified Fc fragments of heterologous (human) IgG in either soluble or aggregated form to the cultures. In these experiments, Fab fragments were ineffective (Berman and Weigle, 1977). Analogous experiments were later performed in order to compare the proliferative response of normal spleen cells in the presence of either homologous or heterologous (human) IgG. A comparable stimulation index was found in both situations. Since it was previously shown that most Fc receptor-bearing spleen cells are B cells, it was concluded that B cells could be stimulated equally well by homologous IgG, provided that the Ig molecules possessed the characteristic conformation of antigen-bound antibody (Berman et al., 1979). Similar conclusions have been reached in studies of the IgM Fc receptor on murine lymphocytes, where it was found that rosette formation could be inhibited by antigen-IgM antibody complexes and pentameric polymers of Fc fragments of human IgM, but not by native murine myeloma IgM or antigen alone (Lamon et al., 1976).

There are also indications for a role of the Fc receptor in the regulation of T cells during humoral immune responses. Fridman and Goldstein (1974) discovered a suppressor factor for antibody production in spleens of irradiated mice reconstituted with allogeneic thymocytes. This suppressor factor is nonantigen-specific and inhibits the response to both T cell independent and T cell dependent antigens *in vitro* (Gisler and Fridman, 1975). The T cell factor binds only Fc regions of IgG antigen-antibody complexes (Neauport-Sautes et al., 1975) and is expressed on a particular subset of suppressor T cells (Fridman et al., 1977a). It has been recently reported that a suppressor factor with similar properties can be produced in large amounts by hybridoma T cell lines (Neauport-Sautes et al.,

1979). Some T lymphoma cell lines have also been shown to produce an immunoregulatory factor binding IgG molecules and suppressing *in vitro* antibody production by mouse spleen cells (Molenaar et al., 1977; Fridman et al., 1977b). However, their physiological significance is uncertain, since they originate from a tumor and not from normally functioning T cells. Other, more indirect, evidence suggesting a role of the Fc receptor of T cells in the regulation of humoral immunity is the Fc region dependence of the suppression of the induction phase of specific antibody formation by IgG1 antibody (Gordon and Murgita, 1975). This finding is consistent with others which showed that suppressive antigen-antibody complexes of IgG affect the T-B cooperation via the Fc fragment of the antibody molecules (Kappler et al., 1973; Hoffmann et al., 1974; Hoffmann and Kappler, 1978).

2.4.2.5. Antibody-dependent lymphocyte-mediated cytotoxity

Probably via Fc receptor-binding, antibodies can serve as receptors responsible for specific cell-mediated lytic reactions. A minority of the lymphocytes are efficient antibody-dependent killer (K) cells. It is generally supposed that K cells are involved in tumor cell damage, in the immune response to acute and chronic virus infections, in autoimmunity and in transplant rejection. Studies on characterization of the cytolytic cells revealed that the effector cells involved in the antibody-dependent cytotoxicity against heterologous erythrocytes were neither mature T nor mature B cells (Greenberg et al., 1973; Pross et al., 1974). Since they lack both characteristic T cell determinants and endogenously generated surface Ig (which is one of the major B cell markers), they belong to the population of "null cells". On the other hand, it has been clearly established that at least some T cells which bind cytophylic IgG can exert cytolytic activity against IgG-coated erythrocytes (Kimura et al., 1977). Moreover, antibody-dependent cytolytic activity has also been noted for some allogeneically stimulated thymocytes (Rubin and Høier-Madsen, 1977).

Antibody-dependent target cell lysis by lymphocytes requires the presence of the Fc structures on the inducing antibodies (Larsson and Perlmann, 1972; Möller and Shevach, 1972). K cell-mediated cytolysis has been observed only if there was a direct and intimate contact between effector cell and target cell (Biberfeld and Perlmann, 1970; Scornik, 1974). The antibodies inducing this type of cytotoxicity belong mainly to the IgG class. With exception of IgM, no antibodies of the other Ig classes have thus far proved to be related to K cell activity (Perlmann

and Cerottini, 1979). Inhibition studies suggest that Fc receptor affinity of mouse K cells is much stronger for IgG2a than for IgG2b and IgG1 (Greenberg et al., 1975). The K cell activity is greatly dependent on the form of the available IgG antibody. Monomeric IgG binds to K cells with low efficiency; consequently, a relatively high concentration of antibody is necessary for lytic K cell activity (Perlmann and Perlmann, 1970; Segal and Hurwitz, 1976; Hurwitz et al., 1977). In contrast, large IgG aggrates (Sulica et al., 1976) and antigen-antibody complexes (Greenberg and Shen, 1973; Lustig and Bianco, 1976) are effective at low concentrations. K cell-mediated target cell lysis is easily inhibited by small soluble immune complexes of IgG, provided they are formed in moderate antigen excess (McLennan, 1972). Therefore, under physiological conditions, the antigen-antibody ratio might be decisive for the actual K cell reactivity.

The recognition site of the different domains of the IgG molecule for the K cell Fc receptor has been well studied only for human IgG. Most evidence is in favour of the concept that both the C_H2 and the C_H3 domains are necessary for optimal K cell activity (Spiegelberg, 1974; Dickler, 1976).

There are indications that K cell activity can be enhanced by IgM antibodies. Highly purified murine IgM myeloma proteins failed to induce cytotoxicity at any concentration when added alone. However, suboptimal IgG-mediated K cell activity could be enhanced by adding murine IgM myeloma proteins (Perlmann and Cerottini, 1979). This result might suggest that IgM has cytophylic properties for K cells, which would be compatible with the finding of Fc receptors for IgM on both T and non-T lymphocytes in mice (Lamon et al., 1976). K cell activity can also be enhanced by factors of the complement system (C1 and C3) (Peters and Theofilopoulos, 1977; Rouse et al., 1977; Ghebrihwet and Müller-Eberhard, 1978). Both the IgM and the complement mediated amplification might be based upon increasing the intercellular contact between K cell and target.

3. *SERUM IMMUNOGLOBULINS OF THE MOUSE*

3.1. *Compartmentalization of immunoglobulins*

Ig's of the various classes are present in different concentrations and proportions in different parts of the body. Their physical and cytophylic properties restrict the rate of exchange between the intravascular and extravascular compartments, their localization in external secretions and their transplacental transport (Waldmann and Strober, 1969). The intravascular and extravascular distribution of the various Ig classes depends largely on the diffusion coefficient of the protein. Nakamura et al. (1968) reported an inverse relationship between the diffusion coefficient and the ratio of the concentration in serum *versus* extravascular fluid. This is in agreement with the observation that large molecules such as pentameric IgM are found mainly in the serum (Metzger, 1970), whereas monomeric IgG can usually be found in serum as well as in extravascular compartments (Fahey and Robinson, 1963; Bazin and Malet, 1969; Waldmann and Strober, 1969). Up to now, no conclusive experiments have been performed to assess the diffusion rate of murine serum IgA into extravascular body fluids. However, since murine serum IgA is predominantly dimeric (Nash et al., 1970; Vaerman, 1973), it is expected that only a small amount of murine serum IgA will diffuse from the circulation into extravascular compartments. As compared to serum, thoracic duct lymph contains a high concentration of IgA (Mandel and Asofsky, 1968). Quantitation of IgA in samples of blood and thoracic duct lymph from adult mice revealed that the serum content of IgA was approximately 30% of that in lymph from the thoracic duct (Kaartinen et al., 1978). The explanation for this apparent paradox is that a large proportion of this IgA originates from the gut-associated lymphoid tissues and is transported via lymphatics to the thoracic duct and blood (Vaerman and Heremans, 1970). With regard to IgE, it is generally accepted that a large portion of this isotype is normally present in the extravascular protein pool (Waldman, 1969; Ogawa et al., 1971). This is of physiological importance, since the cells mediating reaginic responses are localized within the blood (basophils) as well as in extravascular compartments (mast cells).

The different pool sizes of the various Ig classes are also related to selective transport. In mammals, IgA is the major Ig in external secretions (Tomasi and Grey, 1972). There are indications that, in mice, the secretory IgA is produced by an interrelated system consisting of IgA plasma cells which have

a specific homing pattern to the various mucous membranes and exocrine glands of the body in order to defend the possible portals of entry for antigen (Cebra et al., 1976; Weisz-Carrington et al., 1979a). Furthermore, there are indications in man that polymeric IgA can be selectively transported across the epithelium to the glandular lumen of the various exocrine glands by means of the SC (Tomasi et al., 1965; Brandtzaeg, 1974; Poger and Lamm, 1974). In this context, it is also of interest to note that rat hepatocytes produce SC which is involved in the selective binding and transport of polymeric serum IgA from liver, via bile, to the intestinal lumen (Orlans et al., 1979; Socken et al., 1979).

Like polymeric IgA, pentameric IgM can also bind to SC and there are indications in man that this Ig isotype can also be transported into external secretions in a similar way as IgA (Brown, 1978). However, IgM generally occurs in relatively low concentrations in the external secretions as compared with IgG and IgA (Vaerman, 1973). This might be due to the fact that the binding of SC to IgM is less tight than to IgA polymers (Brandtzaeg, 1975; Weicker and Underdown, 1975). In addition, secretory Ig's complexed with SC are better protected against proteolytic enzymes than are noncomplexed Ig's (Heremans, 1974). Since SC-IgM complexes dissociate readily in intestinal fluids and only a small amount of IgM in human intestinal fluids is SC-linked (Richman and Brown, 1977), it is likely that transported IgM will be degraded more rapidly in these secretions than will be IgA.

With respect to colostrum and milk, IgM, IgG and IgA have been found in these secretions in most mammals, with a predominance of the latter Ig class (Vaerman, 1973). In mice, the predominant Ig in colostrum and milk is IgA. The rest consists mainly of IgG (Fahey et al., 1965; Asofsky and Hylton, 1968), while only small amounts of IgM are found (Guyer et al., 1976). Similar results are found in milk of guinea pigs (Vaerman, 1973). In rats, milk and colostrum are reported to contain high levels of only IgA and IgG. IgM was not detectable (Michalek et al., 1975; McGhee et al., 1975). A more detailed quantitative characterization of murine IgG in milk revealed that, of the four IgG subclasses, IgG1 and IgG2a were present in relatively high concentrations. In contrast, the concentration of IgG2b in milk was very low, whereas IgG3 was undetectable (Guyer et al., 1976). An exception to the predominance of IgA in the external secretions in mice has been reported by Osebold et al. (1975), who found that normal mouse lung lavage fluid contains twice as much IgG1 and IgG2, respectively, as IgA; IgM could not be detected.

Whether the presence of IgG in external secretions is due to local synthesis or to transudation from serum is not fully known. In man and ruminants, ample evidence is available to indicate that IgG in milk originates predominantly from serum (Tomasi, 1976). In disease, the ratios of the various Ig subclasses in secretions can change. It has been observed in man that, in secretory fluids of individuals with a selective IgA deficiency, SC complexed IgM can be present in significant amounts (Tomasi and Grey, 1972). This is in accord with experiments with neonatally thymectomized rats. Such animals have severely reduced or even undetectable levels of secretory IgA in saliva. In contrast, they have considerable amounts of secretory IgM after local immunization (Ebersole et al., 1979a). There are also indications for a compensatory role of IgM in secretions in mice if the IgA production is faulty. In the lactating mammary gland of athymic nude mice, the majority of plasma cells contain IgM, while normal mice show a predominance of IgA positive cells (Weisz-Carrington et al., 1979b).

In most mammalian species, including rodents, transfer of passive immunity from mother to young occurs via a selective transport of IgG antibodies (Brambell, 1970). In rats and mice, IgG is the only class of antibodies which can be transported from colostrum or milk into the circulation of young suckling animals, even though these fluids also contain IgA and IgM antibodies (Brown, 1978). By infusing the γ-globulin fraction of immune serum into the gastrointestinal tract of young suckling mice and measuring specific antibody activity in the circulation, it was found that IgG is selectively absorbed by the intestinal mucosa (Brambell, 1966). Studies in neonatal rats revealed that monomeric as well as aggregated complexes of homologous IgG can attach to the intestinal epithelial cells of the jejunum via Fc receptors. These receptors bind only IgG, not IgA and IgM (Borthistle et al., 1977). Autoradiography and competitive inhibition experiments have shown that the specific binding of mouse IgG and its Fc fragment to the luminal surfaces of rat enterocytes is a saturable process and that the capacity to absorb IgG is limited mainly to the first 3 weeks of life (Borthistle et al., 1978). Binding could be substantially reduced by treatment of the intestinal loops with trypsin (Borthistle et al., 1976). This finding might explain the limited period in which the rat intestine can absorb IgG, since an abrupt increase in the concentration of pancreatic enzymes in the intestine occurs at about 3 weeks of life (Mosinger et al., 1959). In maturing mice, the capacity of the intestine to absorp IgG was observed to be limited to the first two weeks of life (Brambell, 1970). Studies on the feeding of neonatal mice with mouse mye-

loma proteins indicate that, of the four IgG subclasses, mainly IgG1 and IgG2a bind to the intestinal wall and can enter the circulation. The order of IgG subclass affinity correlates with the subclass levels found in milk of lactating mice. Also in this study IgM and IgA myeloma proteins were shown to be unable to pass the intestinal wall (Guyer et al., 1976).

3.2. Metabolism of circulating immunoglobulins

The pool sizes of the different Ig's are also related to their pattern of metabolism. With respect to the overall synthetic rates of the major Ig's in mice, we are aware of only one conclusive report (Fahey and Sell, 1965). By quantitation of the various serum Ig's and determination of their respective catabolic rates, the synthetic rates of IgM, IgA, IgG1 and IgG2 have been estimated. It was found that the rate of synthesis for each Ig isotype was of the same order of magnitude (range, 25-50 mg/kg/day). The rate of Ig synthesis is largely influenced by the antigenic load of the environment in which the animals are kept. In germ free (GF) and specific pathogen free (SPF) maintained mice, the production rate is low, while conventional and immunized mice show a large amount of Ig production (Fahey and Robinson, 1963; Sell and Fahey, 1964). For instance, it was estimated that the synthetic rate of IgG in conventionally kept mice could be 50 times higher than in GF animals.

After release from the Ig synthesizing cells, Ig's belonging to each of the classes or subclasses have their own characteristic rate of disappearance from the circulation. On determining the total clearance from the body, conventionally kept mice showed a mean survival half-life for IgG of 4.5 days (Humphrey and Fahey, 1961; Fahey and Robinson, 1963; Sell and Fahey, 1964; Tee et al., 1965; Bazin and Malet, 1969). It has been shown that increasing the IgG concentration in serum (via either hyperimmunization, intravenous infusion or development of a myeloma tumor) accelerates the catabolic rate of IgG (Humphrey and Fahey, 1961; Fahey and Robinson, 1963). Studies in which fragments of IgG have been used indicate that, in fact, the Fc fragment was responsible for the regulation of the catabolic rate of IgG (Fahey and Robinson, 1963; Spiegelberg and Weigle, 1965). As indicated in Table I, IgG subclasses have different half-lives; for IgG1, this is in the range of 4 to 9.7 days, for IgG2a 5.1 to 8.3 days, and for IgG2b 2.7 to 3.2 days (Fahey and Sell, 1965; Bazin and Malet, 1969) and for IgG3 4 days (Grey et al., 1971). No subclass specific regulation of the catabolic rates of IgG1, IgG2a and IgG2b isotypes has been

reported. In other words, the fractional catabolic rate of each of the IgG subclasses is influenced by the serum levels of the others. Alterations in IgM and IgA serum levels did not affect the catabolism of the IgG subclasses (Fahey and Sell, 1965). By following the clearance from serum after infusion of IgM antibodies or IgM myeloma proteins, it was found that IgM has a half-life in the range of 0.2 to 0.6 days (Table 1). In similar experiments, IgA myeloma proteins were shown to have a half-life of 0.5 to 1.3 days (Fahey and Sell, 1965; Bazin and Malet, 1969). The regulation of the catabolism of IgM and IgA is different from that of IgG, since the clearance rate of both isotypes from serum is independent of their serum concentrations. Also changes in the various IgG subclass serum levels did not affect the IgM and IgA catabolism (Fahey and Sell, 1965).

TABLE I. HALF-LIVES (IN DAYS) OF MURINE IMMUNOGLOBULINS

	NIH-WS(1)	XVII(2)	BALB/c(3)	B6D2F1/HC(4)	unknown(5)
IgM	0.2-0.6	0.5	-	-	-
IgG1	4.0	9.7	-	-	-
IgG2a	5.1	8.3	-	-	-
IgG2b	2.7	3.2	-	-	-
IgG3	-	-	-	-	4.0
IgG-tot	-	-	4.1	-	-
IgA	1.1	0.5	-	-	-
IgE	-	-	-	0.4	-

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Recently, passive transfer of homologous IgE antibodies into mice revealed a half-life of IgE in serum of 10.5 hours, as measured by passive cutaneous anaphylaxis in rat skin (Peeters and Carter, 1978). The half-life of circulating rat IgE is independent of its serum level and of the total serum IgG concentration (Tada et al., 1975). In contrast, it has been found in man that the fractional catabolic rate of IgE is inversely related to the serum concentration (Waldmann et al., 1976). This might be related to the unique biological properties of IgE, since this isotype binds to extravascularly localized cells mediating reaginic reactions. Mathematical analysis of the metabolism of IgE revealed that the rate of disappearance of radiolabelled IgE from the serum fits most optimally with a model based upon both intravascular and extravascular catabolism of this Ig class (Iio et al., 1978).

In Table II, the serum levels of the various Ig heavy chain isotypes are given for some conventionally kept mouse strains. Although there is a large variation among the different strains of mice, the IgG1 and IgG2a serum levels usually predominate over those of IgG2b and IgG3 at adult age. This can also be expected, because the synthetic rate of the various IgG subclasses are within the same range, while IgG1 and IgG2a have a relatively low catabolic rate as compared with IgG2b and IgG3. The same holds true for the serum levels of IgM and IgA: as compared with IgG1 and IgG2a, their relatively rapid clearance from the serum roughly correlates with their low serum concentrations (c.f. Tables I and II).

3.3. Antigenic load and the serum immunoglobulin level

The serum Ig level is to a large extent dependent on the antigenic load of the respiratory and digestive tracts. This is apparent from studies in which serum Ig levels of mice raised under GF, SPF and conventional conditions were compared. Under SPF conditions, DBA/2 mice have 10 times more serum IgA and 4 to 5 times more serum IgG than GF DBA/2 mice of the same age (Van Snick and Masson, 1980). These differences are even larger if conventional and GF mice are compared. For conventional NIH-WS mice, the γ-globulin serum level was 20 times higher than in GF mice of the same strain (Sell and Fahey, 1964). Especially the IgG1, IgG2 and IgA serum levels of GF mice are low. However, the serum IgM levels of such mice are normal or increased (Fahey and Sell, 1965; Asofsky and Hylton, 1968; Nash et al., 1969; Benveniste et al., 1971a; 1971b; Natsuume-Sakai et al., 1977). The influence of the antigenic load on the serum Ig level was

further demonstrated by inoculating GF animals with pathogenic agents. Such contaminated mice usually show a sharp rise in the serum IgG1 and IgG2 levels, followed some time later by an increase in the IgA level (Benveniste et al., 1971a). However, serum Ig level measurement does not fully reflect the Ig-synthesizing activity of the immune system. This is due to the different half-lives of the various Ig's and the release of a portion of the synthesized Ig's in excretions and extravascular body fluids. Such influences can be avoided by determining the numbers of cytoplasmic Ig-containing (C-Ig) cells in the various lymphoid organs. By using of this approach, a clear relationship has been showed between the extent of stimulation by external antigens and the total number and class distribution of C-Ig cells in C3H mice. Comparison of GF mice reared on a synthetic diet and housed in bedding-free cages, normal GF mice and normal SPF mice revealed an increasing number of C-Ig cells depending on the extent of antigenic stimulation. As compared with other Ig classes, a relative preponderance of IgM-containing cells was found; this was most pronounced in GF mice reared on a synthetic diet and housed in bedding-free cages (Benner et al., 1980). From the above-mentioned data, it is apparent that the conditions of housing as well as the antigenic load of the mice have to be known for comparison of serum Ig levels in different mouse strains.

3.4. Serum immunoglobulin levels during ontogeny and aging

Follow-up studies in newborn CBA, C57BL, C3H and BALB/c mice revealed that they can accumulate significant levels of IgM in their sera within one month after birth. The serum levels of IgG and IgA, on the other hand, are low at the end of this period (Fahey and Barth, 1965; Kalpaktsoglou et al., 1973; Haaijman et al., 1977). Within 10-24 hours after birth, no IgA or IgM could be detected in the serum of either strain of mice studied. However, by using the Mancini technique, measurable amounts of both Ig isotypes appeared in the serum of C3H and BALB/c mice 3 days after birth (Kalpaktsoglou et al., 1973). In all strains, IgG was present in low but detectable amounts in serum of mice born 10-24 hours previously. It is proposed that this IgG in serum is due partly to prenatal acquisition of maternal IgG via the placenta (Fahey and Barth, 1965; Kalpaktsoglou et al., 1973). During the first 2 weeks of life, suckling mice show a relatively rapid increase and predominance of IgG in their sera, probably due to a selective intestinal uptake of maternal IgG from the milk (see 3.1.). However, serum IgG concentrations mostly show a sharp decrease 1 week after wean-

ing. By that time, ingested maternal IgG becomes degraded by proteolytic enzymes in the gut, while the young's own immune system has not yet developed the capacity to compensate for this loss (Fahey and Barth, 1965; Kalpaktsoglou et al., 1973). After one month of age, the serum concentrations of all Ig classes steadily increase. Adult levels of most Ig classes and subclasses are generally reached at the age of 3 to 6 months (Kalpaktsoglou et al., 1973; Haaijman et al., 1977; Natsuume-Sakai et al., 1977). For IgM, the adult range is already reached within 2 months of age, but, for IgA, this range is usually reached in a period ranging from 6 to 12 months of age. No apparent increase or decrease in the IgM and IgA levels occurs after reaching the adult serum levels. For the various IgG subclasses, there are some differences in the rate of appearance of adult serum levels in different mouse strains. C57BL, C3H and BALB/c mice have maximal serum concentrations of IgG1 at 3 to 6 months of age (Kalpaktsoglou et al., 1973; Natsuume-Sakai et al., 1977), in contrast to CBA mice, which still show increasing levels after 30 months (Haaijman et al., 1977). Adult IgG2a levels are reached between 3 and 12 months of age in all four strains. After that time, these levels do not change very much. With respect to IgG2b, again some differences have been noted. CBA and C57BL mice showed a steady increase during the observation periods of 20 and 30 months (Haaijman et al., 1977; Natsuume-Sakai et al., 1977), while C3H and BALB/c mice reached a constant level within 3 months (Kalpaktsoglou et al., 1973; Natsuume-Sakai et al., 1977). For IgG3 the only data available are from CBA mice. It was observed that the serum concentration of this IgG subclass reached adult values at 6 months of age and remained at this level up to 30 months of age. In advanced age CBA mice, an increasing variation among individual Ig levels occurs (Haaijman et al., 1977). Our own observation with respect to the quantitation of the various Ig isotypes in the sera of aging C57BL mice is in accord with this finding (see Chapter 4, section 4.1.).

Investigations in humans have demonstrated that the serum levels of IgG and IgA increase during aging, in contrast to the IgM levels which remain constant after reaching the adult values (Radl 1980). The increase in the IgG level has been reported to be restricted to the subclasses IgG1 and IgG3 (Radl et al., 1975). These authors also reported an age-related increase in variability among individual subjects, notably with regard to IgM and the IgG subclasses, but not for total IgG.

3.5. *Serum immunoglobulin levels in different mouse strains*

Large differences in serum levels of various Ig classes and subclasses can be found among different mouse strains, even when factors such as antigenic stimulation and age are the same. For instance, a follow-up study of BALB/c and C3H mice showed that BALB/c mice generally have higher serum Ig concentrations than C3H mice. In particular, IgM and IgG1 are clearly elevated in BALB/c mice from one month of age onwards (Kalpaktsoglou et al., 1973). In another laboratory, a comparative study was done with BALB/c, C3H and C57BL mice. Also in this study, BALB/c mice were found to have the highest serum Ig levels. Furthermore, it was noted that the most prominent serum Ig in BALB/c mice is IgG1, while this is IgG2b in C57BL mice (Natsuume-Sakai et al., 1977). Recently, Van Snick and Masson (1980) published serum Ig levels of various mouse strains (C57BL, 129/SV, DBA/2, C3H and BALB/c). The mice used were more than 20 weeks of age and were raised under low antigenic pressure (SPF). They found that different mouse strains can have clearly different serum Ig levels of the various classes and subclasses. For instance, DBA/2 mice were found to have relatively low serum IgM levels, while 129/SV mice showed relatively high IgG and IgA levels in their sera.

Even within a particular mouse strain a large variation can occur in serum Ig levels. This has been shown for Swiss mice by selective breeding for high and low responsiveness to various antigens. These mice could be separated into a high and low responder strain which showed not only a difference in height of antibody responses but also in serum Ig content. The genetic influence on the serum Ig levels was clearly shown for IgM, IgG1, IgG2 and to a lesser extent for IgA. Cross breeding experiments revealed a relatively simple inheritance pattern for IgM, in which the high and low responder genes codominate. However, for the IgG subclasses, a more complicated pattern in which high responder genes seemed to dominate was observed (Lieberman et al., 1972).

Since serum Ig levels are influenced by various factors (antigenic load, age and genetic background), a large variation in serum Ig levels of mice can be expected in the literature on this subject. This is the more true because no generally accepted absolute standard for mouse Ig's is available. Moreover, the reliability of expression of Ig concentrations in absolute values depends largely on the quality of the antiserum used, the nature of the reference standard and the nature of the Ig's which have to be quantitated (discussed in Chapter 6). To give

TABLE II. SERUM IMMUNOGLOBULIN LEVELS IN BALB/c, CBA, C57BL, C3H AND SWISS MICE KEPT UNDER CONVENTIONAL CONDITIONS

Ig (sub)-class	Serum level mg/dl	age months	Quantitation method	Reference
BALB/c				
IgM	15-20	1-12	Mancini	Natsuume-Sakai et al., 1977
IgG1	12-195			
IgG2a	11-133			
IgG2b	11-59			
IgA	5-150			
IgM	30-50	1-10	Mancini	Kalpaktsoglou et al., 1973
IgG1	20-35			
IgG2a	10-25			
IgG2b	15-73			
IgA	10-23			
IgG3	10-20	unknown	Mancini	Grey et al., 1971
IgM	100	3.5	unknown	Hudson and Hay, 1976
IgG1	650			
IgG2a	420			
IgG2b	120			
IgA	26			
IgM	60-130	2	Mancini	Fenton and Havas, 1975
IgG1	40-120			
IgG2	60-490			
IgA	10-30			
IgM	50	unknown	Mancini	Ansbaugh et al., 1974
IgG	500	3	unknown	Fahey and Robinson, 1963.
IgM	570-648	3	Mancini	Bazin et al., 1971
IgA	81-97			
CBA				
IgM	22-65	unknown	Mancini, Rocket	Kaartinen et al., 1978
IgG2a	87-400			
IgA	12-57			
IgM	14	unknown	Mancini	Ansbaugh et al., 1974
C57BL				
IgM	17-70	1-20	Mancini	Natsuume-Sakai et al., 1977
IgG1	5-68			
IgG2a	5-83			
IgG2b	4-112			
IgA	2-69			
IgM	17	1.5	radio-immune inhibition	Briles et al., 1979
IgG1	148			
IgG2a	373			
IgG2b	185			
IgG3	40			

Ig (sub)-class	Serum level mg/dl	age months	Quantitation method	Reference
C3H				
IgM	16-30	1-18	Mancini	Natsuume-Sakai et al., 1977
IgG1	35-95			
IgG2a	15-67			
IgG2b	8-20			
IgA	4-23			
IgM	15-25	1-10	unknown	Kalpaktsoglou et al., 1973
IgG1	5-20			
IgG2a	5-20			
IgG2b	15-90			
IgA	10-26			
IgM	50-210	1-2	unknown	Vaerman, 1973
IgG1	130-590			
IgG2	70-220			
IgA	70-370			
IgM	535-582	3.5	unknown	Vaerman, 1973
IgG1	78-119			
IgA	170-279			
IgM	23-27	3	Mancini	Anderson and Barrett, 1979
IgG1	120-146			
IgG2a	263-345			
IgG2b	37-55			
IgA	40-48			
Swiss				
IgM	30-60	unknown	Mancini	Lieberman et al., 1972
IgG1	70-500			
IgG2	100-600			
IgA	60-110			
IgM	15-17	2	Mancini	Irwin and Knight, 1975
IgG1	180-232			
IgG2	235-300			
IgA	76-97			
IgM	22-26	2	Mancini	Elin, 1975
IgG1	265-297			
IgG2	349-387			
IgA	52-62			
IgM	25-40	unknown	cellulose-acetate electro-immuno diffusion	Bluestone et al., 1973
IgG	372-377			
IgA	13-42			

In this table specifications concerning substrain and origin have been omitted. For this information the reader is referred to the references.

an indication of this variability and a reference for the strains of mice used in the experiments reported in this thesis, a table of the Ig levels of conventionally reared strains in which factors such as age and genetic background (as far as available) are mentioned, has been prepared (Table II). More data on serum Ig levels of normal mice can be found in the reports of Sell and Fahey (1964), Fahey and Barth (1965), Nash et al. (1969), Bazin and Doria (1970), Rozing et al. (1977) and Van Snick and Masson (1980).

4. *T CELL REGULATION OF THE HUMORAL IMMUNE RESPONSE*

4.1. T cell dependence of immunoglobulin production

The humoral immune system can respond to antigenic stimulation with the production of antibodies belonging to different classes and subclasses. The actual Ig (sub)class distribution of the antibodies depends upon the type of antigen, the antigen dose, the route of immunization, whether or not an adjuvant is used, and the availability of T cells. Generally, antigens can be subdivided into two classes. The antigens which induce antibody formation without the help of T cells. They are called T-*in*dependent antigens. If the antibody production requires T cells or is very much helped by T cells, the antigens are referred to as T-dependent antigens. T-independent antigens elicit predominantly antibody responses of the IgM class (Andersson and Blomgren, 1971). T-dependent antigens, as are most conventional antigens, usually induce antibodies of different classes and subclasses. The extent of T cell help required for antibody production is different for the various Ig H-chain isotypes. This is apparent from studies in which the antibody production (i.e., numbers of antibody-forming cells or serum antibody titers) and/or serum Ig levels have been measured in T cell-deprived animals.

The effect of T cell depletion upon the humoral immune response *in vivo* has mostly been studied in the following models:

a. Adult thymectomized mice treated with anti-thymocyte serum
b. Adult thymectomized, lethally irradiated, bone marrow or fetal liver reconstituted mice
c. Neonatally thymectomized mice
d. Congenitally athymic nude mice
e. Lethally irradiated mice infused with mixtures of B and T cells.

a. Adult thymectomized mice treated with anti-thymocyte serum

In order to eliminate virtually all T cells from adult thymectomized (ATx) mice, they are used at least 6 weeks after surgery in order to fully deplete short-lived T cells. In addition, the mice are treated with anti-mouse thymocyte (or anti-mouse brain) serum (ATS) shortly before they are used in experiments. This treatment with ATS is necessary to deplete the recirculating T cells. Since these recirculating T cells have a long life span, ATx only hardly affects immune responses to T-dependent antigens like sheep red blood cells (SRBC) (Kappler et al., 1974; Cantor and Simpson, 1975). Recently, it has been calculated that after ATx the maximal lifespan of T cells capable of mediating delayed

type hypersensitivity reactions against SRBC is about 50 months (Van der Kwast and Benner, 1978). After administration of small doses of ATS such long-lived T cells are eliminated rapidly (Lance et al., 1973; Araneo et al., 1975; Cantor and Weissman, 1976). However, treatment with ATS *in vivo* cannot deplete all long-lived T cells. It has been shown that some T cells from the thoracic duct can not be eliminated by ATS (Miller and Sprent, 1971). Therefore, if ATS has been used for T cell depletion it cannot be completely ruled out that some residual T cells have helped humoral immune responses which were supposed to occur in the absence of T cells.

b. Adult thymectomized, lethally irradiated, bone marrow or fetal liver reconstituted mice

Instead of treating ATx mice with ATS, T cells can also be eliminated by ATx followed by lethal irradiation and reconstitution with ATS-treated bone marrow cells or with fetal liver cells as source of stem cells. Studies in nonthymectomized, lethally irradiated and reconstituted mice indicate that the B cell compartment can recover to their normal level within 3-4 weeks after irradiation, while full recovery of the T cell population takes 3-6 months (Nossal and Pike, 1973; Van Muiswinkel et al., 1975; Rozing and Benner, 1975). Because of the lack of a thymus in ATx mice, the infused stem cells are not capable of giving rise to the T cell population.

c. Neonatally thymectomized mice

The third way of T cell deprivation is thymectomy during the first day of life. In order to exclude the possibility that some maternally derived T cells interfere with the experimental observations, the neonatally thymectomized (NTx) mice should be treated with ATS before use. However, a disadvantage of NTx is that there is often a low survival of the thymectomized mice. This can be due either to maternal neglect and cannibalism or to the development of wasting disease. The latter occurs more frequently after ATS treatment (Humphrey et al., 1964; Basch, 1966; Avasthi and Anderson, 1971). The point about incomplete elimination of peripheral T cells by ATS, made under a, also holds for the NTx model.

d. Congenitally athymic nude mice

Congenitally athymic nude mice do not develop a thymus during pre- and postnatal life as the result of a genetic defect (Pantelouris, 1968). Since they develop a B cell system which has in general similar potentialities as normal mice (Wortis, 1974), these mice have been considered as a very useful animal model to study the T cell (in)dependence of immune respon-

ses. However, despite the lack of thymus tissue, some T cell like cells have been demonstrated in adult nude mice (Raff, 1973). Since no conclusive evidence is available which rules out the possibility that such cells participate as helper T cells in the humoral immune response, also for this model some caution seems to be warranted.

e. Lethally irradiated mice infused with mixtures of B and T cells

The requirement for T-B cooperation in Ig production can also be assayed by using mice, whose immune system has been destroyed by lethal irradiation. The relevant B and/or T cell populations are mixed together with antigen and then transferred into the irradiated recipients. This procedure is called adoptive transfer. The immune response mediated by the transferred lymphocytes can be assayed by measuring the numbers of antibody-forming cells in the spleen or the antibody levels in the serum.

The above five different animal models have been used for studying the T cell dependence of the production of the various Ig's by measuring numbers of antibody-forming cells, serum antibody titers and serum Ig levels. Based upon these systems, evidence has been obtained that B cells committed to IgG antibody production require more T cell help than B cells committed for the production of IgM antibodies (Dresser, 1972; Mitchell et al., 1972; Davie and Paul, 1974; Tingle and Shuster, 1974). Of the four IgG subclasses the production of IgG1 antibodies is most dependent on T cell help (Taylor and Wortis, 1968; Torrigiani, 1972). Furthermore, the IgA (Clough et al., 1971; Benner, et al., 1974; Van Muiswinkel and Van Soest, 1975) and IgE antibody responses (Michael and Bernstein, 1973) are highly dependent on T cell help.

However, the data about the T cell dependency of the antibody production of the various Ig classes and subclasses are not always in agreement with the serum Ig levels in T cell deprived mice. In NTx mice normal or even raised serum IgA and IgG1 levels have been found (Humphrey et al., 1964; Fahey et al., 1965; Benveniste et al., 1969). ATx mice which have been treated with ATS, generally show similar IgG2a, IgG2b, and IgA serum concentrations as shamoperated animals (Bankhurst et al.,1975). These reports are in agreement with our own determinations of serum Ig levels in NTx, ATx, and shamthymectomized (STx) C57BL/KaLwRij mice at 3-4, 9 and 21 months of age. Except for the initially increased serum IgG1 levels of NTx and ATx mice at 3-4 months of age, no clear differences in IgM, IgG1, IgG2a, IgG2b, IgG3 and IgA serum concentrations among the 3 groups have

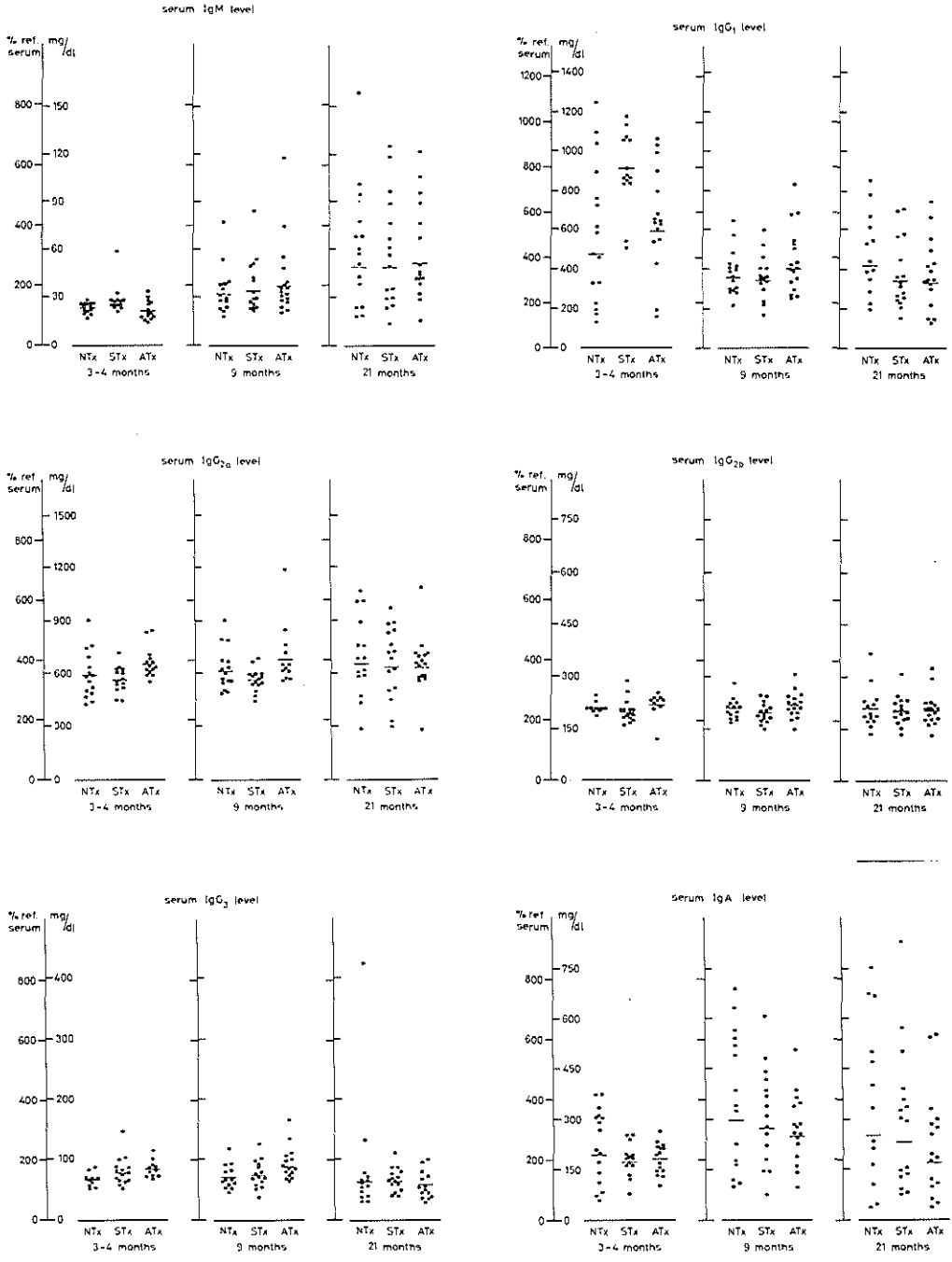

Figure 3. Serum Ig levels in conventionally kept NTx, STx, and ATx C57BL/KaLwRij mice at 3-4, 9 and 21 months of age. The individual serum levels are expressed in both absolute (mg/dl) and relative values (percentage of 5-month-old barrier maintained SPF C57BL/KaLwRij NMS). The horizontal bars represent geometric means of all the individual serum levels from each group of mice.

been noticed at the various ages tested (Fig. 3).

In athymic nude mice, usually normal or enhanced serum IgM levels are found, together with decreased IgG and IgA levels (Luzzati and Jacobson, 1972; Manning and Jutila, 1972; Bloemmen and Eyssen, 1973; Crewther and Warner, 1973; Pritchard et al., 1973; Bankhurst et al., 1975). In contrast, normal serum IgA levels have been reported by Gershwin et al. (1975), while the occurrence of clearly decreased as well as excessively high IgG1 serum levels repeatedly have been observed (Brogren et al., 1977; Okudaira et al., 1977). So far, the underlying cause for these discrepancies is unclear. Some conflicting data might originate from insufficient elimination of T cells in some studies. Other from differences in genetic background, age and antigenic load.

4.2. Generation and function of helper T cells

The first direct evidence that T cells are required for optimal B cell function was presented by Claman et al. (1966), who showed that antibody production to SRBC in irradiated mice was greater following transfer of a mixture of T and B cells than in mice receiving either B or T cells. Two years later, Miller and Mitchel (1968) showed that antibody formation to SRBC by neonatally thymectomized, irradiated and bone marrow reconstituted mice could be restored by T cells from thymus and ductus thoracicus. Also for *in vitro* antibody formation to T-dependent antigens, cooperation between T and B cells is required. Primary *in vitro* antibody responses of B cells to SRBC could only be elicited if SRBC-primed T cells were added to the cultures (Hartmann, 1970). Furthermore, in the hapten-carrier system it has been shown that hapten-specific B cells can only mount an effective antibody production in the presence of carrier-specific T cells and the corresponding hapten-carrier conjugate (Mitchison, 1969; Raff, 1970; Mitchison, 1971). Also in antibody formation against haptenated allogeneic cells T cells play an important role (Janeway et al., 1975; Lake and Mitchison, 1976; Vogt and Simpson, 1979). T cells primed to major histocompatibility (MHC) antigens exhibited a clear helper function during anti-hapten antibody responses. These helper T cells were shown to exert their activity only in combination with the alloantigen used for priming.

Recently two different types of helper T cells have been described for the B cell response to the widely used hapten-carrier complex dinitrophenyl-keyhole limpet hemocyanine (DNP-KLH). In *in vitro* experiments Tada (1978) showed that two subsets of carrier-specific helper T cells can be obtained by passage through

a nylon wool column. Both subsets of helper T cells can help DNP-primed B cells. However, if they were added together to the B cells, a synergistic effect upon the anti-DNP antibody formation was noted. Comparable results were obtained by Swierkosz and coworkers (1979). During the first phase of the *in vitro* anti-DNP response of B cells, helper T cells help the antigen-reactive B cells. The genetically restricted helper activity could be replaced by nonspecific concanavalin A (ConA)-induced helper factor. In a later phase the response was helped by T cells which could not be replaced by ConA factor. Both types of helper T cells had the same phenotype as was noticed by Tada, and also in this case both T helper cell populations synergized.

Presently, there are indications that antigen-specific helper T cells can determine the generation of antibodies belonging to a certain Ig class, allotype and even idiotype.

Ig class specific T cells
Data from *in vitro* cultures of rabbit lymphocytes suggest that helper T cells for IgE antibody synthesis might be different from those for IgG antibody production since different adjuvants can selectively induce secondary antibody responses of either the IgE or the IgG class (Kishimoto and Ishizaka, 1973).

Using an adoptive cell transfer system it has been shown that also in the mouse the way of immunization can cause some dissociation between IgE and IgG antibody responses (Hamaoka et al., 1973; Hamaoka et al., 1974). However, because a number of interrelated and complex variables (e.g., dose of the antigen, nature of the antigen, type of adjuvant, etc.) determine the extent to which IgG and IgE antibodies are produced following immunization, these data have to be considered with caution (Hamaoka et al., 1974). In addition, in the B cell splenic focus assay, it has been demonstrated that an individual antigen(virus)-specific T cell has the capacity to enable primary virus specific antibody responses of more than a single Ig H-chain isotype (Pierce et al., 1978).

Some evidence for a separate T cell regulation of IgA in the mouse has been presented by Elson and coworkers (1979). They studied the effect of ConA-activated T cells from different tissues upon the polyclonally induced production of IgM, IgG and IgA by cultured mouse lymphoid cells. Variation in the regulatory T cell activity was only found for IgA. In particular, T cells from Peyer's patches were found to deliver a high level of T helper activity for IgA synthesis as compared with spleen or peripheral lymph nodes.

Allotype-specific T cells
Allotype-specific helper T cells are reported to exist in hybrid mice. These T cells specifically help a family of B cell clones bearing common allotypic C_H structures. This was shown in adoptive transfer experiments for the secondary IgG2a antibody response to DNP-KLH. Parental helper T cells induced less hapten-reactive hybrid B cells to produce specific antibodies than syngeneic hybrid helper T cells did. In addition, in the presence of parental T cells, hybrid B cells only produced IgG2a antibodies of the allotype corresponding to that of the parent from which the helper T cells were derived (Herzenberg et al., 1976).

Idiotype-specific T cells
Specific helper T cells can probably also selectively activate B cells bearing a certain idiotype. By studying T-dependent anti-hapten antibody responses in mice, it was noticed that preferentially certain clones of hapten-specific B cells were activated instead of an activation of all B cells binding that hapten (Woodland and Cantor, 1978; Hetzelberger and Eichmann, 1978b; Eichmann et al., 1978). Eichmann (1974) found that during the anti-streptococcal humoral immune response in A/J mice, a specific enhancement of the antibody production against the A5A idiotype could be achieved by infusion of heterologous (guinea pig) IgG1 antibodies (directed to murine A5A antibodies) before immunization. This phenomenon was dependent on antigen-specific helper T cells, which selectively cooperate with A5A positive B cells (Eichmann and Rajewski, 1975; Black et al., 1976).

In another idiotypic system it has been found that BALB/c mice respond to PC with antibodies which were virtually all of an idiotype (T15) similar to that of the PC-binding myeloma protein TEPC-15 (Cosenza, 1976). This finding has been correlated with the fact that during the T cell dependent antibody response to PC, the majority of the helper T cells which are involved, bear the T15 idiotype (Cosenza et al., 1977a; 1977b).

After immunization of A/J mice with azophenylarsonate (Ars) coupled to a protein carrier, a large part of the anti-Ars antibodies possess an idiotype which is produced independently of the origin of the carrier. This idiotype, termed cross reactive idiotype (CRID), can be identified by heterologous anti-idiotypic antibodies (Nisonoff et al., 1977). B cells producing this idiotype need helper T cells which are also recognized by this antiserum. Selective elimination of these helper cells from the total repertoire of antigen-specific helper T cells before challenge with Ars-KLH did not reduce the total titer

of anti-Ars-antibodies in the serum of animals adoptively transferred with KLH-primed helper T cells and Ars-primed B cells. However, in the sera of the recipient mice hardly any anti-Ars antibodies of the CRID idiotype could be found. It was concluded that idiotype-specific helper T cells are necessary for the production of CRID antibodies against Ars (Woodland and Cantor, 1978).

It has been proposed that for the dominance of a particular idiotype in an antibody response in fact two types of helper T cells are required, one recognizing the antigen and the other recognizing the idiotype of the B cell. Both T cell subpopulations can exert their helper function independently of each other. However, in addition to antigen-specific T cell help, idiotype-specific helper T cells may be responsible for the selective production of antibodies of that particular idiotype. It is suggested that both T cell subpopulations are required for an optimal antibody response (Eichmann et al., 1978; Hetzelberger and Eichmann, 1978b; Woodland and Cantor, 1978; Bottomly and Mosier, 1979).

For various test systems also nonspecific helper T cells have been described by several authors. It has been shown for DNP-KLH that during coculture of irradiated carrier-primed spleen cells and unprimed cells nonspecific helper T cells are generated which can support the production of IgM antibody to DNP conjugated to several different carriers (e.g., KLH and apoferritin) (Julius and Augustin, 1979). Allogeneically stimulated T cells can help antibody synthesis *in vitro* to antigens which are irrelevant for the activation of these T cells. This is called the positive allogeneic effect. For instance, it has been found that the lack of primary *in vitro* anti-SRBC antibody formation by purified B cells could be restored by addition of normal allogeneic spleen cells to the cultures (Hirst et al., 1970; Munro and Hunter, 1970). Limiting dilution analysis revealed that from these allogeneic spleen cells in fact the alloantigen-specific T cells helped the B cell response (Corley et al., 1978).

There are indications that specific and nonspecific helper T cells are different cell populations. Priming of spleen cells with KLH produced helper T cells which were able to stimulate the response of B cells to both DNP-KLH and (in the presence of KLH) to heterologous erythrocytes. Limiting dilution of the KLH-primed helper T cells revealed that the frequency of helper cells which help the response to TNP-KLH segregated independently from the helper cell which is involved in the

response to the heterologous erythrocytes (Marrack and Kappler, 1975). Waldmann and coworkers (1976) confirmed and reinforced this conclusion by using in a similar experimental protocol irradiated antigen-activated helper T cells with the purpose to exclude effects of proliferation.

4.3. Generation and function of suppressor T cells

A large body of evidence has accumulated which revealed that T cells can also suppress antibody production and thereby regulate the humoral immune response (Gershon, 1974). Both specific and nonspecific suppressor T cell activities have been described. As helper T cells, suppressor T cells have been reported to be able to regulate the humoral immune response at the level of antibody class, subclass, allotype and idiotype.

Evidence for suppressor T cells regulating the antigen-specific IgM antibody response has been reported for both T-dependent and T-independent antigens. An example of the latter type is the work of Baker and colleages who studied the regulation of the response to type III pneumococcal polysaccharide (SSSIII) (Baker et al., 1970; 1973; Markham et al., 1977). The IgM response in normal mice after immunization with this antigen was enhanced if these animals were deprived of T cells by treatment with ATS. For T-dependent antigens like TNP-KLH it was observed that *in vitro* induction of antigen-specific suppressor T cells can be achieved by adding high concentrations of KLH to the cultures. These cells were able to abrogate selectively the primary IgM response of normal spleen cells to DNP-KLH (Kontiannen and Feldman, 1976).

With respect to the IgG antibody response also suppressor T cells have been reported. For instance, antigen-specific suppression of IgG antibody production has been observed in several mouse strains which were immunized with various antigens like deaggregated fowl immunoglobulin (Basten et al., 1974) or haptenated complexes of synthetic copolymers of the amino acids L-glutamic acid, L-alanine and L-tyrosine (GAT) or of L-glutamic acid and L-tyrosine (GT) (Kapp et al., 1975; 1977; Debré et al., 1976). Mice which become tolerant for such agents generate antigen-specific suppressor T cells which can suppress the total IgG response to these antigens. Furthermore, *in vivo* and *in vitro* it has been shown that antigen-specific suppressor T cells regulate the avidity of IgG antibodies during a secondary humoral response against T-dependent DNP-conjugated proteins. Antigen-activated T lymphocytes given shortly before booster immunization

selectively suppressed B cell clones producing high avidity IgG antibodies during the late phase of the immune response (Warren et al., 1976; Warren and Davie, 1977). Recently an IgG2a-specific suppression has been described. Some congenic mouse strains of C57BL background produce low levels of IgG2a antibodies after SRBC immunization both during the primary and secondary immune response. The authors claim that the suppressor T cells which are responsible for this effect are specific for both the IgG2a subclass and the antigen SRBC (Seman and Zilberfarb, 1979).

Class-restricted suppression has also been reported for IgE. The suppression is mediated by non-antigen-specific suppressor T cells (Watanabe et al., 1976; 1978) and is specific for IgE since concomitant IgG1 antibody production is not affected after adoptive transfer of suppressor T cells. There are indications that the murine IgE suppression can be mediated by two subsets of suppressor T cells; one is normally present in untreated mice, and another arises after hyperimmunization. After adoptive transfer these two types of suppressor T cells act synergistically in suppressing IgE antibody formation (Itaya and Ovary, 1979).

In mice no IgA-specific suppressor T cells have been found so far. However, some patients with a selective IgA deficiency have T cells which can prevent the production of only this Ig class. Coculturing of ConA-activated T cells from such patients with normal lymphocytes caused a suppression of the IgA synthesis, whereas the IgM and IgG production remained unaffected (Waldmann et al., 1977).

Allotype suppression
With regard to the production of antibodies of a particular allotype, it has been reported that in hybrid mice perinatally injected with antibodies specific for one parental allotype, the corresponding allotype is chronically suppressed (Jacobson and Herzenberg, 1972; Herzenberg and Herzenberg, 1974). If this is done for the maternal IgG2a allotype suppressor T cells are generated which, in a hapten-carrier adoptive transfer system can suppress selectively the IgG2a antibody formation of the maternal allotype, whereas the IgG1 production remains unaffected (Herzenberg et al., 1976).

Idiotype suppression
In various test systems evidence has been obtained that idiotype-specific suppressor T cells can occur. In the A5A system Eichmann (1976) demonstrated that low doses of the IgG2 fraction

of guinea pig anti-A5A idiotype antibodies in mice have the capacity to induce antigen-specific idiotypically related suppressor T cells, capable of maintaining a chronic suppression of the production of A5A positive antibodies to Streptococcal-A proteins.

The dominant occurrence of the T15 idiotype during the anti-PC response in BALB/c mice can be altered by the administration of anti-T15 antibodies to neonatal or adult mice. This results in suppression of PC-specific clones bearing the T15 idiotype (Cosenza et al.,1977b). In adoptive transfer experiments it was shown by Bottomly et al. (1978) that KLH-primed T cells from adult suppressed mice are unable to cooperate with PC-primed B cells in generating T15 positive antibodies to PC-KLH. In fact this inability was caused by suppressor T cells preventing an effective T-B cooperation. The generation of these suppressor T cells by anti-T15 antibodies required the presence of both the T15 idiotype and the original carrier. Selective removal of these suppressor cells restored the help for T15 positive antibody production to PC in adoptively transferred mice. This indicates that neither anti-T15 treatment by itself, nor the induced suppressor T cell eliminates the helper T cell population or the B cell clones involved in anti-PC T15 positive antibody formation (Bottomly et al., 1978). Also on the basis of *in vitro* experiments it has been claimed that anti-T15 antibodies, together with specific antigen, generate idiotype-specific suppressor T cells (Kim, 1979). Coculturing of fresh normal BALB/c spleen cells with spleen cells preincubated with anti-T15 antibodies and antigen, almost completely abolished the anti-PC response by the normal spleen cells. The suppressor cell was identified as a T cell. However, the generation of T15 idiotype-specific suppressor T cells could not be confirmed by Julius and Heusser. They observed that selective suppression of T15 positive anti-PC antibody production by anti-T15 antibodies could also be induced in BALB/c nude mice. Furthermore, they were unable to transfer suppression of the T15 idiotype by means of T cells from BALB/c mice which had been injected with monoclonal anti-T15 antibodies shortly after birth (Julius and Heusser, to be published).

In the Ars-system, adult mice can be maintained in an idiotypically suppressed state by injecting heterologous (rabbit) antibody directed to the CRID idiotype, followed by repeated antigen (KLH-Ars) injection. Mice which were adoptively transferred with ascitic lymphoid cells from such idiotypically suppressed animals also failed to express the CRID idiotype during the anti-Ars antibody formation (Ju et al., 1977;

Nisonoff et al., 1977). T cells from idiotypically suppressed mice revealed an enhanced percentage of cells forming rosettes with autologous erythrocytes coated with $F(ab')_2$-fragments of the rabbit anti-CRID antiserum as compared with nonsuppressed mice (Owen et al., 1977a). After removal of these rosette-forming T cells, the residual cells did not show suppression of the CRID antibody formation in adoptively transferred animals. Addition of these rosette-forming T cells to anti-Ars-reactive B cells in adoptive transfer experiments caused a severe depression of the CRID expression (Owen et al., 1977b). These results indicate that idiotype-specific suppressor T cells can regulate the CRID antibody formation to the hapten Ars.

Such a T cell dependent suppression of anti-idiotypic antibody formation has also been established under more physiological conditions. It has been claimed that an auto-anti-idiotypic antibody response occurs during the immune response to the T-independent antigen TNP-ficoll (Goidl et al., 1979; Schrater et al., 1979). This auto-anti-idiotypic response is dependent on T cells since T cell-deprived mice (nude mice as well as thymectomized, irradiated, bone marrow reconstituted mice) did not show anti-idiotypic antibody formation during the anti-TNP response. The anti-TNP response in these animals was more persistent than in normal mice. The production of antibody-forming cells in the T cell-deprived animals could be abrogated by administration of the relevant anti-idiotypic antibodies (Schrater et al., 1979). During the anti-TNP response in BALB/c mice an idiotype is expressed which is related to the TNP-binding myeloma protein MOPC-460. This idiotype was found during the response to several T-independent TNP-conjugates. Removal of T cells from spleen cell cultures increased the number of antibody-forming cells secreting antibodies of the MOPC-460 idiotype. Addition of T cells from TNP-primed normal BALB/c mice abrogated this idiotype expression. These suppressor T cells were shown to have the capacity to bind specifically the MOPC-460 idiotype. Addition of such T cells to cultures of TNP-primed B cells prevented the production of this idiotypic antibody by the primed B cells (Bona and Paul, 1979).

Apart from the specific suppressor cells also nonspecific suppressor T cells have been described for the humoral immune response. Primed T cells, on culture with (T,G)AL show specific helper activity for this antigen during the first day of culturing. However, if the cells remain in culture for a long time, the ability to mediate specific help to B cells is lost when tested in *in vivo* assays. Instead, they exert a strong inhibitory effect on IgM antibody formation against both (T,G)AL and com-

pletely irrelevant antigens like SRBC (Taussig, 1974b). Janeway et al. (1975) described that in an allogeneic mixed lymphocyte culture (MLC) cytotoxic T cells as well as suppressor T cells are generated which can both abrogate the antibody formation in the culture. The activity of the suppressor T cells was not confined to the anti-alloantigen immune response. They were also capable of suppressing secondary antibody responses to antigens like DNP-KLH. This suppressor effect of alloantigen-activated T cells on antibody formation has been called the negative allogeneic effect.

Another way to induce nonspecific suppressor T cells is culturing of normal spleen cells in the presence of mitogenic concentrations of ConA. A marked inhibition of the primary and secondary IgM and IgG antibody production to various antigens (heterologous erythrocytes, TNP-SRBC, TNP-KLH) was noticed when ConA-treated spleen cells were added to cultures of normal spleen cells. Also in this system the induction of suppressor activity by ConA occurs relatively late (Dutton, 1975). These ConA-induced suppressor T cells are not genetically restricted in their activity since they inhibit the secondary response to SRBC by cultured allogeneic spleen cells as effectively as by syngeneic spleen cells (Dugan et al., 1977).

4.4. T cell factors

Helper factors

Experiments have been reported concerning soluble T cell products which can occur in supernatants and lysates of antigen-primed T cells. These factors possess helper T cell replacing activities in the humoral immune response. Such T cell replacing factor(s) (TRF) can be obtained from an allogeneic MLC (Schimpl and Wecker, 1972), from mitogen-activated normal lymphoid cells (Wecker et al., 1975), and from antigen-stimulated cultures of primed cells (Waldmann, 1977). In view of their molecular weight, the factors are not likely Ig's (Hübner et al., 1978). They have been shown to augment IgM and IgG antibody responses to various antigens (Schimpl and Wecker, 1975). In fact these T helper factors have been divided into two types, one which is antigen-specific while the other is nonspecific (Klein et al., 1976).

Using various T-dependent antigens, Feldmann and Basten (1972a) discovered that helper T cells can release a factor capable of triggering primed B cells *in vitro* to produce antibodies. The factor was restricted in the sense that only syngeneic B cells could be activated. The factor could only exert its activity if it was administered to the cultures together with the relevant antigen. An *in vivo* active antigen-specific TRF has been des-

cribed by Taussig (1974). This factor is produced during *in vitro* incubation of primed T cells with synthetic antigens like (T,G)AL, and can be found in the supernatant within the first day of culturing. Via adoptive transfer experiments it was shown that the factor can help only those B cell clones which are specific for (T,G)AL. Although the factor bears MHC determinants, it has been shown *in vivo* as well as *in vitro* that it is equally effective in inducing T-dependent antibody responses in syngeneic and allogeneic spleen cells (Taussig, 1974a; Taussig et al., 1975; Taussig and Munro, 1976; Howie and Feldmann, 1977). Furthermore, the factor is characterized by having antigen-binding capacities and Ig-like determinants. This was concluded from the observation that a heterologous antiserum against mouse-IgM can bind the factor (Howie and Feldmann, 1977). Waltenbaugh (to be published) obtained a helper factor from cyclophosphamide-treated mice primed with GT. This factor specifically enhances the primary immune response to this antigen *in vitro* as well as *in vivo*. It was found that also this factor bears MHC determinants (Benacerraf and Germain, 1978).

Another *in vivo* acting antigen-specific helper factor can be derived from supernatants of allogeneically activated T cells. By injecting nude mice with such supernatants it was shown that the factor has the capacity to replace helper T cells during primary and secondary antibody responses of B cells to histocompatibility antigens, and not to antigens like SRBC (Kindred and Corley, 1977). Such a TRF from supernatants of allogeneically activated T cells was not active *in vitro* (Waterfield et al., 1979). Allospecific T cells, maintained for 3 years in culture by sequential restimulation, were tested for their ability to induce a positive allogeneic effect, i.e. help of virgin B cells in producing antibodies to SRBC *in vitro*. It was found that such T lymphocytes could function as helper cells, provided the B cells to be activated had the same MHC haplotype as the stimulator cells used in the original MLC combination. An equal stimulation index could be achieved by the supernatants of these continuously activated T cell cultures. However, such supernatants did not exhibit strain specificity. In fact it was already discerned in 1972 that in an allogeneic MLC T cell factors can be released in the supernatant, which fail to display any degree of antigen specificity, neither in its induction, nor in its action during antibody responses to T-dependent and T-independent antigens (Schimpl and Wecker, 1972; Feldmann and Basten, 1972b).

At present data are available showing that allogeneic factors can not replace all helper T cell functions. For instance, after administration to DNP-primed B cell cultures, allogeneic helper factor does not help a secondary IgG response

to DNP when the hapten is presented in a soluble complex with an irrelevant carrier protein. Only if the antigen is present in a particulate form (e.g., on the surface of macrophages or complexed with antibodies) nonantigen-specific triggering of the IgG antibody response could be achieved by this factor (North et al., 1977; Kemshead and Askonas, 1979). Another observation in this context is that helper factors from allogeneically or ConA-stimulated spleen cells can only enhance the IgM and not the IgG antibody production in nude mice to T-dependent antigens like heterologous erythrocytes and various hapten carrier complexes (Kindred et al., 1979).

Recently the nonspecific component of T cell replacing factors has been debated (Bernabé et al., 1979). Helper factor obtained by ConA treatment of cell populations which were enriched for T cells reactive to antigens like SRBC or horse red blood cells (HRBC) enables nude spleen cells to mount a considerable primary antibody response *in vitro* to these antigens. After negative selection of T cells specific for SRBC or HRBC, the ConA-induced helper factor was selectively incapable of helping nude spleen cells to mount a primary humoral immune response to SRBC and HRBC, respectively. In addition, removal of specific helper activity from ConA supernatants was achieved by absorbing them with antigen-antibody complexes. From these results the authors conclude that nonspecific helper supernatants are in fact a polyclonally induced mixture of various specific helper factors, derived from a whole repertoire of activated T cells (Bernabé et al., 1979).

Stimulation of cultured T cells which were previously primed with the antigen (T,G)AL, revealed an antigen-specific helper factor in the supernatant which could replace the helper function of thymocytes in the production of antibodies to this antigen in irradiated and bone marrow reconstituted mice. The factor could be completely removed by passage through a Sepharose immune absorbent containing anti-idiotypic antibodies to (T,G)AL. This indicates that the helper factor from (T,G)AL-primed T cells bear idiotypic determinants of antibodies specific for this antigen (Mozes and Haimovich, 1979). Also for the A5A system a factor has been described which bear idiotypic determinants. This factor can be found in the supernatants of ConA-stimulated T cell cultures in which these T cells were obtained from mice presensitized with IgG1 anti-A5A idiotypic antibodies. This factor carries the A5A idiotype and is most likely involved in the helper activity of A5A-specific helper T cells during the anti-streptococcal antibody response (Eichmann, 1978).

Suppressor factors

Like helper T cells, suppressor T cells can secrete soluble factors. In most experiments described below, suppressor T cells are proposed to inhibit the humoral immune response by releasing factors which suppress the functioning of helper T cells. Some of these suppressor molecules are genetically restricted in their action and have the capacity to bind the priming antigen. Others are nonspecific and can suppress responses to several antigens, and of B cells from various mouse strains.

With respect to the antigen-specific factors, Tada and coworkers (1976) found an antigen (KLH)-specific suppressor factor capable of suppressing selectively the secondary IgG antibody response to DNP-KLH by primed spleen cells *in vitro*. The suppressor factor was not released from T cells, but could be extracted from sonicated thymocytes and spleen cells previously primed with KLH. The target of this T cell substance was the helper T cell since no effect was seen in the absence of these cells (Taniguchi et al., 1976). Further characterization of this T cell product revealed a heat-labile nature, MHC determinants, no Ig determinants and genetic restriction of its activity (Tada et al., 1976). Similar antigen-specific T cell-derived suppressor factors for IgG antibody production have been extracted from thymus and spleen of nonresponder strains of mice which were immunized with GAT or GT (Kapp et al., 1977; Waltenbaugh et al., 1977). However, in contrast to the Tada factor, this T cell product mediates its suppressor activity by stimulating GAT-or GT-specific suppressor T cells. Furthermore, it was found that its activity is not strain specific (Waltenbaugh et al., 1977). With respect to the GAT-specific suppressor factor it was found that it can exert its activity only in association with the specific antigen (Kapp et al., 1977; Germain et al., 1978). Although the factor lacks conventional Ig C_H and C_L determinants, it has been found recently that it has a cross reactive idiotypic determinant in common with GAT-specific antibodies (Germain et al., 1979).

Antigen-specific suppressor T cell factors can also be obtained from hybridoma T cell lines. Such a cell line was obtained by Kontianen et al. (1978) by hybridization of thymoma cells and suppressor T cells primed with KLH. These authors found a suppressor factor in the supernatant with a specific combining site for the antigen (KLH), with MHC determinants, and with Ig-like determinants. A conventional Ig molecule structure was excluded on the basis of the relatively low molecular weight. *In vitro* the factor mediates a selective suppression of IgM and IgG antibody formation to DNP-KLH. No suppression was seen for

T-independent antigens. In a similar way Taniguchi and Miller (1978) successfully fused a T lymphoma cell line with human gamma globulin (HGG)-specific suppressor T cells. Sonicates from several of these hybridomas mediated specific as well as non-antigen specific suppression of adoptive secondary response to DNP-HGG. However, only one cell line with specific suppressor properties appeared to be stable during long term culturing. A SRBC-specific suppressor factor produced by a hybridoma T cell line has been reported by Taussig (1979). This factor inhibits the primary (IgM) antibody response against SRBC *in vitro*. This T cell product has no Ig determinants, binds SRBC and carries determinants of the MHC complex.

IgE-specific suppressor factors have been found in the supernatants of cultures of T cells which were primed previously with DNP-conjugated mycobacteria. In adoptively transferred mice, the supernatant causes a profound inhibition only of the IgE production in the secondary anti-DNP antibody response. The factor bears MHC determinants and is genetically restricted in its activity. Furthermore, it was shown that it can bind specifically to DNP-primed B cells, among them B cells positive for IgE (Kishimoto et al., 1978). Mice sensitized with allergic agents (e.g., DNP conjugated to worm extract) can produce immune sera and ascitic fluid which can suppress a secondary IgE anti-DNP response after isologous adoptive transfer (Tung et al., 1978). Also in this experimental set up the suppressive activity was found to be genetically restricted, since only an effective inhibition of the IgE immune response could be achieved if the suppressive serum had been derived from donors which were compatible for the MHC complex with the adoptively transferred cells (Katz and Tung, 1978). Recently it has been discovered that passively infused allogeneic cells or MLC supernatants have a similar capacity to induce such a selective IgE suppression. Also in this case the suppressor substances were only effective in combination with syngeneic cells (Katz, 1979).

With respect to the suppression of allotypes in mice, it has been reported by Herzenberg and colleagues (1976) that a supressor factor can be found in supernatants of spleen cell cultures of hybrid mice which were suppressed for the maternal allotype of IgG2a. The factor acts on helper T cells which are necessary for the production of this IgG2a allotype.

Nonantigen-specific suppressor T cell products have been reported to occur in the supernatants of cultures of normal spleen cells after stimulation with high concentrations of ConA (Pierce and Kapp, 1976). The suppressive factors in such supernatants

have been shown to be potent inhibitors of IgM and IgG antibody responses to both T-dependent and T-independent antigens like SRBC, DNP-ficoll or TNP-LPS. Further characterization of these factors revealed no antigen-binding capacities nor any Ig-like determinants (Tadakuma et al., (1976). A nonantigen-specific T cell factor has also been detected *in vitro* by adding ovalbumin-immune spleen cells to cultures of SRBC-primed spleen cells. The factor could only be produced in the presence of soluble ovalbumin and has the capacity to cause a complete suppression of the anti-SRBC antibody response. It has been proposed that suppressor factors produced under such circumstances participate in the termination of humoral immune responses *in vivo* (Thomas et al., 1975).

Recently Kontianen reported a nonantigen-specific suppressor T cell factor produced by a stable hybridoma T cell line, which had been produced by fusion of antigen-specific suppressor T cells and thymoma cells (Kontianen et al., 1978). Also this factor was shown to inhibit both IgM and IgG antibody responses. Another well documented nonspecific suppressor T cell factor has been described by Fridman and coworkers. From extracts of spleens of lethally irradiated BALB/c mice injected with C3H thymocytes a T cell factor was isolated which inhibits the *in vitro* IgM and IgG antibody responses of spleen cells to T-dependent as well as T-independent antigens. When such alloantigen-activated T cells were cultured *in vitro*, the factor was released into the supernatant (Gisler and Fridman, 1975; Neauport-Sautes et al., 1975; Gisler and Fridman, 1976). The factor only binds antigen-antibody complexes containing IgG. This occurs via the Fc part of the IgG. It is proposed that this T cell product is related to a soluble form of the T cell Fc receptor for IgG, which would be expressed on nonantigen-specific suppressor T cells (Fridman et al., 1977a). By the same workers it has been reported that a similar factor can be produced by a T cell hybridoma cell line which was made by fusion of T lymphoma cells and SRBC-primed spleen cells or alloantigen-activated T cells (Neauport-Sautes et al., 1979).

4.5. Pathways of T cell regulation

Humoral immune responses are mostly the result of delicately balanced interactions of several cell types. T cells constitute the major cell type involved in immune regulation. In general, three major types of T cells are distinguished, helper T cells, suppressor T cells and amplifier T cells. The latter subpopulation of T cells can participate in the regulatory network of T

cells involved in antibody formation by driving antigen-reactive B and/or T cells to extra rounds of proliferation.

With respect to helper T cells and suppressor T cells it has been shown that they belong to discrete subpopulations with their own characteristic phenotype, each incapable of generating cells of the alternative phenotype (Rich and Pierce, 1974; Feldmann et al., 1975; Stout and Herzenberg, 1975). The distinct nature of both cell types has been clearly shown in transfer experiments in which both cell types were allowed to repopulate separately syngeneic, lethally irradiated T cell-deprived mice. As tested with SRBC, such animals display only the immune function associated with the transferred T cell subset (Huber et al., 1976).

It is proposed that both the helper and suppressor T cells are already committed to their effector function before antigen contact. In spleen cell cultures ConA can activate helper as well as suppressor T cells in the absence of antigen. Administration of each of the two activated T cell populations separately to SRBC-stimulated spleen cell cultures caused a stimulation or suppression of the specific antibody formation, depending on the T cell population added. After selective elimination of one of the T cell subpopulations *before* polyclonal stimulation, the other remains which, after ConA treatment, either stimulates or inhibits the humoral immune response, depending on which cell population had been eliminated (Jandinsky et al., 1976).

One of the pathways in the suppression of B cell activity is the direct interaction between suppressor T cells and the B cells which mediate the humoral immune response to T-independent antigens like SSSIII and polyvinyl pyrrolidone (Baker et al., 1970; Kerbel and Eidinger, 1972). Depletion of the T cell population by ATS treatment markely enhanced the antibody production to these antigens. Since no T cell help is required for the antibody formation to such T-independent antigens, it was likely that the immediate target for this normally occurring suppression is the B cell (Baker et al., 1974). However, other experiments indicate that the primary IgM response to SSSIII is influenced not only by suppressor T cells, but also by amplifier T cells (Baker et al., 1973). The latter cell type was proposed to exist since infusion of peripheral circulating lymphocytes into T cell-deprived mice could cause an additional enhancement of the anti-SSSIII antibody response. In normal mice it was found that enhancement of the antibody response to SSSIII by administration of ConA 2 days after immunization depends directly on the function of amplifier T cells, which are suppressed during

the early phase of the primary IgM response because of the presence of suppressor T cells. If these suppressor T cells are removed at the time of immunization, the amplifier T cells will stimulate the proliferation of SSSIII-specific B cells. At present it is supposed that the suppressor T cell is the central cell controlling the magnitude of the humoral immune response to SSSIII; at the one hand by limiting the activity of the amplifier T cells and at the other by suppressing the B cell activity (Markham et al., 1977a; 1977b).

B cells responding to T-dependent antigens can also be directly influenced by amplifier T cells (Muirhead and Cudkowicz, 1978). It was shown in adoptive transfer experiments that initially carrier-primed helper T cells constitute the major cell type which determine the magnitude of a primary IgG anti-TNP antibody response. In a later phase the B cell response could be enhanced by amplifier T cells if the antigen was still present. These cells provided an additional positive signal for the sensitized B cells to proliferate. Both types of T cells can act synergistically (Muirhead and Cudkowicz, 1978).

The amplifier T cell has also been described to play an important role in the generation of active helper and suppressor T cells (Feldmann et al., 1977; McDougal et al., 1979). Both the helper and the suppressor activity during an *in vitro* primary IgM response to TNP-KLH requires a T-T interaction with the amplifier T cell for their induction (Feldmann et al., 1977). Especially the interaction of the amplifier T cell with the suppressor T cell is proposed to play an important role in the feedback control of the humoral immune response. During a primary *in vitro* anti-SRBC response a particular subset of antigen-stimulated helper T cells induces a nonimmune amplifier T cell to participate in specific suppressor activity (Eardly et al., 1978; Cantor et al., 1978). The suppressed B cell activity is at least in part the consequence of inhibition of the T helper activity (Eardly et al., 1978). There are indications that this mechanism of feedback control by T cells is particularly relevant for the duration and intensity of IgE-mediated hypersensitivity. This is apparent from the observation that selective elimination of helper and amplifier T cells abolished the suppression of the IgE antibody response in adoptively transferred mice (Watanabe et al., 1977).

The most frequently described interaction among T cells is the influence of suppressor T cells on helper T cell activity. For instance, strains of mice that are low responder for GAT or GT are suppressed in their IgG antibody responses to these anti-

gens. This is at least in part due to antigen-specific suppressor T cells which act on helper T cells (Kapp et al., 1974; Kapp et al., 1975; Debré et al., 1975; Debré et al., 1976). The deficiency was not due to an inability to generate helper T cells, since under appropriate conditions nonresponder mice were shown to develop antigen-specific helper T cells (Kapp et al., 1975; Debré et al., 1976; Benacerraf and Germain, 1978). Also *in vitro* it has been shown that suppressor T cells can interact with helper T cells. Cultures of spleen cells and thymocytes are a rich source of antigen-specific suppressor T cells if during culture a high concentration of the inducing T-dependent carrier (e.g., KLH) is present. These suppressor cells inhibit the IgM response of unprimed B cells to a hapten-carrier complex of the same carrier (e.g., TNP-KLH) via the helper T cells which are necessary for this response (Kontianen and Feldmann, 1976).

Investigations into the mechanism of allotype suppression in hybrid mice revealed that the target for the suppressor T cells involved in this phenomenon is not the B cell itself, but rather an allotype-specific helper T cell, which is required for the production of that allotype (Herzenberg et al., 1976). This was based upon the observation that mice which were suppressed for the maternal allotype of IgG2a lack the specific T helper activity, which is necessary to trigger B cells producing that particular allotype. The suppression must be due to complete elimination of functional helper T cells, since removal of the suppressor T cell population from spleens of suppressed mice did not restore the ability of the B cells to produce antibodies of the suppressed allotype after adoptive transfer. However, recent data suggest that the B cell can also be directly prevented from the production of immunoglobulins of a particular allotype. This can be concluded from the observation that infusion of allotype-specific suppressor T cells in syngeneic hybrid nude mice results in ceasing of the corresponding naturally occurring IgG2a allotype in the serum of these animals (Jacobson, 1978).

Suppressor T cell - helper T cell interactions have also been reported for the regulation of production of idiotypic antibodies. In the A5A system a selective suppression of A5A-specific helper T cells by suppressor T cells occurs in mice pretreated with low doses of guinea pig IgG2 anti-A5A idiotypic antibody. Once generated, such specific suppressor T cells prevent effectively the formation of A5A positive antibodies to Streptococcal-A vaccin, since T cell elimination from a suppressed spleen cell population enables the A5A idiotype

positive B cells to produce antibody if purified helper T cells are added (Hetzelberger and Eichmann, 1978).

As for allotypic suppression, there are indications that idiotypic suppression might be due to a direct influence of suppressor T cells upon the B cells. In the Ars system it has been demonstrated that mice pretreated with anti-CRID generate suppressor T cells which specifically recognize the $F(ab')_2$ fragments of CRID antibodies (Owen et al., 1977a). It has been shown that such suppressor cells are involved in suppression of CRID antibody formation (Owen et al., 1977b) which might suggest that in T cell-mediated suppression of idiotypic antibody formation the recognition of Ig-structures (e.g., $F(ab')_2$ fragments) is involved. Another indication for such interaction has been described for the regulation of the expression of the MOPC-460 idiotype during anti-TNP responses to various T-independent antigens. Depletion of T cells from suspensions of spleens cells from immunized mice, before culturing *in vitro*, significantly increased the production of MOPC-460 positive anti-TNP antibodies. Adsorbtion of these T cells on plastic plates coated with the MOPC-460 myeloma protein revealed a subpopulation of T cells specifically binding this protein. If such cells were recovered from the plates and added to T cell-depleted spleen cell cultures responding *in vitro* to TNP coupled to Nocardia mitogen, a marked inhibition was noticed for the production of the antibodies idiotypically related to MOPC-460 (Bona and Paul,,1979).

5. *HETEROGENEITY OF SERUM IMMUNOGLOBULINS*

5.1. *Heterogeneity of B cell clones during antibody formation*

Antibody responses are generally characterized by a marked degree of structural and functional heterogeneity. Since individual B lymphocytes produce a homogeneous product (Klinman, 1969), it is assumed that such heterogeneity is the consequence of the stimulation of different clones of B lymphocytes. This heterogeneity in antibody molecules can be expressed both functionally (antigen-binding avidity) as well as physicochemically. As far as the avidity is concerned, it has been shown that the antibodies produced early in the response have a great variability in efficiency of binding the antigen (Eisen and Siskind, 1964). Later, predominantly high avidity antibodies are produced (Miller and Segre, 1972; Hucket and Feldmann, 1973; Werblin et al., 1973).

Also with respect to the physicochemical properties, the repertoire of antigen-specific antibodies to most antigens is highly diverse. In the isoelectric focusing assay, a complex spectrum of bands of serum antibodies is found, even when mice are immunized with relatively simple haptenic antigens (Kreth and Williamson, 1973). At present, some antibody responses which are restricted in their heterogeneity are known. This is because of the fact that only a limited B cell pool is stimulated. An example of such a restricted B cell response is the antibody production against PC in BALB/c mice. Over 90% of all PC specific antibodies bear the T15 idiotype (Cosenza, 1976; Claflin and Cubberley, 1978). Although this antibody response is primarily restricted to the T15 idiotype, physicochemical heterogeneity can still exist, because clones producing this idiotype can belong to different Ig isotypes. This is apparent from the isoelectric spectrum of T15 positive anti-PC antibodies. In the first place, the T15 positive antibodies belong to different Ig H-chain isotypes, i.e., IgM, IgG and IgA (Gearhart et al., 1975). In addition, three major subsets of T15 positive IgG antibodies which belong to the subclasses IgG1, IgG2 and IgG3, respectively, have been distinguished. Even in antibodies belonging to one particular subclass, physicochemically different antibody molecules can be produced, since, within each IgG subclass, at least two, but often more, related sets of homogeneous bands are detected in isoelectric focusing (Claflin and Cubberley, 1978).

Another well-known example of a restricted antibody response is the immune response to bacterial antigens such as pneumococcal and streptococcal polysaccharides in rabbits and mice (Braun and Jaton, 1974). Animals with high serum titers usually have electrophoretic restrictions of the specific antibodies, with a clonal dominance of one or a few clonotypes. Structural and functional analysis revealed a molecular uniformity and thus a monoclonal origin of these antibodies, i.e., they were shown to belong to a single Ig class, L-chain type, allotype and idiotype.

The heterogeneity of the antibody response is largely dependent on T cells. Thymectomy of mice causes an acceleration of the age-associated loss of IgG and high affinity antibody production to T dependent DNP-conjugated carriers (Weksler et al., 1978). Furthermore, in microcultures of primed B cells responding to TNP-Ovalbumin, only a restricted physicochemical heterogeneity of hapten-specific antibodies has been assessed if limited numbers of helper T cells were available. When large numbers of T cells were added, the isoelectric spectrum of supernatant antibodies became very heterogeneous and the precise number of clones could no longer be firmly established (Phillips and Waldmann, 1977). Also *in vivo* it has been found that mice largely depleted of T cells were incapable of producing a broad antibody spectrum in response to T-dependent antigens. Adult thymectomized mice which had been treated with ATS were capable of developing considerable antibody titers to SRBC after repeated hyperimmunization with this antigen. Isoelectric focusing of these sera, however, revealed that the antibodies produced were less heterogeneous than the antibody spectrum found in sera of normal mice (Doenhoff et al., 1979).

5.2. *Paraproteinaemia in lymphoreticular malignancies*

The heterogeneity of antibody production is directly reflected in the heterogeneity of serum Ig's. Involvement of only a few antigen-reactive B cell clones in an antibody response will be manifested in a restricted heterogeneity of the serum Ig spectrum. Dominance of a single B cell clone can lead to excessive production of a particular Ig, which will appear in the serum as a homogeneous Ig component or paraprotein. In Table III (according to Radl, 1976), an overview of conditions frequently associated with homogeneous Ig components is presented. In this thesis, a homogeneous Ig component will be referred to as H-Ig if the underlying cause is unknown and its appearance is transient. In situations were the component is persistent

and is related to a malignant or a benign disorder of the immune system, it will be referred to as a paraprotein. Consequently, the condition in which the paraprotein occurs is called a paraproteinaemia.

TABLE III. OCCURRENCE OF HOMOGENEOUS IMMUNOGLOBULINS IN SERA OF MAN AND SOME OTHER ANIMAL SPECIES

Condition	Human	Animal model	Paraproteinaemia
1. B-cell neoplasia	(a) Myeloma, Waldenström's macroglobulinaemia, lymphoma-heavy chain dis.	Dog, cat, cow-myeloma rat, mouse-plasma cell tumor	(a) Obligatory
	(b) Lymphosarcoma, chron. lymphatic leukemia, amyloidosis	Mouse-"plasma cell leukemia", lymphoma, SJL/J dis.	(b) Facultative
2. Immunodeficiency diseases	Wiskott-Aldrich syndrome, severe combined immunodeficiency		Often transient
3 Early ontogenesis with excess stimulation	Intrauterine infections		Transient
4. Reconstitution of the immune system after bone marrow transplantation	Immunodeficiency diseases; aplastic anemia, leukemia, pretreatment with an immunosuppressive regimen	Irradiated mouse, -"- Rh. monkey	Transient
5. Aging	Idiopathic paraproteinaemia (monoclonal benign gammapathy)	Mouse (C57BL/KA)	Frequency: in man from 0% in the 3rd decade up to 19% in the 10th decade; in C57BL mouse from 0% in the 1st month up to 60% in the 30th month
6. Autoimmune diseases	chronic cold agglutinin dis.,chronic autoimmune liver dis.	Mouse (NZB)	
7. Infections,tumors, liver and skin diseases	Reactive paraproteinaemia? lichen myxedematosus, mycosis fungoides	Aleutian mink dis., hamsters with xenogeneic tumors?	
8. Immunization with haptens and polysaccharides	E.g., dextran, levan	Rabbits, mice	Genetically determined.

From Radl, 1976.

The best known group of lymphoreticular malignancies accompanied by paraproteins represent a disorder of the B cell lineage of the immune system. Lymphomatous diseases of B cells include neoplastic transformation at various stages of differentiation. In these cases, the production of a paraprotein depends on the differentiation stage in which the neoplastic event occurs and the capacity for further maturation into an Ig secreting cell. In man, paraproteinaemia has been primarily associated with lymphocytoid-plasmacytic or plasmacytic tumors (Waldenström macroglobulinemia, multiple myeloma), although neoplastic events in earlier differentiation stages of the B cell lineage

(lymphocytic lymphomas, chronic lymphocytic leukemia) can also be associated with paraprotein production (Warner et al., 1974). With respect to the latter, various lymphoma-like tumors which are accompanied by the production of paraproteins have been described in mice. Rask-Nielsen and coworkers, for instance, described lymphoreticular malignancies with a plasmacytoid morphology (according to their own nomenclature: plasma cell leukemia) which give rise to the appearance of paraproteins in serum. This may be because either the neoplastic cells indirectly induce a dysfunction in the Ig-synthesizing apparatus or the neoplastic cells themselves mature into Ig-secreting plasma cells (Rask-Nielsen et al., 1960; Clausen et al., 1960; Ebbesen and Rask-Nielsen, 1967; Rask-Nielsen et al., 1968; Rask-Nielsen and Ebbesen, 1969).

A relatively high frequency of paraproteinaemia occurs in certain mouse strains. Aging NZB and NZB-hybrid mice are extremely susceptible to autoimmune diseases (De Vries and Hijmans, 1966, 1967) and often develop lymphoid malignancies accompanied by IgM paraproteinaemia (East, 1970; Sugai et al., 1973). Paraproteinaemia also frequently occurs in SJL/J mice. During senescence, these mice show a high incidence of spontaneous reticulum cell tumors (Dunn and Deringer, 1968). Serum electrophoresis revealed that, at the early onset, these malignancies are often accompanied by restricted heterogeneity of Ig's and/or the occurrence of paraproteins which are predominantly of the IgG class (Wanebo et al., 1966; McIntire and Law, 1967; Haran Ghera et al., 1973). However, although these tumors probably originate from B cells (Carswell et al., 1976; Lerman et al., 1976), it is not likely that there is a direct correlation between the development of a reticulum cell sarcoma and paraproteinaemia. This is based on observations in C57BL/KaLwRij mice. These mice also show an age-related pathology which is determined to a large extent by the prevalence of developing reticulum cell sarcomas (Blankwater, 1978; Radl et al., 1978). Transplantation experiments have shown that, in cases were both a reticulum sarcoma and a paraproteinaemia occurred, the paraproteinaemia could not be propagated in young syngeneic recipients (Radl et al., 1979). In the same study, lymphoblastic lymphomas were also tested for a direct relationship with concomittant paraproteinaemia. In one case, serial transplantation into young recipients revealed that the paraproteinaemia (IgM, κ) could also be propagated. In addition, it was established by immunofluorescence that the IgM paraprotein originated from neoplastic B cells (Radl et al., 1979).

The relationship between paraprotein production and tumor development is more evident in the case of the plasmacytic tumors, since there the neoplastic event is usually directly related to the Ig-secreting cell population. Although spontaneous neoplastic transformation in plasma cells (plasmacytomas) is quite a rare event in mice (Dunn, 1957), they have been intensively studied because some mouse strains are highly susceptible to artificial induction of these tumors (Potter, 1972). In BALB/c mice, it was discovered that intraperitoneal implantation of solid plastic materials or intraperitoneal injection of adjuvants, mineral oils or pure alkenes (e.g., pristane) induced the formation of granuloma tissue in which plasmacytoma could arise (Merwin and Algire, 1959; Merwin and Redmon, 1963; Anderson and Potter, 1969). When this was done, 60-70% of the mice developed a plasma cell tumor within 5 to 8 months (Potter, 1972; 1973; 1975). A high susceptibility to plasmacytoma induction has also been found in NZB and (NZB x BALB/c)F1 hybrid mice (Goldstein et al., 1966; Warner, 1971; 1975).

Many of these plasmacytomas can be continuously propagated by serial transplantation of neoplastic cells into syngeneic recipients. The take-frequency of stable cell lines is usually near 100%. After transplantation, such cells show continuous proliferation; consequently, their products accumulate in the serum as paraproteins. Very high serum levels of these paraproteins are reached in some cases. In such a state, the levels of some of the other serum proteins (e.g., albumin and Ig of other classes and subclasses) are reduced. Mice which receive a transplant of neoplastic plasma cells always have shortened lifespans.

A large proportion of all mouse plasmacytomas produce paraprotein of the IgA class. This is probably due to the fact that these tumors are induced in the vicinity of the digestive tract, an important site of localization of potentially IgA-producing cells. In addition to IgA-producing plasmacytomas, plasmacytomas producing paraproteins of IgM and all subclasses of IgG have also been documented. (Potter, 1972; Warner, 1975). Plasmacytoma-derived paraproteins are thought to represent ordinary Ig molecules belonging to the normal repertoire of antibodies. An indication for this is the observation that some of these paraproteins have binding activity for haptenic antigens derived from the environment of the mouse (microbes, food, bedding) (Potter, 1970; 1971; 1977). Furthermore, it has been noted that there can be a great similarity between PC- and dextran-binding paraproteins, on the one hand, and normal antibodies, on the other, with respect to idiotype and cross reactive specificities

(Lieberman et al., 1974; Weigert et al., 1974; Hansburg et al., 1976).

The most common form of plasma cell neoplasia in man is multiple myeloma. This condition is primarily related to infiltration of neoplastic cells into the bone marrow. However, there are indications that B lymphocyte clonal ancestors of the malignant plasma cells are not restricted to the bone marrow, since, in the peripheral blood of patients with multiple myeloma, a proportionally expanded population of cells was found to be idiotypically related to the products of the neoplastic plasma cells in the bone marrow (Mellstedt et al., 1976; Kubagawa et al., 1979). This is not analogous to the artificially induced plasmacytomas in mice, since the localization of the primary tumors is mainly restricted to the peritoneal cavity. Another discrepancy between the plasma cell tumors in mice and man is the fact that murine plasmacytomas predominantly produce IgA paraproteins, while a preponderance of IgG has been noted in multiple myeloma in man (Zawadzki et al., 1967). However, these discrepancies might be due to the method of induction of the plasma cell tumors in mice. A murine equivalent of human myeloma has been recently described in C57BL/KaLwRij mice (Radl et al., 1979). That spontaneously arising tumor produced an IgG2a (κ) paraprotein with anti-DNP antibody activity. The bone marrow and spleen of the mouse were found to be heavily infiltrated with neoplastic cells containing cytoplasmic IgG2a. This myeloma cell line could be continuously propagated without loss of the capacity to produce the original paraprotein.

Salmon and Seligmann (1974) have advanced a hypothesis which states that the development of myeloma requires two hits. The first hit is due to antigen and leads to monoclonal B cell proliferation. This may represent a preneoplastic state. Such clones may involute or they may be subsequently transformed into myeloma. The second hit (which may occur months or years later) is the oncogenic or mutagenic stimulus (e.g., oncorna virus) which transforms a susceptible subclone in this population. This subclone then undergoes neoplastic growth.

Once transformed that subclone will initially follow an exponential growth curve. However, with time, this curve will reach a plateau. At this point, only a small percentage of the tumor cells are actively proliferating. Salmon and Seligmann proposed that this slowdown of the expansion of tumor cells is the result of some form of feedback inhibition. Evidence supporting such a feedback regulation can be derived from experiments with murine lymphoid tumors.

5.3. *Immunoregulation of neoplastic B lineage cells*

Although malignant lymphoid tumors represent an irreversible imbalance in the immune system, it has been shown that, despite their progressive character, some of these tumors still retain some responsiveness to host immune regulatory influences. Specific suppression of the development of several B cell lymphomas has been observed in mice which were immunized with the paraproteins of these tumors or were infused with the corresponding anti-idiotypic antibodies before inoculation of an otherwise lethal number of tumor cells (Sugai et al., 1974; Haughton et al., 1978). Inhibition of the growth of a transplanted plasmacytoma in mice can occur if the animal mounts a humoral immune response to the paraprotein of that plasmacytoma (Meinke et al., 1974; Eisen et al., 1975). Recently, a direct humoral influence on the paraprotein production of plasmacytoma cells has been observed *in vitro*. Culturing the anti-DNP antibody (IgA) producing plasmacytoma MOPC-315 with homologous or heterologous DNP-conjugated γ-globulin complexes specifically inhibited IgA antibody synthesis and secretion by this plasma cell tumor (Abbas and Klaus, 1978).

T cells can also modulate the paraprotein production of plasmacytomas. Allotype specific suppressor T cells which can regulate the IgG2a allotypic antibody levels can also suppress the growth of an IgG2a producing plasmacytoma cell line (Bosma and Bosma, 1977). Furthermore, allogeneically stimulated, MHC specific T cells can abrogate the antibody production of cultured Ig-secreting tumors such as MOPC-104E (IgM) and MOPC-315 (IgA) (Abbas, 1979). Rohrer and Lynch (1978) have shown that the progressive differentiation of MOPC-315 from nonsecreting lymphocytoid cells into Ig-secreting plasmacytes in intraperitoneally implanted diffusion chambers can be modulated by specific helper and suppressor factors of T cell origin. In a later study, it was reported that the paraprotein production by this tumor is susceptible to feedback regulation, since isologous immunization with the paraprotein induces idiotype-specific T cells which, after adoptive transfer, suppress IgA secretion by MOPC-315 cells (Rohrer et al., 1979). When MOPC-315 cells were subcutaneously or intravenously inoculated into mice which contained antigen-specific helper T cells or suppressor T cells, the progressive character of the tumor cells was initially promoted and antagonized, respectively. With time, only inhibition of the tumor growth occurred in both groups of mice (Rohrer and Lynch, 1979).

In conclusion, several lymphoid tumors can be manipulated by

immunoregulatory mechanisms. However, the physiological significance of these effects is still unclear.

5.4. *Paraproteinaemia in nonmalignant conditions*

H-Ig or paraproteins have also been observed in individuals without a B cell malignancy. In man as well as in mice, an increasing incidence of serum Ig's of restricted heterogeneity accompanied by H-Ig and/or paraproteins has been observed during aging. Evaluation of clinical data revealed that, in cases of permanent paraproteinaemias, the benign forms (i.e., without clinical signs of malignancy) are about 100 times more frequent than those caused by B cell neoplasia. Their frequency increased from 0% in the third decade of life to 19% in the tenth decade (Radl et al., 1975). Such persistent forms of paraproteinaemia in apparently healthy individuals have been given many different names, but here the term *idiopathic paraproteinaemia* (IP) (Radl and Masopust, 1964) will be used. In contrast to malignant paraproteinaemia, IP does not influence the life span of the affected individuals. With respect to the class distribution of the idiopathic paraproteins, the majority belongs to the IgG class (Zawadzki and Edwards, 1972; Waldenström, 1973; Radl et al., 1975; Axelsson, 1977).

Comparative investigations of various mouse strains revealed a great variability with respect to the onset and incidence of H-Ig in the sera of aging mice. The highest frequency was found in C57BL/KaLwRij mice, where H-Ig components have been detected as early as 3 months of age. In contrast, CBA and BALB/c mice have a very low incidence of H-Ig components, which are not seen before 21 months of age. In a follow-up study, it was established that the components were persistent and not associated with malignancy in the majority of the animals. Although no exact estimation was made, it was suggested that the ratio between benign and malignant paraproteinaemias in C57BL mice might be much higher than 100 : 1, a figure which has been reported for man. The class distribution of these permanent H-Ig components showed a predominance of IgG2a followed by IgG1, IgG2b, IgM, IgG3 and IgA in order of decreasing incidence (Radl et al., 1978). Because of the very high frequency of these persistent benign H-Ig components in the C57BL/KaLwRij mice, this strain has been chosen for studying to what extent these components can be considered as the murine counterpart of the human IP. Comparison of the characteristics of human IP and the persistent components in C57BL/KaLwRij mice revealed no essential differences. Briefly, the criteria for murine IP are:

1. Presence of a paraproteinaemia which persists for at least 6 months;
2. The level of the paraprotein does not exceed a concentration of 400 mg/dl in serum;
3. The serum level of other Ig classes and subclasses is not or only slightly affected by the paraprotein;
4. The condition of IP is not accompanied by signs of malignancy or Bence Jones proteinuria.

As in malignant proteinaemias, the B cell clones responsible for IP can be further propagated in young healthy syngeneic recipients by bone marrow or spleen cell transfer. However, in contrast to malignant clones, the "take"-frequency of transplanted IP clones decreases with each subsequent transplantation and is limited to about 4 generations (Radl et al., 1978; 1979).

During the studies on the incidence of H-Ig components in aging C57BL/KaLwRij mice, it was found that a minority of these components (10%) was transient and usually disappeared within 1 to 4 months (Radl et al., 1978). Transient H-Ig are a usual finding during the reconstitution process after a successfull bone marrow transplantation. Children with severe combined immunodeficiency (SCID) disease developed multiple transient H-Ig after receiving a suitable bone marrow graft (Radl and Van den Berg, 1973). The same phenomenon was observed in rhesus monkeys which were lethally irradiated and reconstituted with syngeneic or autologous bone marrow cells. For a number of these transient H-Ig, antibody activity towards antigenic stimulation could be established (Radl et al., 1974; Van den Berg et al., 1976). In lethally irradiated and bone marrow reconstituted mice, similar changes in Ig heterogeneity have been observed during the observation period of 1.5 to 3.5 months after reconstitution (Van Muiswinkel et al., 1976). In clinical as well as animal studies, these H-Ig were most frequently found during the first months of the reconstitution period. They gradually changed into a heterogeneous Ig spectrum as the reconstitution proceeded.

5.5. *Influence of the T cell system on the development of homogeneous immunoglobulin components*

There are several indications that restrictions of the Ig heterogeneity can be the consequence of an impairment of the T cell system. H-Ig are frequently found in patients with immunodeficiencies such as Nezelof syndrome, Di-George syndrome and Wiskott-Aldrich syndrome and bone marrow grafted patients recovering from SCID (Hitzig and Jako, 1971; Radl et al., 1973; 1976; 1979). In general, conditions under which these H-Ig

arise do not represent a defect in B cell functioning, but an impairment in the T cell system. In both the reconstitution process of bone marrow grafted SCID patients and lethally irradiated and reconstituted mice, it has been found that there are differences in the rate of recovery of the T and B cell compartments. In reconstituted mice, B cells reach their normal numbers within 4-6 weeks after bone marrow transplantation, while the T cells are still below their normal level at 30 weeks after reconstitution (Nossal and Pike, 1973; Rozing and Benner, 1976).

A direct relationship of the influence of T cells on the occurrence of H-Ig has been demonstrated in lethally irradiated mice which received a syngeneic T cell depleted bone marrow graft. In such animals, different grades of T cell impairment were induced by either thymectomy before irradiation and reconstitution or infusion of T cells in STx animals during the first week of recovery. As controls, mice which were only shamthymectomized before irradiation were used. During the reconstitution period, the highest H-Ig incidence occurred in the group of ATx mice and the lowest in the group of T cell-infused mice (Van Muiswinkel et al., 1976).

Thymectomy of neonatal and young adult mice also substantially increased the occurrence of Ig's of restricted heterogeneity, transient H-Ig and IP during aging (Radl et al., 1979; 1980b). This effect of thymectomy was observed in mice which are prone to develop H-Ig and/or paraproteins (C57BL/KaLwRij) as well as in mice which normally show a low incidence of these abnormalities (CBA/BrARij).

On the basis of above mentioned observations, a three-stage hypothesis has been worked out for the development of IP during aging (Radl, 1979b):

Stage 1. Age-related immunodeficiency
During aging, involution of the thymus and a genetically determined selective gradual decay of certain T cell subpopulations leads to alterations in the T cell functions. The progress and extent of these changes are under the influence of some extrinsic factors such as environment, chronic antigenic stimulation and virus infection.
Stage 2. Restricted heterogeneity of Ig, transient paraproteins
As consequences of the above-mentioned changes cooperation with and control of B cells by the T cells become impaired. The resulting imbalance in the immune system network leads to a restriction of heterogeneity of the immune response and excessive

clonal proliferations with an overshoot production of H-Ig antibodies.

Stage 3. Persistent paraproteinaemia

The repeated monoclonal and oligoclonal expansions result in a greater probability for either a spontaneous or a virus-induced mutation of the regulatory genes within a given B cell clone. This clone, originally turned on by an antigenic stimulus, will continue to proliferate and secrete its Ig product even after that stimulation has disappeared. This intrinsic defect in cell regulation is different from that in B cell malignancies. IP can be considered to represent a benign tumor of a B cell line.

With respect to this hypothesis, it can be mentioned that, apart from the absence of clinical signs of malignancy, the benign character of the IP has been further substantiated by the recent finding that, while increasing the frequency of IP, thymectomy does not substantially influence the incidence of lymphoreticular neoplasms or malignant paraproteinaemias in aging C57BL and CBA mice (Radl et al., 1980b). Therefore, it is likely that there are different pathways for the development of the two types of B cell tumors.

In conclusion, the finding of Ig's with restricted heterogeneity and of transient H-Ig and idiopathic paraproteins in high frequencies in individuals with a T immune system impairment supports the concept that T cells play an important role in the regulation of the heterogeneity in the synthesis of Ig's. The development of such benign disorders of the Ig-synthesizing apparatus is also dependent on the genetic background.

6. *EVALUATION OF THE METHODS USED FOR CHARACTERIZATION OF THE SERUM IMMUNOGLOBULIN SPECTRUM*

6.1. *The rocket electrophoresis method as a quantitative electroimmunoassay*

6.1.1. *Formation of precipitates*

Principle

Rocket electrophoresis can be used as a quantitative method which involves electrophoresis of antigen in an antibody containing gel (in our case, agarose). During application of an electric field, the antigen moves into the gel and forms antigen-antibody complexes which aggregate to form visible stationary precipitates if an equivalent ratio has been achieved between antigen and antibodies. At first, precipitating complexes occur at the sidelines of the pathway in which the antigen travels, since antigen excess at the front prevents precipitation of the antigen-antibody complexes. The successive consumption of antigen during formation of the sidelines finally results in a precipitation area which has the appearance of a rocket. Its length is related to the antigen concentration (Laurell, 1966; Merrill et al., 1967).

Application

In general, rocket electrophoresis is applicable as quantitation method only if the antigen has an electric charge which is other than that of the antibody molecules in the gel. Ig levels in a test sample cannot be estimated quantitatively as easily as most other proteins, since their mobility is very similar to that of the antibodies in the gel. However, carbamylation of the amino acid groups of the Ig's under investigation by potassium cyanate before electrophoresis appears to be a satisfactory method for resolving this problem. Partial carbamylation (in our case, 30 minutes at 37°C in potassium cyanate excess) increases the charge of the Ig molecule, while only a slight decrease in antibody affinity occurs (Weeke, 1968). Under high pH conditions (pH 8-9), the antibodies in the gel remain essentially stationary during electrophoresis, in contrast to carbamylated Ig's, which move to the anode.

Appearance of rocket shaped precipitates

Prolongation of the electrophoresis time after the final size of the rocket has been reached does not usually change the height of the rocket. However, immune complexes of relatively low molecular weight might still slowly diffuse through the gel without changing the shape of the rocket. Only the sharpness of the sidelines of the rocket will decrease. Comparison of peak heights

and area measurement of rockets of serially diluted samples revealed, in our hands, a greater accuracy for the latter method. Therefore, the area of the rocket has been used as the quantitation criterion. The accuracy of measurement of the surface of the rocket diminishes when the sidelines become faint. This diffusion of the precipitate can be reduced by adding 1-3% polyethyleneglycol (PEG, MW 6000-7500) to the agarose before casting the gel. This enhances complex formation and immobilizes large particles, since the free water content and the space in the gel available for migration is reduced (Zeppezauer and Brishammar, 1965; Harrington et al., 1971; Adams and Jerry, 1974).

The shape of the rocket largely depends on experimental conditions such as quality of the gel, buffer, electric field, etc. In the context of a general description of the rocket electrophoresis method, the extensive evaluation of these factors by Laurell (1972) should be reviewed. Here, only some general aspects of the antigen and the corresponding antiserum will be mentioned in relation to the most frequently occurring rocket forms. In principle, the shape of a rocket is mainly determined by (a) the molecular weight of the antigen and (b) the ratio between the concentrations of antigen and antibody.

As described in Chapter 2, there is a great diversity in the molecular form of the different Ig's. IgA can occur in different molecular forms (monomeric or polymeric, with or without SC and/or J-chain), while native IgM molecules are pentameric. IgG, on the other hand, occurs only in the monomeric form. The more polymers present in an Ig sample, the more the normal and clear-cut type of precipitate (narrow rocket-like precipitates with sharp sidelines) will change into one which is more difficult to interpret. For instance, the occurrence of highly polymeric molecules in secretory IgA can lead to rockets which have a smudged inner demarcation. In extreme cases, precipitation zones instead of lines can also be obtained. In such cases, erroneous measurement of the area of the rocket can result, since not all precipitates contribute to the outer sidelines. This precipitate trailing of the antigens is due to blocking of the gel pores by large immune complexes. By diminishing voltage (thus decreasing the reaction speed) and prolongation of the electrophoresis time, together with high dilution of both antigen and antibodies, most of this trailing phenomenon can be avoided.

The ratio between antigen and antibody can also influence the shape of the rocket. For most antigens, there is an optimum range for maximum sharpness and minimum precipitate trailing.

In general, rockets between 1 and 4 cm in height are considered to represent as closely as possible the amount of antigen applied. Rockets less than 1 cm high can have distinct and sharp sidelines, but they are too small for accurate area measurement. On the other hand, rockets higher than 4 cm often develop blunt peaks and oval precipitate lines because of the fact that not all antigen molecules have been optimally bound during the usual electrophoresis time of 18-20 hours. Consequently, these types of rockets are also inappropriate for quantitation.

As stated above, the strength of the antiserum can also contribute to the type of the rocket. A relatively weak antiserum can still cause erroneous rockets within the appropriate range of 1 to 4 cm in height. In this case, the affinity or concentration of effective antibodies for the antigen is so low that visible precipitates can be achieved only if a relatively high concentration of antiserum is applied. However, a high protein level of antibodies might interfere with the space that is available for migration in the gel, which, in turn, can cause precipitate trailing and hairy rocket borders. Increasing immune complex formation via PEG is a possibility to reduce the required antiserum concentration; however, the effect is relative, since PEG tends to decrease the pore size of the gel. Another advantage of adding PEG to the antibody containing gel is that otherwise nonprecipitating antibodies can be involved in the precipitation of antigens (Adams and Jerry, 1974).

In summary, it is clear that reliable estimations of the Ig content in test samples can be made only if sufficient knowledge is available on physical characteristics such as diffusion constant, reaction speed and strength of the antigen-antibody interaction. In this context, the molecular composition of the Ig's under investigation and the ratio between the amounts of antibody and antigen are important factors in determining the form of the rocket. However, the reliability of a quantitation of the Ig concentration in a test sample depends not only on the development of well-shaped rockets but also on the antigenic heterogeneity of the Ig's and the specificity of the available antisera. These aspects of Ig quantitation by rocket electrophoresis will be discussed below.

6.1.2. Quantitative determinations of immunoglobulins

Essential concepts

Reliable quantitation of Ig levels and expression in absolute values is extremely difficult. This is apparent from the discrepancies among the results of different laboratories which tes-

ted one particular serum sample of human origin for its content of various Ig classes and subclasses by using different techniques (Rowe et al., 1972). Similar discrepancies were found when using one particular quantitation method for various human serum samples (Ferguson et al., 1974). Evaluation of these discrepancies revealed three important variables which have to be properly considered before an Ig level can be expressed in absolute values. These variables are:

(a) the antigenic specificity and antigen binding capacity of the applied antiserum;
(b) the nature of the antigen, which includes the antigenic and molecular heterogeneity of the Ig's under investigation;
(c) the nature of the reference standard which is used for expressing the results in absolute values.

For accurate determination of Ig levels, these three variables must be closely related to each other. This means that, in principle, a useful specific antiserum must cover the antigenic spectrum of the corresponding Ig class or subclass in both the test sample and the reference standard. In addition, the molecular and antigenic heterogeneity of the Ig class or subclass in the reference standard should be related as closely as possible to that of the test sample.

Antiserum properties and immunoglobulin heterogeneity

The reliability of most immunoassays for characterization of Ig's depends primarily on the quality of the antiserum used. In the studies reported in this thesis, the murine Ig classes and subclasses were tested in immunoprecipitation techniques such as immunoelectrophoresis, immunofixation, Ouchterlony and rocket electrophoresis. Therefore, the first requirement for a suitable antiserum was its Ig precipitating capacity. Other requirements which had to be fulfilled were: high antibody titer and a high grade of purity and specificity. For mice, antisera against IgM, IgG1, IgG2a, IgG2b, IgG3 and IgA are commercially available. However, the quality can vary from one supplier to another and also between different batches from the same supplier. Therefore, it is imperative to test an antiserum thoroughly in different immunoassays before it is applied in the experiments for which it is ultimately destined. In our case, antisera for determinations of Ig classes or subclasses were tested against normal mouse serum (NMS) in immunoelectrophoresis for isotype specificity and precipitating capacities. Furthermore, the antiserum was tested by rocket electrophoresis in different antigen (NMS)-antibody ratios in order to determine whether only one precipitate would develop. After these tests, the optimal conditions for electrophoresis (antigen-antibody

ratio, application of PEG, current conditions, etc.) were adapted to the nature of the Ig's in the test samples and the reference standard in order to obtain measurable rockets.

Most heterologous antisera of commercial origin are raised against artificially induced murine myeloma proteins, since these are easy to isolate in large quantities and in a pure state. However, since serum Ig molecules are physicochemically heterogeneous, even within a certain Ig class or subclass, such antisera might be inappropriate for quantitation. This is because there is the possibility that such antisera are restricted in their capacity to recognize the full spectrum of antigenic determinants of the Ig class or subclass under investigation. If various specific antisera are available, each raised against a different myeloma protein of the same Ig (sub)class, it is desirable to check this possibility by comparing results after separate testing of the same heterogeneous sample with all of these different antisera. Alternatively, the chance of obtaining erroneous data can be reduced by pooling such antisera before use in quantitative analyses. This problem was avoided by using NMS as a reference stanard. In this standard, the absolute Ig levels of the various classes and subclasses were determined via calibration against myeloma preparations of known protein content (c.f. Table IV).

Characteristics and calibration of the reference standard
As stated above, the accuracy of quantitation of Ig levels is dependent on the reference standard from which the absolute values are derived. In man, serum Ig levels are usually measured by using an appropriate internal standard. In this internal standard, the Ig levels are calibrated against an international standard from the World Health Organization (Rowe et al., 1972). However, no internationally accepted standard of comparable nature is presently available for mouse Ig's. Although preparations consisting of one or more myeloma proteins of various Ig classes and subclasses are commercially available, their use for directly expressing experimental data in absolute values is not recommended. The reason for this is twofold. Firstly, their antigenic spectrum is not as heterogeneous as that of normal serum Ig's. Secondly, standard preparations of mixtures of purified myelomas are very expensive. The usual procedure for expressing serum Ig levels in absolute values includes an intermediate step in which a pool of NMS is used. Such an intermediate reference (secondary) standard consists of material comparable to that in the test samples. By calibrating this internal standard to different myeloma preparations, it is possible to make rough estimations of the absolute values for concentrations of different Ig classes and subclasses in test samples.

TABLE IV. SPECIFICATION OF ANTISERA AND PRIMARY STANDARDS USED FOR DETERMINATION OF THE IMMUNOGLOBULIN CONCENTRATIONS IN THE CBA AND C57BL NMS SECONDARY STANDARDS.

ANTISERUM BATCH	IMMUNOGEN	PRIMARY STANDARD	CBA NMS[x] (mg/dl)	C57BL NMS (mg/dl)	OUCHTERLONY TEST FOR ANTI-IDIOTYPE ACTIVITY
RAM/IgM (Nordic;44-878)	heterogeneous IgM cryoglobulins	MOPC-104E (Meloy[xx])	43.3	18.8	neg[xxx]
GAM/IgG1 (Nordic;33-977)	MOPC-21	MOPC-31C (Meloy)	238.3	114.7	neg
GAM/IgG2a (Meloy;B104-88570)	LPC-1	RPC-5 (Meloy)	370.1	ND	neg
GAM/IgG2a (from Dr. J. Radl)		spontaneous C57BL myeloma	ND	169.9	neg
GAM/IgG2b (Meloy;B105-77028)	MPC-11	MOPC-195 (Meloy)	110.9	86.6	neg
GAM/IgG3 (Bionetics; 8405-18/BC022)	J-606	FLOPC-21;[xxxx] normal C57BL NMS	64.0	49.7	ND
GAM/IgA (Meloy;B106-78033)	MPC-110	TEPC-15 (Meloy)	178.8	89.4	neg
RAM/IgA (from Dr. J. Radl)	TEPC-15	TEPC-15 (Meloy)	211.1	ND	pos

[x] The CBA reference serum was a pool of sera of conventionally kept mice of approximately 1 year old. The C57BL reference serum was a pool of sera of SPF barrier maintained 5-month-old mice.

[xx] Meloy reference standard J401, Springfield, Ohio, USA.

[xxx] Neg refers to negative, pos to positive, and ND to not determined.

[xxxx] The IgG3 level in the CBA and C57BL reference serum was measured against a secondary standard of normal C57BL serum with known IgG3 content. The IgG3 level of this standard had been determined previously by Dr. J. Radl by comparison with an isolated IgG3 paraprotein (FLOPC-21).

Factors leading to erroneous quantitation of murine Ig's. In introducing a secondary standard, the main error in absolute quantitation resides in the accuracy of calibration of this standard from primary reference preparations. We used a preparation from Meloy Laboratories (Springfield, Virginia, USA) consisting of a mixture of myeloma proteins and containing

one myeloma protein from each Ig class and subclass (c.f. Table IV). However, if antisera which have been raised against a single myeloma protein are used, one has to consider the possibility that the determination of Ig levels in the secondary NMS standard are based on erroneous calibration data, especially if that same myeloma protein is present as a reference in the primary standard. This is because of the fact that, in addition to antibodies specific for H-chain isotype determinants, such an antiserum usually also contains antibodies against idiotypic determinants of the myeloma protein. Consequently, this can lead to erroneously high values in determinations for the corresponding Ig class or subclass in the NMS standard. An example of such an anti-idiotype activity is presented in Table IV for the quantitation of IgA in the secondary CBA standard. In a combination of GAM/IgA (TEPC-15) and a primary standard of TEPC-15, the serum IgA content in the CBA standard was calculated to be 211.1 mg/dl, in contrast to a value of 178.8 mg/dl when a different combination was used. Therefore, we used only antisera raised against different myeloma proteins which were the same as those found in the Meloy preparation. In addition, all antisera used for calibration of the CBA and C57BL NMS standards were tested for anti-idiotype activity. A small amount of each antiserum was absorbed with NMS and tested for the absence of these antibodies by Ouchterlony immunodiffusion, using the same myeloma proteins as present in the Meloy standard in separate combinations. No anti-idiotype activity was found in any of the antisera used (c.f. Table IV).

Concerning the quantitation of secretory IgA in milk, it is possible that some underestimations have been made because the molecular composition of IgA in our standard (NMS) differs in two important respects from the IgA molecules in milk:

(a) Due to the binding of a secretory piece to secretory IgA molecules, there are some antigenic differences between secretory IgA and serum IgA. We used an antiserum which was raised to the IgA myeloma protein MPC-110. Since myeloma proteins lack the characteristic configuration of secretory IgA molecules, it is likely that the antibody repertoire of the antiserum will not optimally recognize secretory IgA.

(b) Proportionally more IgA molecules occur in highly polymeric forms in secretions than in serum. By precipitate trailing, such polymers can cause rockets which do not completely reflect the IgA concentration actually present in the test sample.

For the other Ig's in milk (mainly IgG), such quantitation problems would not be expected with NMS as a reference, since IgG most likely diffuses from the blood into the milk and IgG in milk is monomeric.

Another factor which can affect the outcome of Ig quantitation is the possible prevalence of heterologous antisera to precipitate Ig's of a particular allotype. Most commercially available antisera to murine IgG subclasses are raised against BALB/c myeloma proteins. It is generally assumed that IgG subclass-specific hetero-antisera recognize species specific antigenic determinants common to all mice. This assumption is not always justified, since antisera raised against BALB/c myeloma proteins have been shown to bind more IgG from mouse strains which have the same allotype as BALB/c mice than IgG of other mouse strains in some instances. Such an allotypically restricted capacity of antisera to precipitate IgG was primarily found for IgG2a. Especially the efficiency of binding of C57BL IgG2a molecules was extremely low (Epstein and Gottlieb, 1977).

No insufficient binding of IgG1, IgG2b or IgG3 proteins has been noted in the various strains of mice studied with our antisera. However, for IgG2a specific antisera (obtained from Meloy laboratories), also a relative deficiency in precipitating C57BL Ig's as compared with BALB/c and (allotypically similar) CBA Ig's was found. Therefore we employed an antiserum specific for the C57BL IgG2a *allotype* in experiments in which IgG2a levels had to be determined in mice with a C57BL background (see Chapter 4, section 4.1. and Appendix publication IV). In addition, a pool of normal C57BL NMS instead of a CBA standard was used as the secondary standard in these cases.

Finally, the occurrence of a paraprotein in the serum might influence the quantitation of the Ig class in which such a component occurs. This might be because the antigenic and molecular heterogeneity of such a sample is different from the NMS reference standard. This possibility was tested for IgM by adding known amounts of a MOPC-104E paraprotein to serum samples containing a normal heterogeneous IgM. For the rocket electrophoresis, it was found that the IgM concentration was directly related to the amount of paraprotein added, even if half of the IgM in the sample consisted of MOPC-104E molecules. Thus, at least with the antiserum we used for quantitation of IgM, no clear effect of the presence of a paraprotein on the quantitation of that Ig class was found.

In conclusion, a number of factors have to be considered for an adequate determination of Ig levels. Some difficulties (such as anti-idiotype activity) can be avoided by expressing the Ig levels as a percentage of a secondary standard consisting of NMS. However, in order to enable comparison with other data in the literature, the levels are also given in absolute values (mg/dl).

6.2. *Qualitative analysis of serum immunoglobulins*

Assessment of homogeneous immunoglobulins
Two criteria have to be fulfilled in the demonstration of a H-Ig or paraprotein. These are:
(a) Homogeneity of the constant part of the molecules of the suspected Ig component. Thus, the component must belong to only one H-chain isotype, allotype and L-chain type.
(b) Homogeneity of the variable part of the Ig molecules. Thus, it must possess an unique idiotype and, if antibody activity can be demonstrated, it must bind to only one antigen.

In the isoelectric focusing assay, a H-Ig or paraprotein will give rise to only a few closely related bands. This microheterogeneity in bands is due to postsynthetic changes in the biosynthetically homogeneous product such as deamination of some amino acids in the amino-terminal part of the molecules (Williamson et al., 1973). However, the different physicochemical and serological techniques for establishing the homogeneous nature of a paraprotein are not always possible to perform and a selection is usually made in order to detect and to characterize a paraproteinaemia. For the studies reported in this thesis, we have used agar electrophoresis according to the method of Wieme (1959), immunoelectrophoresis (Scheidegger, 1955) and the technique of immunofixation (Cejka and Kithier, 1976).

As can be seen in Figure 4, a serum sample separates into several fractions after electrophoresis: the albumin-, α_1-, α-$_2$, β_1-, β_2- and γ-globulin fractions. Initially, the sera are screened for paraproteins by Wieme- and immunoelectrophoresis. The criterion for designation of a paraprotein in a serum sample after Wieme electrophoresis is the occurrence of a narrow homogeneous extra band in the β-γ region. With this sensitive technique, paraproteins in concentrations of 50 mg/dl or more can be detected (Radl, 1979). A disadvantage of this technique is that it may be difficult to assess some paraproteins of the IgM and IgA classes and fast moving components of the IgG class which migrate into the α_2-β regions during electrophoresis. In this region, paraproteins can be masked by overlapping non-Ig serum proteins (c.f. Fig. 4).

By using specific antisera to Ig classes, subclasses and L-chain types in the immunoelectrophoresis technique, detection of paraproteins (indicated by agar electrophoresis) can be confirmed and in some instances extended. The criterion for a paraprotein in this technique is a symmetric deviation of a precipitin line in the same region as had been detected by agar

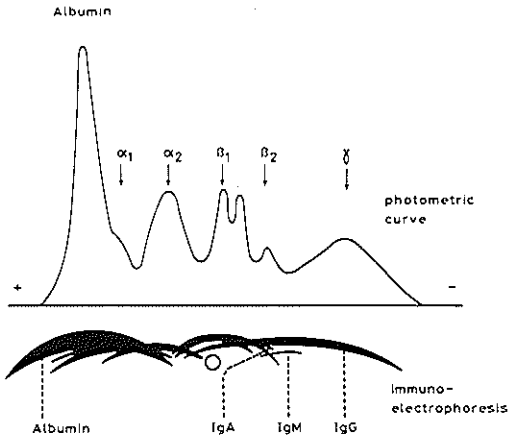

Figure 4. Simplified scheme of a photometric and immunoelectrophoretic pattern of human serum (modified from Weir, 1973).

electrophoresis. Paraproteins can be most easily detected in immunoelectrophoresis, if agar with a high electroendosmosis is used. In that case, all Ig's will drift towards the cathode to form long asymmetric (heterogeneous) precipitin lines. Moreover, this has the advantage that the precipitin lines are not distorted by the starting well. For optimal detection of paraproteins, uncontrolled variations in electrophoretic conditions (e.g., deviations in current) between different "runs" can be excluded by performing the complete classification analysis of one serum sample on one glass slide. Another advantage of this arrangement is the possibility to include NMS as a reference point to which each test sample can be compared. Furthermore, the detection of minor abnormalities (which are often seen for IgM) is easier if the Ig's under investigation are precipitated in duplicate from two troughs which are parallel to the line of electrophoretic migration of the test sample.

Paraproteinaemia can not always be established with certainty in agar electrophoresis and/or immunoelectrophoresis. This may be because either the Ig pattern does not show a clear deviation characteristic for a homogeneous component or the serum H-Ig level is too low. This latter may be the case, e.g., for IgG2b and IgA in barrier maintained (SPF) animals (c.f. Appendix publication VI). In such cases, immunofixation on the agar electrophoretic plates was also performed. After overlaying the gel with strips of acetate cellulose containing antisera with anti-Ig

or anti-Fab activity, distinct precipitin bands develop in case of paraproteins. Heterogeneous Ig's, on the other hand, form only a diffuse precipitate pattern. In an optimal antigen(Ig)-antibody ratio, this method can detect paraproteins at concentrations which are too low to be detected by agar electrophoresis. Using monospecific antisera, this technique offers the possibility to identify class, subclass and L-chain type of the paraproteinaemia. As in immunoelectrophoresis, the detection of paraproteins is not hampered by overlapping non-Ig proteins in the sample. A disadvantage of immunofixation is that it is an expensive assay, since relatively large amounts of antisera with high antibody titers are necessary for optimal detection.

A restriction in the heterogeneity of serum Ig's will be manifested in these techniques as a general reduction in the normally broad and heterogeneous Ig spectrum. Then, some Ig populations are reduced and others are increased in a rather homogeneous way. This is detected in immunoelectrophoresis as a reduction in both length and asymmetric form of an Ig precipitin line. In immunofixation, small mono- or oligoclonal Ig components can often be detected. Such an imbalance can be accompanied by an unusual ratio between the amount of κ and λ L-chains. In that case, immunoelectrophoretic analyses may reveal heavier precipitin lines of Ig's of the λ type than found for normal, heterogeneous serum Ig spectra. Normally, λ L-chains constitute about 5% of the total Ig population (see Chapter 2). Therefore, antisera specific for λ L-chains hardly form visible precipitates with normal mouse serum.

7. *INTRODUCTION AND DISCUSSION OF THE EXPERIMENTAL WORK*

Characterization of the serum Ig spectrum can be valuable in clinical diagnosis, since the level and the heterogeneity of serum Ig's may change in disease. Particularly, disorders of the immune system can be accompanied by changes in serum Ig levels (e.g., selective IgA deficiency, severe combined immunodeficiency (SCID) disease) and serum Ig heterogeneity (as in Nezeloff, Di George and Wiskott-Aldrich syndromes) (Ammann and Hong, 1973; Radl, 1976; Waldman et al., 1977). Deviations in the serum Ig spectrum can be due to abnormalities in the T as well as the B cell lineage, since:

(a) The progeny of B cells actually represent the Ig-synthesizing apparatus; and
(b) T cells regulate the synthesis of most Ig classes and subclasses.

Immune system disorders can also affect the serum Ig spectrum in experimental animals. The purpose of the experimental work presented in this thesis was to obtain quantitative and qualitative data on murine serum Ig's and the cellular background of deviations from the normal spectrum.

The serum Ig levels of normal untreated mice reported by different authors are much varied (c.f. Table II). This might be due partly to differences in age and antigenic experience of the animals (data of which are not always mentioned) or due to limitations of techniques used for Ig quantitation. As outlined in Chapter 6, many different factors may affect the outcome of the quantitation of Ig's (e.g., specificity spectra of the antisera used, allotype specificity, nature of the reference). Because we have realized these problems, a special attention was paid to the technical aspects of the qualitative and quantitative studies performed for this thesis.

Appendix publication I deals with serum and secretory Ig levels (IgM, IgG1, IgG2 and IgA, respectively) in AKR, C3H, CBA and C57BL mice. This was of interest because only one report on the concentrations of IgM, IgA and all four IgG subclasses in milk of mice is presently found in the literature (Guyer et al., 1976). However, this paper gives no information on the mouse strain used and the extent to which the concentrations of the various Ig H-chain isotypes can vary in milk among different strains of mice. We were especially interested in AKR mice, because these mice have been reported to have very low serum IgA levels (Potter and Lieberman, 1967) and are known to be in-

fected with Gross murine leukemia virus (GMuLV) throughout life. Because of this infection, they develop a malignant lymphoma from which they die between 6 and 14 months of age (Hays, 1973; Klein, 1975). There are several indications in man for a relationship between selective IgA deficiency and congenital virus infections (Soothill et al., 1966; Lawton et al., 1972) and between IgA deficiency and malignancy (Ammann and Hong, 1973). Comparison of the results of the 4 mouse strains used confirmed the observation of Potter and Lieberman that AKR mice have proportionally low IgA levels in the serum. The same was found for milk of AKR mice. The levels of IgM, IgG1 and IgG2 in serum and milk of AKR mice were quite comparable with those of the other strains. However, the low IgA level in AKR mice is probably not a major predisposing factor in the development of lymphoma. This view is based upon studies on the relationship between the serum IgA level and the susceptibility to lethal GMuLV infection, which are still in progress. Using 20 congenic lines derived from the F2 generation of a cross breeding of AKR and C57L mice, we could not find a positive correlation between the serum IgA level and the incidence of the GMuLV-induced lymphoma. Instead, we found a positive correlation between the low serum IgA level and the Ig-1^d allotype (Taylor, Cherry, Mink and Benner, to be published).

In Appendix publication II, the cellular background of the low IgA serum level of AKR mice was investigated by transplantation of AKR bone marrow cells into C3H mice and *vice versa*. C3H mice were chosen for this purpose for two reasons. Firstly, the AKR and C3H strains differ greatly in serum IgA content; this enables characterization of the IgA levels in the sera of the chimeric mice as "AKR-type" or "C3H-type" (Potter and Lieberman, 1967; Appendix publication I). Secondly, AKR and C3H mice are compatible for the MHC and this favors a successful grafting of allogeneic bone marrow cells. By quantitation of the recovery of the serum IgA level after allogeneic bone marrow transplantation, it was established that AKR hemopoietic stem cells can give rise to a C3H-like IgA level. This IgA was of the AKR allotype. On the other hand, after transplantation of C3H bone marrow cells into lethally irradiated AKR mice, only a low serum IgA level was found in these chimeric mice. Apparently, in this experimental set up, the serum IgA level is primarily determined by the genetic background of the host and not by the genetic background of the B cells. Since a normal catabolic rate of IgA was found and no autoimmunity to IgA could be demonstrated in AKR mice, we conclude from the above studies that the low serum IgA level in AKR mice can be determined by two, possibly independent, factors. One of these is related to the Ig-1^d allotype and the other is an irradiation-resistant factor capable of preventing

the transplanted bone marrow cells from producing high serum IgA levels.

The influence of T cells on the serum Ig spectrum was studied in mice which were devoid of a thymus. Two models were used: congenitally athymic nude mice and adult thymectomized mice.

Appendix publication III describes the influence of the absence of the thymus on the serum concentrations of IgM, IgG1, IgG2a, IgG2b, IgG3 and IgA as well as on their heterogeneity in 6, 40 and 110-week-old nude mice. With respect to the serum Ig levels, the T cell-deficient state of athymic nude mice is known to have a profound effect on the serum concentrations of most Ig classes and subclasses. Conventionally kept nude mice aged between one and six months generally show reduced serum levels of IgG and IgA (Pantelouris, 1978).

No long-term observations on serum Ig levels in untreated nude mice have been described in the literature. This is probably due to the fact that it is impossible to keep these animals alive and healthy for a long period if they are raised under conventional conditions. However, such investigations are made possible by maintaining nude mice under low pathogenic conditions. Appendix publication III deals with barrier maintained SPF nude mice kept for up to 110 weeks of age. With the exception of IgG1, the data from the literature were confirmed, in that serum levels of IgG2a, IgG2b and IgA were lower in athymic nude mice than in their euthymic (heterozygous) littermates. For the IgG1 and IgG2 serum levels in nude mice, there is a discrepancy between their production after intentional immunization and their serum concentrations. Nude mice generally produce primarily antibodies of the IgM class (Pritchard et al., 1973; Wortis, 1974). Therefore, it was not expected to find in the 40-week-old group that a considerable number of the nude mice tested had serum levels of IgG1 and (to a smaller extent) IgG2a and IgG2b which were equal to or even increased as compared with the heterozygous littermates. This discrepancy might be related to nonspecific Ig synthesis which generally accompanies specific antibody production (Avrameas et al., 1976; Rosenberg and Chiller, 1979). Another possible cause might be the hepatitis virus infection which was found in virtually all 40-week-old nude mice. It is known that such an infection in nude mice can induce considerable IgG antibody production (Tamura et al., 1978). Although this second possibility seems to be attractive, the possibility that the high serum levels of IgG1 of nude mice are independent of infection has to be considered, since similar observations have been made in germ free nude mice (Okudaira et al., 1977).

In Appendix publication IV, the deficiency in the Ig-synthesizing apparatus of nude mice was further analysed by comparing the levels of various serum Ig's and their corresponding numbers of cytoplasmic Ig-containing cells (C-Ig cells) in the lymphoid organs. This was done in untreated and thymus transplanted nude mice as well as in untreated heterozygous littermates. At 4, 8, 12 and 32 weeks after thymus transplantation, the influence of the recovery of the T cell system on the number of C-Ig cells was determined. This experimental set-up was of interest because the long-term effect of thymus transplantation in nude mice on the number of C-Ig cells in various lymphoid organs and the Ig class distribution of the C-Ig cells are unknown. In addition, there are no available data in the literature on the kinetics of the recovery of the Ig-synthesizing apparatus in nude mice after thymus transplantation, although it is known that transplantation enables them to develop normal serum levels of all Ig classes (Wortis, 1974). In this study, it was shown that the mean serum Ig levels of the various classes and subclasses in untreated athymic nude mice roughly correlated with the mean number of the corresponding C-Ig cells, if corrected for pool size and metabolic rate. IgM clearly predominated in the nude mice. In recently thymusgrafted nude mice, a sharp increase in serum Ig levels as well as in C-IgG1, C-IgG2 and C-IgA cells was observed. Two months after thymus transplantation, the values for thymus transplanted nude mice even surpassed those of the heterozygous littermates. Especially for IgG1 and IgG2 was a clear simultaneous increase found in the number of C-Ig cells in spleen and bone marrow and in the serum Ig concentration. Since the C-Ig cell number in the other lymphoid organs were hardly increased in the same period, these results are compatible with the view that the spleen and bone marrow are the major sources of serum IgG1 and IgG2 in young adult mice.

Besides the effect of the athymic state on the quantities of serum Ig's, we investigated the influence of this condition on the heterogeneity of the serum Ig's. Disorders of the immune system are accompanied by the production of H-Ig components and/or paraproteins. The paraproteins can be of a benign or a malignant character. Benign paraproteinemias are far more frequent than the malignant ones. The T cell system plays an important role in the regulation of the heterogeneity of serum Ig's. In the serum of individuals with a T system impairment, restrictions in the Ig heterogeneity and the occurrence of H-Ig components are frequent findings (Radl, 1979a). A direct correlation between the severity of the T system impairment and the frequency of H-Ig components was demonstrated in adult thymectomized, lethally irradiated, reconstituted mice with different

grades of T cell deficiency (Van Muiswinkel et al., 1976). In this investigation, T cell depleted mice showed a high H-Ig incidence during the observation period of 3 months, in contrast to animals which received infusions of T cells after irradiation and reconstitution.

In Appendix publication V this observation was extended by comparing the influence of previous thymectomy, splenectomy and the combination of thymectomy and splenectomy on the occurrence of H-Ig in lethally irradiated, bone marrow reconstituted mice. The splenectomy was incorporated into the experimental design because it has been claimed that the absence of the spleen can affect the cooperation between T and B cells in adoptive antibody formation (Bucsi et al., 1972; Wargon et al., 1975). Both the total incidence and the Ig class distribution of H-Ig were determined in the various experimental groups. During the follow-up period of 9.5 months, most H-Ig components appeared to be transient; this is in accord with clinical and experimental studies of individuals who received a successful bone marrow graft (Radl and van den Berg, 1973; Radl et al., 1974). It was further found that thymectomy alone can increase the H-Ig incidence. The peak incidence occurred between 1.5 and 3.5 months after bone marrow transplantation. In this period, the H-Ig components belong primarily to the IgG1 and IgG2 subclasses. Later the H-Ig incidence of the IgM class also increased. H-Ig of the IgA class were found only sporadically. The predominance of the components of the IgG class in operated as well as sham-operated mice might be for two closely related reasons:

(a) the IgG antibody production is relatively T cell dependent as compared with IgM (Dresser, 1972; Mitchell et al., 1972; Davie and Paul, 1974; Tingle and Shuster, 1974); and
(b) an imbalance between T and B cells can cause a dysregulation of the T cell dependent Ig production.

The latter is based on the observation that, after lethal irradiation and bone marrow reconstitution, the B cell population recovers at a faster rate than does the T cell population (Nossal and Pike, 1973; Rozing and Benner, 1976). The reason for the low incidence of H-Ig components of the IgA class in the various groups of mice is unclear. However, technical limitations might contribute to this result, since H-Ig in the IgA class might be easily masked under the electrophoresis conditions applied (see Chapter 6, section 6.2.).

The influence of the T cell system on the heterogeneity of the Ig production was also investigated in aging athymic nude mice. In view of the marked increase in the incidence of H-Ig in thymectomized, irradiated, bone marrow reconstituted mice (Ap-

pendix publication V) and the increase in the incidence of H-Ig and/or nonmalignant paraproteins during aging in both humans and mice (Radl, 1980), relatively frequent disturbances in Ig heterogeneity were expected to occur in nude mice during senescence.

Indeed, the results of such studies presented in Appendix publication III confirm this expectation. In that study, an increased incidence of H-Ig components was found in the sera of 40- and 110-week-old nude mice as compared with their euthymic littermates. Since the H-Ig were predominantly found in the IgG1 subclass, we questioned whether these components could account for the frequent occurrence of unexpectedly high IgG1 serum levels in nude mice. However, statistical analysis of such a potential influence revealed no significant correlation. This finding is in accord with observations of Radl et al. (1975), who also could not detect a marked influence of IP on serum Ig levels in advanced age man.

In Appendix publication VI, the onset and Ig class distribution of H-Ig was further investigated in a follow-up study of individual nude mice, their heterozygous littermates and mice of the background strains into which the nude gene was introduced (BALB/c and CBA). It was found that the incidence in the heterozygous mice was intermediate between that of the nude mice and the corresponding background strain. This result suggests that the thymus of mice heterozygous for the nude gene is functionally inferior to that of the homozygous background strain. In 13-month-old nude mice, 46% of the H-Ig components persisted for more than 6 months. Such a persistent occurrence of the same component is one of the criteria for the diagnosis of IP (see Chapter 5, section 5.4.). Therefore, it is likely that athymic nude mice are highly susceptible to developing IP. In agreement with results of Appendix publications III and V, determination of the class distribution of the H-Ig and/or paraproteins of nude mice revealed a predominance of the IgG1 subclass, although such disturbancies could also be found in the IgM, IgG2a, IgG2b, IgG3 and IgA H-chain isotypes. The incidence of H-Ig and nonmalignant paraproteins is not only enhanced in case of homozygosity in the nude gene. The genetic background can also affect their incidence at other levels. This has been found in man (Meyers et al., 1972) as well as in various mouse strains (Radl et al., 1978). The latter authors reported that a high incidence of H-Ig and IP occurs among C57BL mice, in contrast to the BALB/c and CBA strains, which show a low incidence and only at advanced age.

In conclusion, the studies reported in this thesis and the reviewed literature show that both the serum Ig level and the serum Ig heterogeneity are highly dependent on the genetic background and T cell function of an individual. Ig's of different classes and subclasses are unequally affected by deficient T cell function. When T cell impairment occurs, the serum levels of IgM and IgG3 are hardly affected, but the levels of the other Ig classes and subclasses are generally altered. In nude mice, this is usually reflected in an increased IgG1 level and decreased levels of IgG2 and IgA. Deviations from the normally heterogeneous serum Ig spectrum due to T cell impairment can become manifest as restricted heterogeneity, transient H-Ig components and idiopathic paraproteins. Especially the IgG1 subclass is predisposed to undergo such changes in heterogeneity.

8. *SUMMARY*

The vertebrate immune system depends on two major subpopulations of lymphoid cells: T and B lymphocytes. T cells develop in the thymus and mediate cellular immune responses. B cells differentiate to a large extent in the bone marrow. After antigenic stimulation, the B cell's progeny of plasma cells produce antibodies which mediate humoral immune responses. An optimal humoral immune response to most antigens requires the interaction of B and T cells. These latter can regulate B cell activity positively as well as negatively. The majority of the antibodies produced are released into the blood stream. Their activity can usually be found in certain serum globulin fractions. These globulins mediating immunity are called immunoglobulins (Ig's). Ig molecules in the serum of the adult mouse belong to the IgM, IgA, IgG1, IgG2a, IgG2b, IgG3, IgE and IgD classes. Their concentration in serum depends largely on age, antigenic stimulation genetic background and the development of the thymus dependent limb of the immune system. The experimental work on the serum Ig spectrum of mice which is presented in the publications appended to this thesis deals mainly with the latter two aspects.

Appendix publication I concerns an investigation on to what extent AKR mice are deficient for IgA in serum and secretions. This was of interest because, in man as well as in animals, IgA deficiency frequently is related to autoimmunity and malignancy and AKR mice are predisposed to develop lymphoma between 6 and 14 months of age. Quantitation of the IgM, IgG1, IgG2 and IgA levels in serum and milk of 5-month-old AKR, C3H, CBA and C57BL mice showed that, of these mouse strains, AKR have the lowest serum and secretory IgA levels. However, these levels were not far below those of the CBA mouse, which is a long-lived strain and exhibits no specific immune pathology. Therefore, it is unlikely that the lymphoma development in AKR mice is closely related to the low IgA content.

In Appendix publication II the genetic influence on the serum IgA level was further investigated by determining the capacity of AKR bone marrow cells to give rise to serum IgA in lethally irradiated allogeneic hosts. For this purpose, the AKR bone marrow cells were transplanted into C3H mice and *vice versa*. C3H mice were chosen because they have a relatively high serum IgA level (a difference of a factor 4 at 5 months of age; c.f. Appendix publication I) and bone marrow transplantation in this combination is usually successful because of MHC compatibility. It was found that AKR mice reconstituted with C3H bone marrow

cells developed IgA serum levels within the normal (relatively low) AKR range. In C3H mice reconstituted with AKR bone marrow cells, however, high levels of serum IgA showing the AKR allotype were found. Therefore, it is concluded that the low serum IgA concentration of AKR mice is not a reflection of a genetically determined incapacity of the B cell line to produce IgA, but rather a manifestation of a genetically determined capacity to prevent IgA synthesis.

The second aspect studied was the influence of the thymus on the Ig production. T immune system defects are known to influence the serum Ig spectrum by affecting the serum levels as well as the heterogeneity of Ig's.

Appendix publication III describes investigations on the influence of the thymusless state in aging nude mice kept under low pathogenic (SPF) conditions. At advanced ages (40- and 110-week-old, respectively), the nude mice showed characteristic differences in their serum Ig spectra as compared with their heterozygous littermates. Generally, they showed decreased levels of IgG2a, IgG2b and IgA. In contrast, the IgG1 serum levels were often significantly increased. Furthermore, 40- and 110-week-old nude mice showed a high incidence of restricted Ig heterogeneity and H-Ig components which were predominantly of the IgG1 subclass. Investigation of the possible relationship between the occurrence of H-Ig components of IgG1 and the unexpectedly high serum levels of this Ig subclass did not reveal a positive correlation.

In Appendix publication IV the deficiency in the Ig-synthesizing activity in nude mice was further studied by transplanting a neonatal thymus lobe under the kidney capsule of 8-week-old nude mice. In these mice, the recovery was monitored with respect to the number of cells positive for cytoplasmic IgM, IgG1, IgG2 and IgA and the serum levels of the corresponding Ig's. It was found that the initial predominance of cells containing cytoplasmic IgM in untreated nude mice changed into an almost normal percentage distribution of cells containing the various Ig H-chain isotypes. An overshoot of both the number of cells producing IgG1 and IgG2 and their respective serum levels was noted at 8 weeks posttransplantation. Thus, by that time, thymus-reconstituted nude mice have built up serum levels of all Ig classes and subclasses to an extent that is comparable to mice which normally have the disposal of a T cell system.

The heterogeneity of serum Ig's after irradiation and reconstitution and in athymic nude mice was investigated in Appendix

publications V and IV, respectively. In Appendix publication V, the occurrence of H-Ig of various Ig classes and subclasses (IgM, IgG1, IgG2 and IgA) in the serum was determined during the recovery of lethally irradiated mice, reconstituted with syngeneic bone marrow cells. These mice had previously been thymectomized and/or splenectomized. Only thymectomy was found to cause an increase in the frequency of H-Ig components, with a peak incidence between 1.5 and 3.5 months posttransplantation. During the entire observation period of 9.5 months, the components mainly occurred in the IgG class. Most H-Ig were transient and disappeared within some months.

To further investigate the role of the thymus in the occurrence of H-Ig components, the serum Ig spectra of aging nude mice were analysed for the presence of H-Ig. This study is reported in Appendix publication VI. The results reveal an earlier onset and an increased incidence of H-Ig in nude mice as compared with euthymic controls. Determination of the class distribution of the various components showed similar results as were obtained in Appendix publications III and V, namely, a relatively high H-Ig incidence of IgG1. However, H-Ig of the IgM, IgG2a, IgG2b and IgG3 H-chain isotypes were also a frequent finding. A striking observation was that, in 13-month-old nude mice, 46% of the paraproteins persisted for at least 6 months. This persistent occurrence suggests that nude mice are highly susceptible to the development of the benign B cell disorder *idiopathic paraproteinaemia*.

9. *SAMENVATTING*

Het immuunsysteem van gewervelde dieren is afhankelijk van twee subpopulaties lymfocyten, n.l. T en B cellen. T cellen worden in de thymus gevormd en zijn verantwoordelijk voor de cellulaire immuunrespons. B cellen differentiëren voornamelijk in het beenmerg. Na antigene stimulatie ontstaan uit B cellen plasmacellen die de antilichamen produceren die verantwoordelijk zijn voor een humorale immuunrespons. Voor een optimale humorale immuunrespons tegen de meeste antigenen zijn T cellen nodig, die zowel in positief als in negatief opzicht de B cel activiteit kunnen reguleren. Aangezien de meeste antilichamen die geproduceerd worden in de bloedbaan terecht komen, is antilichaam activiteit over het algemeen terug te vinden in bepaalde fracties van serumglobulinen. Deze antilichaam activiteit bevattende globulinen worden immunoglobulinen (Ig's) genoemd. De Ig's in het serum van een volwassen muis behoren tot the IgM, IgA, IgG1, IgG2a, IgG2b, IgG3, IgE en IgD klassen. De serum concentratie van deze verschillende Ig's is sterk afhankelijk van de leeftijd, de aard en intensiteit van de antigene stimulatie vanuit de omgeving, de genetische achtergrond en de volledige ontwikkeling van het T cel systeem. Het experimentele werk dat in dit proefschrift beschreven wordt, is met name gericht op de twee laatstgenoemde aspekten van de serum Ig produktie in de muis.

De serum concentraties van de diverse Ig klassen en subklassen kunnen aanzienlijk variëren in verschillende muizenstammen. In Appendix publikatie I is onderzocht in hoeverre AKR muizen deficient zijn voor IgA in serum en secreten. Deze vraagstelling was gebaseerd op gegevens uit de literatuur dat er bij mens en dier een relatie kan bestaan tussen IgA deficiëntie enerzijds, en autoimmuniteit en maligniteit anderzijds, en het feit dat AKR muizen op een leeftijd variërend van 6 tot 14 maanden een lymfoom ontwikkelen. Kwantitatieve bepalingen van IgM, IgG1, IgG2 en IgA concentraties in serum en melk van 5 maanden oude AKR, C3H, CBA en C57BL muizen toonden aan dat van de geteste stammen AKR muizen het laagste gehalte aan IgA hadden in serum en secreten. Omdat deze waarden niet erg afweken van de concentraties in CBA muizen (muizen met een lange levensduur, zonder duidelijke pathologische afwijkingen van het immuunsysteem), is het niet waarschijnlijk dat de lymfoomontwikkeling in AKR muizen direct gerelateerd is aan hun, in het algemeen lage, gehalte aan IgA.

In Appendix publikatie II is de genetische invloed op de serum IgA concentratie verder onderzocht door te bepalen in hoeverre

transplantatie van AKR beenmergcellen naar letaal bestraalde allogene recipiënten kan zorgen voor IgA produktie in deze recipienten. Voor deze vraagstelling zijn AKR beenmergcellen getransplanteerd naar C3H muizen en *vice versa*, omdat C3H muizen op een leeftijd van 5 maanden een veel hogere (ongeveer 4 keer zo veel) serum IgA concentratie hebben dan AKR muizen (zie Appendix publikatie I) en omdat beenmergtransplantatie in deze combinatie een redelijke kans van slagen heeft (beide stammen zijn MHC compatibel). AKR muizen, die gereconstitueerd werden met C3H beenmergcellen, vertoonden na enige maanden nog steeds serum IgA concentraties die karakteristiek zijn voor normale onbehandelde AKR muizen. C3H muizen gereconstitueerd met AKR beenmergcellen daarentegen, ontwikkelden relatief hoge serum IgA concentraties. Dit IgA had het AKR allotype. De conclusie uit deze waarnemingen was dat de lage serum IgA concentratie in AKR muizen niet zozeer het gevolg is van een genetisch bepaald onvermogen van AKR B cellen om IgA te produceren, maar eerder van het vermogen om IgA synthese te voorkomen.

Het tweede aspect dat ten aanzien van de serum Ig produktie bestudeerd is, betreft de invloed van de thymus op het serum Ig spectrum. Het is bekend dat defecten in het T cel systeem zowel de serum concentraties van de verschillende Ig's kunnen beïnvloeden als hun heterogeniteit.

In Appendix publikatie III is de invloed van het ontbreken van een thymus op het serum Ig spectrum onderzocht in nude muizen van 6, 40 en 110 weken oud die onder laag pathogene (SPF) omstandigheden gehouden werden. Thymusloze nude muizen van 40 en 110 weken oud vertoonden karakteristieke verschillen vergeleken met de heterozygote nestgenoten die wel een thymus hadden. Over het algemeen waren in nude muizen de serum spiegels van IgG2a, IgG2b en IgA verlaagd in tegenstelling tot IgG1, dat vaak in sterk verhoogde concentratie aanwezig was. Bovendien bleken 40 en 110 weken oude nude muizen slechts een beperkt vermogen te hebben om een electrophoretisch heterogeen Ig spectrum te ontwikkelen in de diverse Ig klassen en subklassen. Dit onvermogen werd voor een deel weerspiegeld in de sterk verhoogde incidentie aan homogene Ig (H-Ig) componenten in het serum. Deze componenten waren voornamelijk van de IgG1 subklasse. Er is nagegaan of er een relatie bestaat tussen de aanwezigheid van H-Ig in deze subklasse en de hoge IgG1 serum concentraties in nude muizen. Er kon daarbij geen statistisch significant verband aangetoond worden.

In Appendix publikatie IV is het defect in de Ig synthese van nude muizen verder onderzocht door bij jong volwassen nude muizen een neonatale thymuslob onder het nierkapsel te transplanteren.

In dergelijke muizen werd het verloop van het reconstitutieproces gevolgd aan de hand van zowel het aantal cellen dat IgM, IgG1, IgG2 en IgA in het cytoplasma vertoonden als de serum concentraties van deze Ig's. De aanvankelijk overheersende aanwezigheid van IgM positieve cellen (in onbehandelde nude muizen) veranderde in een vrijwel normale procentuele distributie van de verschillende Ig klassen en subklassen over de Ig producerende cellen. Acht weken na thymus transplantatie werden ten aanzien van zowel het aantal Ig producerende cellen als de serum concentraties van de diverse Ig's hogere waarden gevonden dan in de heterozygote nestgenoten. Op dat moment bezitten de thymus gereconstitueerde nude muizen blijkbaar het vermogen om voor alle Ig klassen en subklassen serum concentraties te ontwikkelen die vergelijkbaar zijn met dieren die normaal over een T cel systeem kunnen beschikken.

In Appendix publikaties V en VI is de heterogeniteit van serum Ig's verder onderzocht in letaal bestraalde, beenmerg gereconstitueerde muizen en in thymusloze nude muizen. In Appendix publikatie V werd nagegaan in hoeverre H-Ig in serum IgM, IgG1, IgG2 en IgA voorkomen tijdens het herstel van muizen waarin na letale bestraling syngeen beenmerg getransplanteerd werd. Deze muizen waren voor de bestraling gethymectomeerd en/of gesplenectomeerd. Alleen als de thymus ontbrak bleek de H-Ig frequentie verhoogd te zijn met een maximum tussen 1,5 en 3,5 maand na transplantatie. Tijdens de gehele observatieperiode van 9,5 maand werden de meeste componenten in de IgG klasse gevonden. De H-Ig componenten die in deze muizen gevonden werden, bleken van tijdelijke aard te zijn, aangezien ze gewoonlijk een paar maanden na detectie weer verdwenen waren.

De rol van de thymus in het ontwikkelen van H-Ig is verder onderzocht door serum Ig spectra te analyseren van verouderende nude muizen. De resultaten van deze analyses zijn vermeld in Appendix publikatie VI. Nude muizen ontwikkelen op relatief jonge leeftijd en in verhoogde mate H-Ig, wanneer ze vergeleken worden met heterozygote nestgenoten. De bepaling van de Ig klasse distributie van de diverse componenten gaf dezelfde resultaten als al in Appendix publikaties III en V vermeld zijn, n.l. een relatief hoge incidentie aan H-Ig in de IgG1 subklasse. Alhoewel in mindere mate, werden ook regelmatig H-Ig componenten aangetroffen in de IgM klasse en in de subklassen IgG2a, IgG2b en IgG3. Slechts incidenteel werd een H-Ig gevonden in de IgA klasse. Een opmerkelijke observatie was dat in 13 maanden oude nude muizen 46% van de aangetroffen componenten reeds tenminste 6 maanden aanwezig was. Dit suggereert dat nude muizen gepredisponeerd zijn voor de ontwikkeling van de goedaardige B cel tumor *idiopathische paraproteinemie*.

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CURRICULUM VITAE

Na het behalen van het diploma HBS-B aan het Heldring College te Zetten in 1970 begon ik de studie Biologie aan de Landbouw Hogeschool te Wageningen. Het kandidaatsexamen heb ik afgelegd in september 1974. In de doctoraalfase van mijn studie heb ik gedurende de praktijkperiode onder leiding van Dr. O.B. Zaalberg op het Medisch-Biologisch Laboratorium in Rijswijk (ZH) onderzoek gedaan naar de optimale kweekomstandigheden van miltcellen. Het diploma Landbouwkundig Ingenieur werd verkregen op 24 januari 1977. Van september 1976 tot begin januari 1977 was ik part time leraar biologie aan de Christelijke Koningin Wilhelmina MAVO te Wageningen. Per 1 februari 1977 trad ik in dienst als wetenschappelijk assistent bij de afdeling Celbiologie en Genetica van de Erasmus Universiteit te Rotterdam. Binnen deze afdeling werd, onder leiding van Prof.Dr. R. Benner en Prof.Dr. O. Vos, het in dit proefschrift beschreven onderzoek verricht.

APPENDIX PUBLICATION I

SERUM AND SECRETORY IMMUNOGLOBULIN LEVELS IN PRELEUKAEMIC AKR MICE AND THREE OTHER MOUSE STRAINS

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SUMMARY

Levels of IgM, IgG_1, IgG_2 and IgA were determined in serum and milk of AKR mice, which spontaneously develop lymphoma at 6-14 months of age. As a reference C3H, CBA and C57BL mice were studied. Of the four mouse strains studied AKR had the lowest serum and secretory IgA levels. The values of the other immunoglobulins in AKR mice were comparable to those of CBA mice. C3H and C57BL mice had significantly higher immunoglobulin levels. Serum of lactating mice showed fairly decreased IgG_1 and IgG_2 levels as compared with non-lactating mice, probably due to transudation into the milk. The serum IgM and IgA levels were not consistently affected by lactation.

INTRODUCTION

There are several indications that in animals and man IgA deficiency is related to autoimmunity and malignancy (1). Furthermore, people who have suffered from a congenital infection with rubella virus (2,3) have a relatively high incidence of selective IgA deficiency. The relationship between IgA deficiency, autoimmunity and malignancy is unclear at present.

AKR mice have a naturally occurring infection with an endogenous virus, the Gross murine leukaemia virus, which is present from birth (4,5). Infection with this virus causes lymphoma in all AKR mice between 6 and 14 months of age, but not necessarily in other mouse strains (4,5). In addition, AKR mice have been reported to have

very low serum IgA levels (6). Concerning immunoglobulin levels in secretions of mice, there is a lack of data in the literature. Serum and secretory IgA are reported to be produced by largely separate populations of immunoglobulin-synthesizing cells (7,8). Therefore, we decided to investigate whether the IgA deficiency of AKR mice only holds for serum, or is general. This paper presents quantitative data on levels of IgA and the other immunoglobulin classes in serum and milk of AKR mice. For comparison similar determinations were done in C3H, CBA and C57BL mice.

MATERIALS AND METHODS

Mice. Five months old female AKR/FuRdA, C3H/fA, CBA/Rij and C57BL/Rij mice were used. The AKR ($H\text{-}2^k$) mice were bred at our own department, the C3H ($H\text{-}2^k$) mice were purchased from the Laboratory Animals Centre of the Erasmus University, Rotterdam, and the CBA ($H\text{-}2^q$) and C57BL ($H\text{-}2^b$) mice were purchased from the Medical Biological Laboratory TNO, Rijswijk, The Netherlands.

Collection of serum and milk. Blood was taken with a capillary pipette from the orbital plexus. After isolation of the serum, the samples were stored at -70°C prior to use. Milk was collected (0.3-0.5 ml during 15 min) 5 to 9 days after parturition, by asparation using a modification of the milking device described by McBurney et al (9). The offspring was separated from the mothers 18 hr before milking. Lactating mice were intraperitoneally injected with 0.4 u.i. Oxytosin (Piton-S$^{(R)}$; Organon, Oss, The Netherlands) per kg body weight 30 min before milking, in order to stimulate milk-flow. After collection the milk was centrifuged twice for 30 min at 27,000 g in order to collect clear milk-serum.

Quantitation of immunoglobulins. The levels of IgM, IgG_1, IgG_2 and IgA in serum and milk were determined by using the rocket electrophoresis method according to Laurell (10). The agarose solution was supplemented with 1% polyethyleneglycol (MW 7000). Before electrophoresis the immunoglobulins were carbamylated according to Weeke (11). For quantitations peak surfaces instead of peak heights were calculated. A large pool of normal mouse serum obtained from approximately one-year-old CBA mice was used as a reference serum. The concentration of IgM, IgG_1, IgG_2 and IgA in the reference serum was determined by comparing with a mouse immunoglobulin reference standard containing myeloma proteins (Meloy Laboratories, Inc., Springfield, USA). The reference serum contained 38.5 mg IgM, 230.2 mg IgG_1, 432.8 mg IgG_2 and 178.8 mg IgA per 100 ml. The antisera to mouse IgM, IgG_1 and IgG_2 were raised in rabbits by Nordic Immunological Laboratories, Tilburg, The Netherlands. The antiserum to mouse IgA was raised in a goat by Meloy Laboratories. All antisera were found to be specific for the respective mouse immunoglobulins as tested by immunoelectrophoresis and rocket electrophoresis.

TAB. 1: Immunoglobulin concentrations in serum of 5-month-old AKR, C3H, CBA and C57BL mice.

Mouse strain	IgM	IgG_1	IgG_2	IgA	Total Ig[a)]
AKR(8)[b)]	18.1±1.7[c)]	133.9±20.4	220.9±19.8	39.5± 1.5	412.4±28.5
C3H (10)	32.7±3.1	258.2±47.2	311.6±19.3	177.5±20.8	780.0±55.2
CBA (7)	26.9±4.7	159.4±25.6	295.0± 9.5	61.9± 5.6	543.2±28.3
C57BL(8)	30.1±1.7	118.1±11.5	485.8±14.3	83.9± 7.4	717.9±19.9

a) Total immunoglobulin concentration was calculated by summing up the figures for the various classes and subclasses.

b) Numbers of mice tested in parentheses.

c) Figures represent the arithmetic mean ± 1 SEM in mg per 100 ml serum. Total immunoglobulin levels of AKR and CBA mice were significantly lower than in C3H and C57BL mice ($p < 0.005$).

Quantitation of protein in milk. Protein concentration in milk was determined by the Lowry assay, as modified by Bensadoun and Weinstein (12).

RESULTS

Serum IgM, IgG_1, IgG_2 and IgA levels were determined in 5-month-old AKR mice. For comparison these Ig levels were also measured in H-2 compatible C3H mice and in CBA and C57BL mice of the same age. AKR serum contained 18.1 ± 1.7 mg IgM, 133.9 ± 20.4 mg IgG_1, 220.9 ± 19.8 mg IgG_2 and 39.5 ± 1.5 mg IgA per 100 ml serum (Tab.1). These figures are within the range of the immunoglobulin levels found in CBA mice, except for IgA. No consistent pattern in the relative amount of the different immunoglobulin (sub) classes was found in the four mouse strains tested. C57BL mice were found to have a relatively high level of IgG_2, while C3H mice were proportionally high for IgG_1 and IgA. Total immunoglobulin concentration was the lowest in AKR mice, while the highest immunoglobulin levels were found in C3H and C57BL mice.

In order to investigate whether the relatively low IgA level in AKR mice also holds for secretions, immunoglobulin levels were compared in the milk of the aforementioned four strains of mice. The

TAB. 2: Immunoglobulin concentrations in serum and milk of 5-month-old lactating AKR, C3H, CBA and C57BL mice.

Mouse strain	Sample[a]	IgM	IgG_1	IgG_2	IgA	Total Ig[b]
AKR(5)[c]	serum	22.8±2.2[d]	41.1± 4.8	84.9±13.1	36.1± 2.8	184.9±14.4
	milk	<0.9	15.3± 2.6	34.8± 6.7	84.3±14.2	134.4±15.9
C3H(5)	serum	31.0±3.7	64.9±11.9	82.2±19.5	50.4±11.3	228.5±25.7
	milk	<0.9	32.7± 6.7	41.8±15.9	107.8±22.9	183.2±28.6
CBA(5)	serum	22.4±1.3	36.4± 7.2	59.8± 3.7	78.0±12.8	196.6±15.2
	milk	<1.2	12.1± 3.0	24.2± 3.8	118.5±12.9	154.8±13.8
C57BL(7)	serum	32.5±2.0	84.9±10.6	168.3±23.2	94.0±16.5	379.7±30.4
	milk	<2.0	39.1± 5.7	78.2±16.1	201.5±15.7	318.8±23.2

a) Serum and milk were collected 5 to 9 days after parturition.

b) Total immunoglobulin concentration was calculated by summing up the figures for the various classes and subclasses.

c) Number of mice tested in parentheses.

d) Figures represent the arithmetic mean ± 1 SEM in mg per 100 ml serum or milk.

effect of lactation upon the serum immunoglobulin levels was also determined in these mice. It was found that the IgA level in milk was the lowest for AKR mice (Tab.2). However, this value was nearly as high as found in CBA mice. Also the IgG_1 and IgG_2 concentration in milk of AKR and CBA mice were of about the same magnitude. The levels of IgG_1, IgG_2 and IgA in milk of C57BL mice were about twice as high as in AKR and CBA mice. In the milk of neither strain of mice IgM was clearly present. Comparison of the concentrations of various immunoglobulin classes and subclasses in milk and serum showed that the ratio between secretory and serum immunoglobulin level was the highest for IgA.

Lactation did not affect the serum IgM and IgA levels, as can be deduced from comparison of Tables 1 and 2. Only in C3H mice the serum IgA level was reduced during lactation. A relatively large decrease of the serum IgG_1 and IgG_2 levels during lactation was a consistent finding. This lowered the total serum immunoglobulin level by at least 50 per cent.

Differences in secretory immunoglobulin levels between various mouse strains might be related to the protein concentration in these secretions. Therefore, the protein concentration was determined in the various milk samples. Milk of AKR, CBA and C57BL mice appeared to contain about equal amounts of protein, but the protein concentration in milk of C3H mice was clearly higher (Tab.3). Consequently, relative immunoglobulin levels (calculated as milligram immunoglobulin per gram protein) in milk of C3H mice (Tab.3) were less high than the absolute immunoglobulin levels (Tab.2). These relative immunoglobulin levels of C3H mice were the lowest of the four mouse strains tested. C57BL mice on the other hand, appeared to have the highest relative immunoglobulin levels.

DISCUSSION

AKR, NZB and SJL/J mice have a high incidence of spontaneously occurring tumors. Since there is a relationship between immunocompetence and malignancy, these strains have been investigated for a possible immunodeficiency at the humoral and cellular level (cf. ref. 13 and 14). While most authors agree that leukaemic AKR mice have a decreased immunocompetence, preleukaemic AKR mice have been reported to have normal or somewhat decreased humoral and cellular immune reactivity (cf. ref. 4 and 13). Potter and Liebermann (6) reported that AKR mice are deficient for serum IgA. In view of these data we considered it worthwhile to quantitate the serum and secretory IgA levels of AKR mice, and to compare that with those of other, low tumor incidence, mouse strains. Of the four mouse strains tested, our AKR/FuRdA mice (which have a medium life span of 32 weeks) were found to be the lowest for IgA in serum (Tab.1) and milk (Tab.2).

TAB. 3: Protein concentration and relative immunoglobulin levels in milk of 5-month-old AKR, C3H, CBA and C57BL mice.

Mouse strain	Protein (g/100ml)	IgM	IgG_1	IgG_2	IgA	Total Ig
AKR	2.4±0.1[a]	< 0.18[b]	9.0±1.8	14.4±3.0	34.8±6.0	58.5± 2
C3H	4.1±0.6	< 0.18	7.8±1.8	10.2±3.6	25.8±6.6	43.8± 7.8
CBA	2.6±0.3	< 0.42	4.2±1.2	9.0±1.8	45.6±7.2	59.4±21.6
C57BL	3.0±0.2	< 0.78	12.6±1.8	25.8±5.4	66.6±7.2	105.6± 9.0

a) Figures represent the arithmetic mean ± 1 SEM.

b) Relative immunoglobulin levels were calculated as milligram immunoglobulin per gram protein. For these calculations the immunoglobulin concentrations from Tab.2 were used.

However, these levels in AKR mice were not far below those of CBA mice, which is a long-lived strain without specific immune pathology (15). Therefore, the low IgA level of AKR mice is probably not a major permitting factor in the development of lymphoma.

As most other mammalian species, mouse serum IgA consists predominantly of molecules sedimenting in the 9S range (16), indicating that it has the dimer form, just like the majority of secretory IgA. The origin of mouse serum IgA was thought to be predominantly the gut associated lymphoid tissue (17). However, studies of Haaijman et al. (18) have shown that the mouse bone marrow also contains large numbers of IgA containing cells, indicating that this organ might substantially contribute to the serum IgA level.

There is a lack of data in the literature concerning immunoglobulin levels in mouse colostrum and milk. It has been shown that mouse colostrum contains IgA, IgG_1 and IgG_2, but no IgM (19,20). Our results show that the same holds for milk, when collected 5-9 days after parturition (Tab.2). Quantitatively, IgA was predominant. Similar results have been reported for milk of rats (21,22) and many other mammals (8). In the neonatal mouse the IgG_1, IgG_2 and IgA from milk are probably resorbed into the blood. This supposition is supported by the observation that in newborn mice the serum IgG_1, IgG_2 and IgA levels increase fastly after birth, and decrease after weaning (20,23).

There is a large body of evidence that the majority of IgA in secretions is synthetized locally. In vitro experiments have shown

that mouse mammary tissue can synthesize IgA (19). In how far transudation of IgA from serum contributes to the secretory IgA in mouse milk is unclear. If there is any contribution, this is not consistently reflected in a decrease of the serum IgA level. On the other hand, serum IgG_1 and IgG_2 level decrease by about 50% during lactation (cf. Tables 1 and 2). This might be due to transudation into the milk. Indeed, there is ample evidence that IgG in milk is predominantly derived from serum (8). This even holds for the large amounts of IgG in bovine colostrum and milk (24).

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APPENDIX PUBLICATION II

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Serum Immunoglobulin Levels in Mice

Determination of the Low IgA Level in AKR Mice by an Irradiation-Resistant Factor

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Abstract. A comparison was made between the serum immunoglobulin (Ig) levels in H-2 compatible AKR and C3H mice. The IgG1 and especially the IgA level in preleukemic AKR mice was much lower than in age-matched C3H mice, while the IgM concentration was hardly different for AKR and C3H. Lethally irradiated AKR and C3H mice reconstituted with syngeneic bone marrow (BM) cells showed a return to serum Ig levels which are normal for these strains. In AKR mice reconstituted with C3H BM cells low IgA levels were observed. On the other hand, in C3H mice reconstituted with AKR BM cells high quantities of IgA appeared, showing the AKR allotype. It is concluded that the low serum IgA concentration in AKR mice is not a reflection of a genetically determined inability of the B cell line to produce IgA, but rather a manifestation of a genetically determined capability to prevent IgA synthesis.

Introduction

Serum immunoglobulin (Ig) concentrations in mammals vary widely according to species, genetic background, age and immune status [10, 21, 31]. During ontogeny [12, 21], after lethal irradiation and reconstitution of mice [17, 30], and after bone marrow (BM) transplantation in children with a severe combined immunodeficiency [27, 33] the Ig's of the various classes appear in the sequence IgM-IgG-IgA. Synthesis of these various Ig's require T cell help to a various extent [2, 19, 26]. Thus, genetically athymic nude mice have normal or enhanced levels of IgM, while levels of IgG1, IgG2 and IgA are severely reduced in most animals [2, 26]. However, in nude mice on a Balb/c background a hyperglobulinemic state for IgG1 can occur [22, own observations]. In thymus-bearing animals enhanced T suppressor cell activity can cause severely decreased serum levels of a single Ig class, e.g. IgA [3] and IgE [5].

AKR and C3H are H-2 compatible strains of mice with largely different levels of the various Ig classes [15, 25]. In 5-month-old mice the IgM level is almost comparable in both strains, whereas the IgG1 and IgG2 level in serum of AKR is about half of that in C3H. The serum IgA level of AKR mice is even less than 25% of

that in C3H mice [15, 25]. Generally, the concentration of an Ig of a particular class depends on the rate of synthesis and the rate of catabolism, which both might be affected by the genetic background of an individual. In this paper we describe BM transplantation experiments in which low IgA AKR mice were reconstituted with BM cells from the high IgA producer C3H and vice versa. These experiments were done to investigate the influence of the genetic background of AKR and C3H mice upon their respective serum Ig levels. The experiments show that infusion of lethally irradiated AKR mice with C3H BM cells is not able to enhance the serum IgA level. This result suggests, that the low IgA level of AKR mice is mainly caused by an irradiation-resistant factor, and not by a genetically determined deficiency in AKR B cells.

Materials and Methods

Animals. AKR/FuRdA (H-2^k), C3H/fA (H-2^k) and (C57BL/Rij × CBA/Rij)F_1 (H-$2^{b/q}$) female mice were used. The animals were bred at the Department of Cell Biology and Genetics and the Laboratory Animals Centre of the Erasmus University at Rotterdam. Blood was obtained by cardiac puncture under Nembutal (Abbott SA, Saint-Rémy-sur-Avre, France) anesthesia (70 mg/kg body weight) or from the orbital plexus under ether anesthesia.

X-Irradiation and Reconstitution. For lethal irradiation 8-week-old mice were exposed to 850 (C3H) or 1,000 rad (AKR) X-irradiation delivered at a dose rate of 32 rad/min by a Philips Mueller MG 300 X-ray machine operating at 250 kV and 11 mA with a Cu filter. The distance to target was 53 cm. Further details are described by *Vos* [32]. The animals were injected intravenously with 2×10^6 femoral BM cells or 5×10^6 fetal liver cells within 3 h after irradiation. Fetal liver cells were derived from embryos at 14–15 days gestation.

Quantification of Ig's. The serum levels of Ig's were measured by rocket electrophoresis according to *Laurell* [11] using 1% agarose (No. 4, Nordic, Tilburg, The Netherlands) in high resolution buffer pH 8.8 (Gelman, Ann Harbor, Mich.). Samples (8 μl) of serum and standard were applied in duplicate and subjected to electrophoresis at a constant voltage of 50 V for 16 h at room temperature. Before electrophoresis the samples were carbamylated overnight according to *Weeke* [35]. A pool of normal mouse serum from (DBA/2 × C57BL/Rij) F_1 was used as a secondary standard. It is well known that expression of the results in absolute values gives much less accurate information than relative quantification [4, 29], because there are no proper reference standards of heterogeneous Ig's of individual classes and subclasses. Only reference standards consisting of myeloma proteins are available for calibration of a secondary standard. Our (DBA/2 × C57BL/Rij) F_1 standard was related to a reference standard which contained the following myeloma proteins: 104 mg/dl IgM, 514 mg/dl IgA and 674 mg/dl IgG1 (Meloy Laboratories Inc., USA). Because of the known problems giving calibration with one particular myeloma protein, only rough estimations can be made about the content of different Ig classes and subclasses in a secondary standard. Therefore, we prefer to give our Ig concentrations in relative units. One unit approximately represents 1 mg.

Specific Antisera. A rabbit antiserum to mouse IgM was obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam). Goat antisera to mouse IgG1 and IgA were purchased from Meloy Laboratories Inc.

The antiserum to the allotype of C3H-IgA was raised in A mice according to the method described by *Mage et al.* [14]. As immunogen the purified myeloma protein TEPC-15 (Bionetics, Kensington, USA) was used. Serum antibody activity and specificity against TEPC-15 and the allotype of C3H-IgA was examined by Ouchterlony immunodiffusion and by immunoelectrophoresis [24]. The unabsorbed antiserum developed clearly visible precipitin lines with TEPC-15 and normal C3H serum in the immunodiffusion test but no precipitate with a serum from nonleukemic AKR mice.

The anti-Thy-1.2 antiserum was obtained from serum of AKR mice immunized with C3H thymocytes according to *Reif and Allen* [28]. The anti-Thy-1.1 antiserum was raised as ascitic fluid in C3H mice after immunization with AKR thymocytes according to the method of *Munoz* [20]. The specific cytotoxic potency of both antisera for corticosteroid-resistant thymocytes was determined in the trypan blue exclusion assay as described previously [18].

IgA Catabolism. A slight modification of the method of *Crewther and Warner* [6] was used. In short, mouse IgA myeloma protein (TEPC-15) was labelled with $Na^{125}I$ (IMS 30, The Radiochemical Centre, Amersham, England) according to the chloramine-T method of *Hunter* [8]. AKR and (C57BL × CBA)F_1 mice were injected intravenously with 3.25 μg of ^{125}I-TEPC-15 (specific activity 5.8×10^7 cpm/μg). 50 μl blood samples were collected and counted for radioactivity at various intervals after injection.

Determination of the Chimeric Nature of Mice Reconstituted with Allogeneic Cells. Two methods were applied to determine the chimeric nature of allogeneically reconstituted AKR and C3H mice. By means of Ouchterlony analysis the allotype of serum IgA of individual mice was tested using an alloantiserum against the C3H IgA-allotype. Precipitation was performed in 1% agarose supplemented with 3% polyethyleneglycol (MW 6,000–7,500).

The trypan blue exclusion test was performed to determine the Thy-1 isotype on thymocytes of chimeric mice, which were reconstituted with bone marrow or fetal liver cells. In a two-step procedure thymocytes (10^7/ml) were incubated with either anti-Thy-1.1 or anti-Thy-1.2 antiserum in melting ice for 30 min. As control thymocytes were mixed with normal mouse serum obtained from AKR mice. After washing in a balanced salt solution the cells were incubated at 37 °C for 15 min with guinea pig complement (Flow Laboratories, Rockville, Md.) which had been absorbed previously with mouse spleen cells. The percentage of dye excluding cells was determined after addition of 0.2% trypan blue to the cell suspension.

Results

Ig Levels in Normal AKR and C3H Mice

The serum Ig levels in H-2 compatible AKR and C3H mice are shown in figure 1. From 6 till 24 weeks of age an increase in

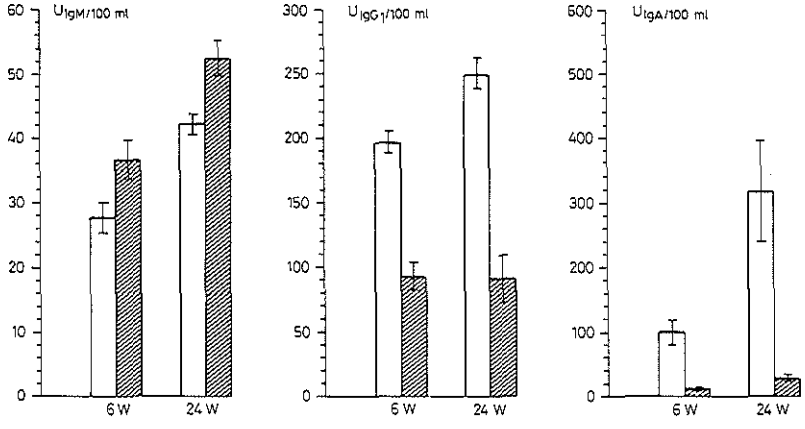

Fig. 1. The level of IgM, IgG1 and IgA in serum of normal C3H (open columns) and AKR (hatched columns) mice. Samples from 6–7 female mice were examined at an age of 6 weeks (6 W) and 24 weeks (24 W). The concentration of each Ig class is given in U/100 ml serum. The results are expressed as arithmetic mean ± 1 SE.

the amount of Ig was observed in the IgM and IgA classes. These data are in agreement with the observations of *Natsuume-Sakai et al.* [21]. The amount of IgG1 showed only a slight increase in C3H mice and was the same in AKR mice aged 6 and 24 weeks. Due to the frequent occurrence of spontaneous lymphoma in AKR older than 24 weeks no determinations were performed in older mice. Interesting are the relative amounts of the different Ig classes in AKR and C3H mice. The IgM levels did not differ significantly at an age of 6 and 24 weeks. However, the amount of IgG1 and IgA was significantly lower in AKR than in C3H mice. This difference between AKR and C3H mice was most apparent for IgA (AKR: 15 and 31 U/100 ml; C3H: 103 and 320 U/100 ml at an age of 6 and 24 weeks, respectively). The difference in IgG1 levels was less striking (AKR: 93 and 92 U/100 ml; C3H: 194 and 251 U/100 ml at an age of 6 and 24 weeks, respectively).

IgA Catabolism

Data about the IgA catabolism were obtained by following the decrease in radioactivity in blood samples obtained at various times after intravenous injection of 3.25 μg ^{125}I-labelled IgA myeloma protein (total activity 189 $\times$ 10^6 cpm) into the low IgA producer AKR and the relatively high IgA producer (C57BL $\times$ CBA)F_1 mice. Within 4 h after injection, the radioactivity in the blood had decreased till 2.5×10^5 – 3×10^5 cpm/50 μl, i.e. 10% of the original value in the case of 3 ml blood. This fast early disappearance has also been reported by others [6, 23] and is probably due to secretion into bile [23]. A logarithmic decrease of radioactivity in blood of both AKR and (C57BL $\times$ CBA)F_1 mice was observed (fig. 2). The rate of decline in both mouse strains was nearly the same. The time to clear half of the level of ^{125}I-IgA was 20.1 h for AKR and 21 h for F_1 mice. It is concluded that there is only a minimal difference between the IgA catabolism in AKR and (C57BL $\times$ CBA)F_1 mice.

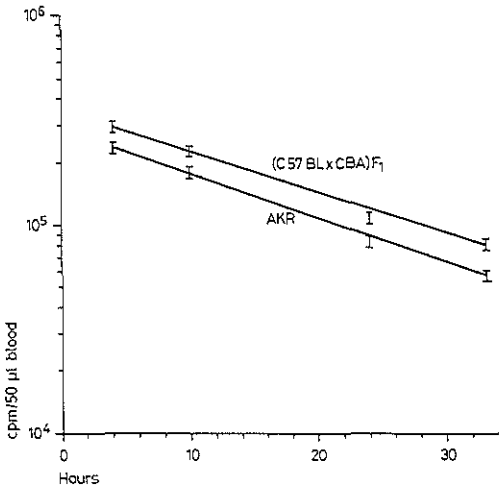

Fig. 2. Catabolism of ^{125}I-labelled IgA myeloma protein (TEPC-15) in (C57BL $\times$ CBA)F_1 and AKR mice. The mean radioactivity count (cpm $\pm$ 1 SE) in 50 μl blood samples is plotted against the time (hours) after injection of a constant amount in 5 mice.

Ig Levels in Irradiated and Reconstituted Mice

Lethal irradiation and reconstitution with syngeneic of allogeneic BM cells induced a sharp decrease of all Ig classes during the first 2 weeks after treatment (fig. 3). In the period between 4 and 9 weeks after irradiation the AKR and C3H mice reconstituted with syngeneic BM cells showed a return to Ig levels normal or slightly subnormal for AKR and C3H mice of the same age. In the same period C3H mice reconsti-

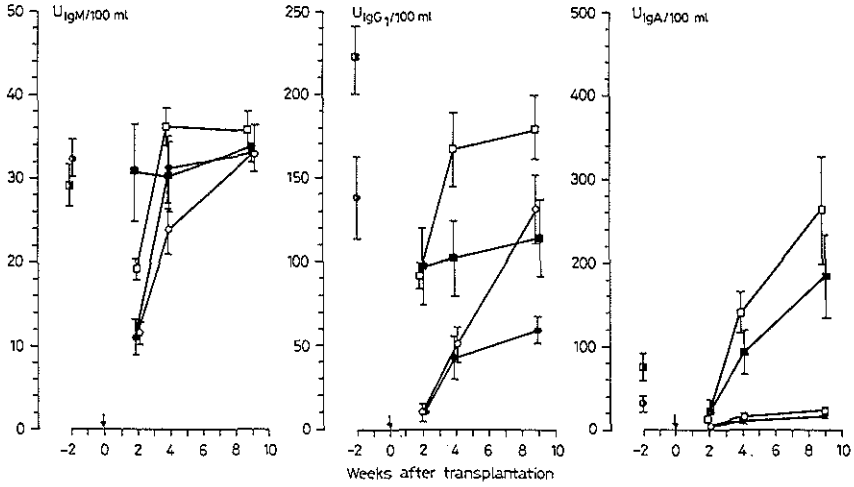

Fig. 3. The level of IgM, IgG1 and IgA in serum of C3H and AKR mice after irradiation and bone marrow (BM) transplantation. 6-week-old animals were bled before irradiation (▣ C3H; ◑ AKR). Irradiation and reconstitution with BM cells (ψ) was performed at an age of 8 weeks. BM transplantation was either syngeneic (□ C3H + C3H BM, ● AKR + AKR BM) or allogeneic (■ C3H + AKR BM; ○ AKR + C3H BM). The concentration of each Ig class is given in units per 100 ml serum. The results are expressed as arithmetic mean ± 1 SE. Each group consisted of at least 6 mice.

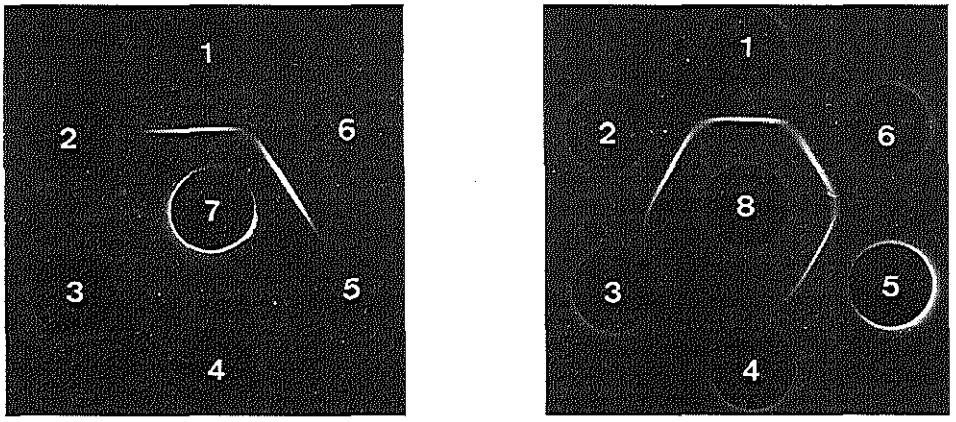

Fig. 4. Results of an Ouchterlony immunodiffusion test to determine the chimeric nature of irradiated AKR and C3H mice at 9 weeks after reconstitution with allogeneic bone marrow (BM) cells. The peripheral holes in the agarose gel were filled with sera from the following reconstitutions: C3H + C3H BM (1), C3H + AKR BM (2), AKR + AKR BM (3), AKR + C3H BM (4), and from untreated nonleukemic AKR mice, 11 months old (5) and untreated C3H mice, 9 months old (6). The central holes were filled with a mouse anti-allotype serum specific for C3H-IgA (7) or a goat antiserum against mouse IgA (8).

tuted with AKR BM cells showed quantities of Ig more or less comparable to normal C3H mice. This observation provides evidence that a genetic defect in AKR hemopoietic stem cells is probably not the cause of the low IgA level in AKR mice. In AKR mice reconstituted with C3H BM cells the serum Ig pattern was the same as in AKR mice reconstituted with syngeneic BM cells.

Chimeric Nature of Mice Reconstituted with Allogeneic Cells

To reveal the origin of the B cell population in chimeric mice an allo-antiserum against the C3H IgA allotype was used. It is shown in figure 4 that serum IgA in C3H mice reconstituted with AKR BM cells lacks the C3H allotype. However, in C3H mice reconstituted with C3H BM cells the C3H allotype was clearly demonstrated. The low IgA level in AKR mice reconstituted with either allogeneic or syngeneic BM cells did not allow a visible precipitation reaction in the Ouchterlony immunodiffusion technique.

To reveal the origin of the T cell population, antisera against the Thy-1 isotype were used in a cytotoxicity assay. The data presented in table I provide evidence for the statement that the T cell population in chimeric mice is also from donor origin. In AKR mice reconstituted with C3H hemopoietic stem cells almost all thymocytes were killed by treatment with an anti-Thy-1.2 antiserum. The same antiserum had no effect on thymocytes from C3H reconstituted with AKR fetal liver cells. The opposite results were obtained when thymocytes of the same animals were treated with anti-Thy-1.1 serum.

Table I. Determination of the Thy-1 isotype on thymocytes of AKR and C3H mice reconstituted with allogeneic hemopoietic stem cells

Thymocyte donors	Serum treatment	% surviving thymocytes[1]
AKR + C3H BM[2] (n = 5)	anti-Thy-1.2	4.1 ± 0.4[3]
	anti-Thy-1.1	85.0 ± 11.2
	NMS	100
AKR + C3H FL (n = 7)	anti-Thy-1.2	5.1 ± 1.2
	anti-Thy-1.1	116.3 ± 6.8
	NMS	100
C3H + AKR FL (n = 12)	anti-Thy-1.2	125.7 ± 8.1
	anti-Thy-1.1	2.5 ± 0.5
	NMS	100

[1] The trypan blue exclusion assay was used to determine the Thy-1 isotype on thymocytes of chimeric AKR and C3H mice 25 weeks after transplantation.
[2] Mice were lethally irradiated and reconstituted with either 2×10^6 bone marrow (BM) or 5×10^6 fetal liver (FL) cells. Number of mice in parentheses.
[3] Figures represent the arithmetic mean ± 1 SEM. The percentage for chimeric mice sometimes reached values higher than 100%, since the number surviving thymocytes treated with anti-Thy-1 sera was compared with cells treated with normal mouse serum (NMS) derived from AKR mice.

Discussion

The experiments reported in this paper show that AKR mice have a relatively low IgA level, in contrast to H-2 compatible C3H mice (fig. 1). In lethally irradiated C3H mice the progeny of AKR BM cells produced quantities of IgA comparable to those produced by cells derived from C3H BM (fig. 3). On the other hand, after lethal irradiation of AKR mice, neither AKR nor C3H BM cells were able to improve the serum IgA level of the recipients. Apparently the height of the serum IgA level in AKR

and C3H mice is determined by the genetic background of the environment in which the progeny of the transplanted hemopoietic stem cells synthesize the Ig's. The recovery of the serum IgG1 level in allogeneically reconstituted AKR and C3H mice was not only determined by the host, but also by the type of hemopoietic stem cells transplanted. These mice attained IgG1 levels intermediate between the high level of syngeneically reconstituted C3H mice, and the low level of syngeneically reconstituted AKR mice (fig. 3). Obviously, these effects are not related to the major histocompatibility complex, since both AKR and C3H are H-2^k. For chickens, evidence has been obtained for a genetic relationship between IgA deficiency and alleles at the major histocompatibility locus [13].

In man, low IgA levels are frequently due to the production of anti-IgA autoantibodies [1]. This is probably not the cause of the low serum IgA level in AKR, since TEPC-15 IgA myeloma protein injected into AKR was found to have the same half-life as after injection into relatively high IgA-producer (C57BL × CBA)F_1 mice (fig. 2). Furthermore, the presence of anti-IgA antibodies in AKR serum could not be demonstrated in Ouchterlony immunodiffusion test, even not when tested against samples containing substantial amounts of AKR IgA or IgA from other mouse strains [data not shown]. Therefore, the low serum IgA level of AKR and the high level of C3H probably reflects a low and high IgA synthetic activity in AKR and C3H mice, respectively.

Most serum IgA is produced by the progeny of B lymphocytes already committed to IgA synthesis. This has been shown in cell transfer experiments using the fluorescent-activated cell sorter to separate B cells with different Ig isotypes [9]. The difference between IgA synthetic activity in AKR and C3H mice might be due to a different rate of development of this B cell subpopulation during ontogeny and after lethal irradiation and BM transplantation. Alternatively, a different IgA synthetic activity might be caused by different proportions of T helper and T suppressor cells in AKR and C3H mice [16]. In chickens it has been shown that enhanced suppressor cell activity indeed can cause IgA deficiency [3].

AKR mice have also been reported to be low IgE producers [5, 34]. This low IgE production is due to nonantigen specific suppressor T cell activity [5]. Just like the low IgA level, the low reagin level of AKR is not linked to the H-2 complex, since the H-2 compatible C3H and CBA mice are capable of persistent IgE antibody production [34]. Several laboratories have shown that elimination of suppressor (T) cells could convert low IgE responders into high IgE responders [5, 7, 34]. *Watanabe and Ovary* [34] were able to do that for AKR mice by means of X-irradiation. However, this procedure was effective only at an early stage of immunization, suggesting that the mature suppressor cells are irradiation resistant. When similar suppressor cells cause the low IgA level of AKR mice, these cells are probably also responsible for the low IgA level in lethally irradiated AKR mice reconstituted with C3H BM cells (fig. 3). Interestingly, in C3H recipients AKR lymphoid cells would not give rise to such suppressor cell activity.

In conclusion, the results presented in this paper suggest that the low serum IgA concentration of AKR mice is not a reflection of a genetically determined inability to

produce IgA, but rather a manifestation of a genetically determined capability to prevent IgA synthesis.

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The authors thank Professor Dr. *O. Vos* and Dr. *J. Radl* for their continuous support and interest. Furthermore, they thank Dr. *F. Klein* for practical advice, Mr. *P. L. van Soest* for excellent technical assistance, Mr. *W. Valen* for the illustrations, Mr. *T. van Os* for expert photography and Mrs. *C. Meijerink-Clerkx* for typing the manuscript.

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APPENDIX PUBLICATION III

SERUM IMMUNOGLOBULINS IN NUDE MICE AND THEIR HETEROZYGOUS LITTERMATES DURING AGING

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SUMMARY

Serum immunoglobulin (Ig) levels were investigated in 6, 40 and 110-week-old congenitally athymic (nude) mice and their heterozygous littermates. Concentrations of IgM, IgG1, IgG2a, IgG2b, IgG3 and IgA were determined by rocket electrophoresis. At 6 weeks of age, IgM was the most prominent serum Ig in both nude and heterozygous mice. Except for IgM and IgG3, some nude mice displayed unquantifiable levels of some of the other Ig classes or subclasses. At this age, the average levels of the various Ig classes and/or subclasses did not differ significantly between the two groups of mice. At the ages of 40 and 110 weeks, most nude mice showed serum Ig spectra in which all classes and subclasses were present. Young (6 wk) and middle-aged (40 wk) nude mice generally showed a wider variation in Ig levels than did their heterozygous littermates. The most striking differences between aged nude mice and aged heterozygous mice were:
(a) the generally decreased levels of IgG2a, IgG2b, and IgA;
(b) the frequent occurrence of increased serum levels of IgG1; and,
(c) the increased incidence of homogeneous Ig components ("paraproteins") in the sera of nude mice.

INTRODUCTION

The serum levels of immunoglobulins (Ig) in nude mice have been the subject of several investigations, because of the possibility to study the influence of a T immune system deficiency on Ig production. In all available reports, conventionally kept nude mice aged between one and six months have been used. In general, these nude mice have comparable serum IgM levels to those of heterozygous littermates, while IgG and IgA levels are depressed (Pantelouris, 1978). However, increased serum levels of IgM in nude mice have been found by Bankhurst, Lambert and Miescher (1975), while a particular stock of nude mice of Gershwin,

Merchant, Gelfand, Vickers, Steinberg and Hansen (1975) showed approximately normal serum IgA levels. No long-term observations on serum Ig levels in untreated nude mice were reported. Such investigations may be possible in specific pathogen free (SPF), barrier maintained nude mice, which have a much longer lifespan than conventionally kept ones (Holland, Mitchell, Gipson and Whitaker, 1978).

Also no information is available on the heterogeneity of the serum Ig's in nude mice. In the sera of mice with a T immune system defect, a restriction in the Ig heterogeneity and an increased frequency of homogeneous Ig components (H-Ig) were observed (Mink, Radl, Van den Berg, Van Muiswinkel and Van Oosterom, 1979). Similar phenomena have also been reported to occur during aging in both humans and mice (reviewed by Radl, 1979). Disturbances in the Ig heterogeneity in the aging nude mice may, therefore, be expected. The following aspects were the subjects of our study in 6, 40 and 110-week-old nude mice and their heterozygous littermates:

(a) the serum levels of IgM, IgG1, IgG2a, IgG2b, IgG3 and IgA isotypes; and
(b) the frequency of H-Ig in the sera of the various groups.

MATERIALS AND METHODS

Mice.

Six, 40 and 110-week-old nude mice and their heterozygous littermates (7 to 24 per age group) were purchased from the Radiobiological Institute TNO, Rijswijk (ZH), The Netherlands. The 6 and 40-week-old mice were the second and third generations after cross-breeding of male nude mice on a CBA background and female BALB/c mice. Nude and heterozygous mice of 110 weeks of age had a CBA background. All animals were from a pathogen-free and barrier-maintained colony. They received sterilized pelleted food (Hope Farms, Woerden, The Netherlands) and sterilized water *ad libitum*. The mice of the 40-week-age group were removed from the barrier colony 2 weeks prior to bleeding and were kept in a conventional animal room. All mice from the 6 and 110 week age groups remained in the barrier-maintained facility until the day of bleeding.

Antisera.

Rabbit antisera to mouse IgM (batch nr. 10-476) and IgG1 (batch nr. 6-576) and a goat antiserum to IgG3 (batch nr. 31-977) were obtained from Nordic Immunological Laboratories, Tilburg, The Netherlands. Goat antisera directed against mouse IgG2a (batch

nr. B104-88570), IgG2b (batch nr. B105-77028) and IgA (batch nr. B106-64392) were purchased from Meloy Laboratories, Springfield, Ohio, USA. All antisera were found to be specific for the respective mouse Ig's as tested by immunoelectrophoresis and rocket electrophoresis.

Serum analysis.
Anaesthetized mice, 7-24 per age group, were bled from the retro-orbital sinus. Serum levels of IgM, IgG1, IgG2a, IgG2b, IgG3 and IgA were determined by rocket electrophoresis (Laurell, 1972) as described previously (Mink and Benner, 1979). As a reference standard, a large serum pool from normal CBA mice was used. The absolute concentrations of the various Ig classes and/or subclasses in the CBA serum pool were determined by comparison with a mouse Ig standard (Meloy Laboratories Inc., Springfield, Ohio, USA). This latter standard was a mixture of the following myeloma proteins: MOPC 104E (IgM, (λ), 117 mg/dl), TEPC 15 (IgA, (K), 507 mg/dl), MOPC 31C (IgG1 (K), 638 mg/dl), RPC 5 (IgG2a (K), 756 mg/dl), and MOPC 195 (IgG2b (K), 513 mg/dl). The IgG3 level in the CBA standard was measured against a secondary standard of normal C57BL serum, which contained 70 mg/dl IgG3 as determined by comparison with an isolated IgG3 (K) myeloma protein FLOPC 21. The optimal electrophoretic conditions for our experiments allowed quantifications down to 2.0 mg/dl for IgM, 2.1 mg/dl for IgA, 3.1 mg/dl for IgG1, 4.4 mg/dl for IgG2a, 3.6 mg/dl for IgG2b and 2.8 mg/dl for IgG3. To avoid calibration errors due to anti-idiotype activity, a small amount of each antiserum was absorbed with normal mouse serum and tested for the absence of these antibodies by Ouchterlony immunodiffusion, using in separate combinations the same myeloma proteins as present in the Meloy standard. No anti-idiotype activity was found.

All serum samples were tested for the presence of homogeneous immunoglobulins (H-Ig) by agar electrophoresis according to Wieme (1959) and by immunoelectrophoresis. In the sera in which the presence of H-Ig could not unambigeously be demonstrated by agar electrophoresis and immunoelectrophoresis, the technique of immunofixation (Cejka and Kithier, 1976) was applied.

Necropsies.
A number of nude mice in this study were killed with CO_2 after the collection of blood. The mice were necropsied in order to asses their general health, as well as to verify their thymusless condition. Tissues from 15 nude mice of the 40-week-age group were fixed in 10 per cent buffered formalin, embedded in paraffin and sectioned for histological examination.

Statistical analysis.
Differences in the concentration of serum Ig between nude mice and heterozygous controls was investigated by application of the Wilcoxon test for two independent samples. The difference in dispersion of serum Ig levels between the two groups of mice was investigated with the Siegel-Tukey test (Lehmann, 1975). Confidence intervals for geometric means of serum Ig levels with and without a H-Ig component in that particular subclass were obtained by assuming a log-normal distribution. Significant differences in geometric means were verified with the Student-t-test.

RESULTS

No structures clearly resembling thymic remnants were present in any of the nude mice examined grossly. In 5 of the 15 cases examined histologically, small cysts were observed in the anterior mediastinum. These cysts were lined by a single layer of flattened or low cuboidal epithelium, and were sometimes associated with several acini of ectopic salivary gland tissue. No lymphocytes were consistently associated with these structures.

Fourteen of the 15 nude mice of the 40-week-age group had histological lesions compatible with mouse hepatitis virus (MHV) infection. These consisted in most cases of mild to severe multifocal hepatic necrosis and syncytial giant cell formation in mucosal epithelium of the colon and cecum. In 6 cases such syncytial giant cells were found in the absence of liver lesions. There was no histological evidence for the presence of any other infectious diseases in these mice.

Ig's of all classes and subclasses were present in the sera of 6-week-old mice heterozygous for the nu-gene, IgM being the most prominent. Comparable concentrations of IgM and IgG3 were found in nude mice of the same age. However, in most nude mice, the IgG1, IgG2a, IgG2b and IgA levels were too low to be quantitated (Fig. 1). Two nude mice showed higher serum levels for all IgG subclasses than did the heterozygous littermates. The occurrence of extremely low IgG and IgA levels in most 6-week-old nude mice made it difficult to properly analyse the potential statistical differences between serum IgG and IgA levels of nude and heterozygous mice. Therefore, the statistical analysis of such differences as indicated in Fig. 1 has to be considered with caution. Analysis of the dispersion of the Ig levels in both groups of mice showed significantly larger variations for IgG2a, IgG2b, IgG3 and IgA in nude mice.

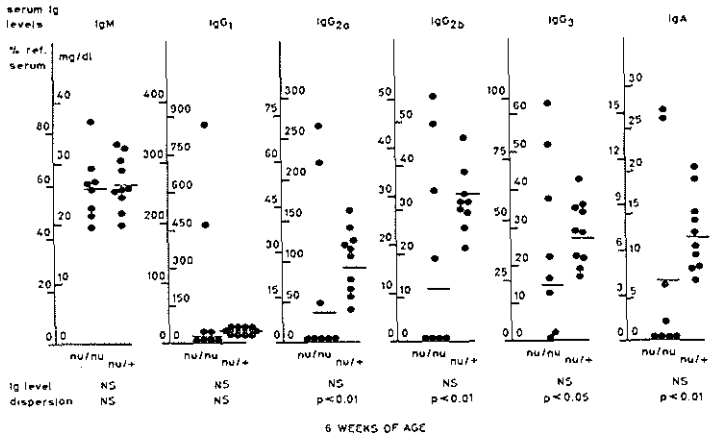

Figure 1. Serum levels in nude mice and their heterozygous littermates at the age of 6 weeks. The individual serum levels are expressed in both absolute (mg/dl) and relative values (percentage of our CBA reference serum). Significances in differences of the geometric average values of serum levels between the two groups of mice were calculated by the Wilcoxon test. No H-Ig components were found in 6-week-old mice. NS means not significant.

At 40 weeks of age, all nude mice had developed clearly detectable serum levels of all Ig classes and/or subclasses (Fig. 2). At this age, nude mice showed normal levels of IgM, while the IgG2b, IgG3 and IgA levels were lower than in the euthymic littermates. A considerable number of nude mice had increased serum levels of IgG1 and IgG2a. Statistical evaluation revealed that this increase was significant for IgG1 ($p < 0.01$) but not for IgG2a. Analysis of the dispersion of the Ig levels in nude and heterozygous mice showed a significantly greater variation for IgG2a in nude mice. For the other Ig classes and subclasses, no significant differences in dispersion were found between nude and heterozyggous mice.

Nude mice aged 110 weeks showed IgM, IgG1 and IgG3 serum levels similar to those of the heterozygous littermates, while their IgG2a, IgG2b and IgA levels were significantly lower (Fig. 3). No IgG1 or IgG2a could be detected in the sera of a few nude mice. As far as the dispersion at the age of 110 weeks is concerned, only the variation of IgG1 levels was found to be significantly greater in nude mice than in the heterozygous littermates (Fig. 3).

Investigations of the heterogeneity of individual Ig classes failed to demonstrate a H-Ig component in sera of 6-week-old animals. The occurrence of H-Ig in the other groups is indicated in Figs. 2 and 3. The incidence of H-Ig in the various groups is summarized in Table I.

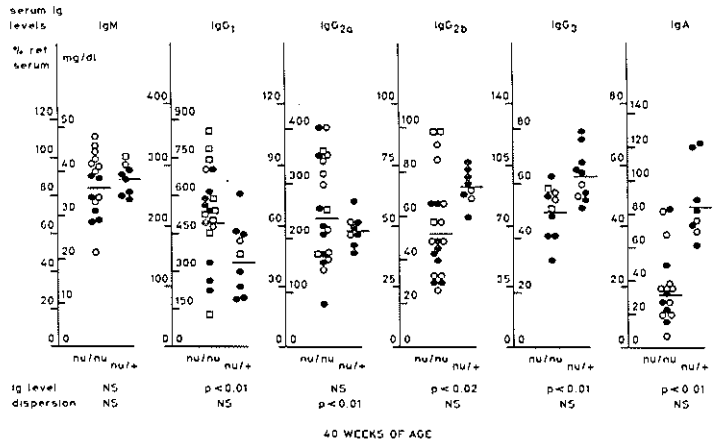

Figure 2. Serum Ig levels in 40-week-old mice; the same conditions as in Figure 1. In addition, an assessment of the heterogeneity of the Ig levels in individual animals is given by using the following symbols: ● Ig of normal heterogeneity in all classes; ○ normal heterogeneous Ig pattern of a given class or subclass, but one or more H-Ig components in another class or subclass; □ Ig class or subclass containing H-Ig components.

At 40 weeks of age, 12 of 24 nude mice showed one or more H-Ig components, in contrast to an incidence of 2/10 in the heterozygous littermates. At the age of 110 weeks, the nude mice displayed a H-Ig incidence of 5/12. Heterozygous control mice of this age also frequently showed H-Ig components (3/7). Two or three simultaneously occurring H-Ig components of different classes or subclasses were found in some nude mice. Moreover, in 3 of the 24 nude mice tested at 40 weeks of age, the occurrence of two different H-Ig components was noted within a single Ig subclass, namely IgG1. No multiple H-Ig were found in the serum of a single mouse in the phenotypically normal control group.

The class and/or subclass distribution of the H-Ig was as follows: of 24 nude mice at 40 weeks of age, 9 animals displayed H-Ig of the IgG1 subclass, 3 of IgG2a, 3 of IgG2b and 2 of the IgG3 subclass. Of 10 heterozygous controls, one animal developed a H-Ig of IgM and another of the IgG1 subclass. At the age of 110 weeks, of 12 nude mice, one animal developed H-Ig of IgM, 4 of IgG1, and 1 of IgG2a, while of the 7 control mice, 1 H-Ig was of the IgG1 and 2 of the IgG2a subclass. No H-Ig of the IgA class was found in either group of mice.

The potential influence of the occurrence of H-Ig components on serum Ig levels was investigated for IgG1 in 40-week-old nude mice. This was done by comparing IgG1 serum levels in mice which contained a H-Ig component within that subclass and IgG1 levels

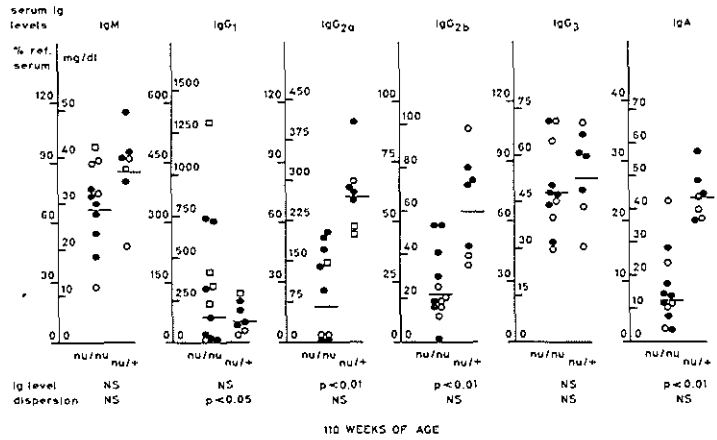

Figure 3. Serum Ig levels in 110-week-old mice; the same conditions as in Figure 2.

in mice which showed no H-Ig component in their sera. The geometric mean of the IgG1 level of the mice which were positive for H-Ig of IgG1 was 510 mg/dl with a 95% confidence interval of 334-779 mg/dl. In mice with a normal heterogeneous Ig spectrum, the average IgG1 serum level was 427 mg/dl (286-639 mg/dl). Statistical evaluation via a student-t-test did not reveal significant differences between the two groups. For the other Ig classes and/or subclasses, this analysis was impossible, due to the relatively small numbers of mice with a H-Ig component in these classes and subclasses (Table 1).

DISCUSSION

Most investigations performed with conventional athymic nude mice under 6 months of age demonstrated low serum levels of IgG and IgA, but normal concentrations of IgM (Pantelouris, 1978). This selective deficiency was attributed to the T immune system defect in the nude mice. Production of antibodies of the IgG and IgA classses is known to be dependent to a large extent on T cell help. IgM production, on the other hand, is less dependent on the help of T cells (Pritchard, Riddaway and Micklem, 1973). The same may be valid for the IgG3 subclass as indicated from work of Bankhurst (1975). Our results, obtained with SPF mice, were comparable with those reported in the literature for 6-week-old nude mice. At this age, IgG1, IgG2a and IgG2b levels are very low, but the IgG3 level is normal (Fig. 1).

At the age of 40 weeks, the nude mice showed clearly detectable serum levels for all Ig classes and/or subclasses. In 110-week-

TABLE I. INCIDENCE AND CLASS DISTRIBUTION OF HOMOGENEOUS SERUM IMMUNOGLOBULINS IN AGED NUDE MICE AND THEIR HETEROZYGOUS CONTROLS

	40 weeks							110 weeks						
	μ	γ1	γ2a	γ2b	γ3	α	total incidence of H-Ig	μ	γ1	γ2a	γ2b	γ3	α	total incidence of H-Ig
nu/nu	0[x]	9	3	3	2	0	12/24[xx]	1	4	1	0	0	0	5/12
nu/+	1	1	0	0	0	0	2/10	0	1	2	0	0	0	3/7

[x] Figures represent the number of mice which display one or more homogeneous immunoglobulin (H-Ig) components in their serum immunoglobulin spectra.

[xx] The total incidence of H-Ig (numerator) found in the number of mice tested in a particular group (denumerator). In both groups of nude mice, H-Ig of more than one class or subclass were found in a single mouse. For this reason, the total incidence is lower than can be deduced from summing up fractional incidences of H-Ig.

old mice (nude mice as well as euthymic littermates), the average levels of most Ig classes and/or subclasses had decreased as compared with those of 40-week-old mice.

An extraordinary finding in all three age groups of nude mice was the occurrence of excessive IgG1 production, a phenomenon that has been described previously for nude mice (Brogren et al., 1977), conventional as well as germfree (Okudaira et al., 1977). This observation contrasts with the generally held view that the T cell dependency of IgG1 production is reflected in reduced serum IgG1 levels in nude mice (Wortis, 1974). However, the presence of T cells is not an absolute requirement for induction and secretion of IgG. Nude mice infected with hepatitis virus have been shown to have the capacity to generate an indirect PFC response after immunization with SRBC (Tamura, Machii, Ueda and Fujiwara, 1978). In our study the majority of the 40-week-old nude mice were infected with this virus. Therefore this infection may (together with a possible genetic predisposition (Okudaira et al., 1977)) account for the significantly raised IgG1 serum levels in the nude mice of this age group as compared with their heterozygous controls. Recently it has also been shown that spironucleosis infection in nude mice increased serum levels of IgG1, IgG2a and IgG2b (Kunstyr, Meijer and Ammerpohl, 1977). Furthermore an appreciable IgG anti-TNP immune response has been detected in nude mice after immunization with TNP-LPS (Humbert, Motta and Truffa-Bach, 1979). Other relevant indications are observations in several other mouse mutants which are immunodeficient in the T cell system (e.g. "lethargic" and "dwarf" mutants). These animals can also display elevated serum IgG1 levels as compared to their normal littermates of the same age (Dung, Lawson and Stevens, 1977; Duquesnoy, Christensen, Pedersen and Kemp, 1975).

Analysis of the dispersion of serum Ig levels showed a clear difference between nude mice and their heterozygous littermates at 6 weeks of age. At this age, nude mice have a wider dispersion for all Ig classes and/or subclasses except for IgM and IgG1. Such a difference might be related to the T immune system defect in nude mice, since 6-week-old euthymic mice have a normal functioning T system. No significant differences in variability between the two groups of mice were present at the ages of 40 and 110 weeks. As compared to younger mice, 110-week-old heterozygous littermates showed a general increase in dispersion of serum Ig levels. This confirms earlier observations of Radl et al. (1975) and Haaijman, van den Berg and Brinkhof (1977) concerning increased variability of serum immunoglobulins in aging man and CBA mice, respectively. A defect in the T immune system is accom-

panied by an increased incidence of restricted heterogeneity of serum Ig's and the occurrence of H-Ig (Radl, 1979). Nude mice at 40 weeks of age also showed an increased incidence of H-Ig as compared to their heterozygous littermates. This difference was no longer found at the age of 110 weeks; this may be explained by an increased incidence of H-Ig components in the heterozygous mice as a consequence of aging (Radl, Hollander, Van den Berg and De Glopper, 1978).

The analysis of the class distribution of H-Ig components revealed a predominance of H-Ig in the IgG class, particularly in the IgG1 subclass (Table 1). We have previously obtained similar results in thymectomized, lethally irradiated mice after reconstitution with syngeneic bone marrow cells (Mink et al., 1979).

The high incidence of H-Ig in the IgG1 subclass in 40-week-old nude mice allowed a statistical analysis of the potential difference between serum IgG1 levels in mice which were positive for H-Ig in that subclass and in mice which showed no H-Ig component. It was found that the presence of H-Ig components within the IgG1 subclass did not alter significantly the serum level of that subclass. This finding is in accord with observations of Radl, Sepers, Skvaril, Morell and Hijmans (1975), who noted that the appearance of idiopathic paraproteinaemia only slightly influenced serum Ig levels in aged man. The observation that the occurrence of H-Ig in a particular class or subclass does not affect the corresponding serum Ig level indicates that the excess production of H-Ig is accompanied by a reduced production of the other heterogeneous Ig's of the same class or subclass.

In conclusion, the results presented in this paper show that nude mice are able to produce Ig's of all classes and subclasses. However, these Ig's are often of restricted heterogeneity or homogeneous. The underlying cause of this H-Ig development in nude mice is presently under investigation.

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APPENDIX PUBLICATION IV

KINETICS OF RECOVERY OF SERUM Ig LEVELS AND OF CYTOPLASMIC Ig POSITIVE CELLS IN VARIOUS LYMPHOID ORGANS OF NUDE MICE AFTER THYMUS TRANSPLANTATION

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SUMMARY

The long-term effects of thymus-transplantation in nude mice was studied with regard to the number of (cytoplasmic immunoglobulin positive) plasmablasts and plasma cells (C-Ig cells) in various lymphoid organs and their immunoglobulin (Ig) class distribution profile. These data were correlated with the serum Ig levels of the same mice. Already four weeks after thymus transplantation the number of C-Ig cells in the spleen of nude mice had increased to the 2-3 fold of that found in normal nude mice and normal heterozygous littermates of the same age. This overshoot already subsided at eight weeks after thymus transplantation. The increase of the C-Ig cell number in the other lymphoid organs tested (bone marrow, mesenteric lymph nodes and Peyer's patches) started later than in spleen, and did not show a clear overshoot. Almost complete recovery of the C-Ig cell pattern to that of normal littermates was found 32 weeks post-transplantation.

Analysis of the Ig class distribution of the C-Ig cells showed that the increase of the C-Ig cell numbers after thymus transplantation in nude mice was almost exclusively confined to IgG1, IgG2 and IgA. The increase of C-IgG1 and C-IgG2 cells in spleen and bone marrow correlated with a simultaneous increase of the serum IgG1 and IgG2 level, suggesting that these organs are the major source of serum IgG in young adult mice.

INTRODUCTION

Hairless mice homozygous for the nu (nude) gene lack a functional thymus (Pantelouris, 1978). These mice fail to respond normally to thymus-dependent antigens like sheep red blood cells, but react as good or even better than normal mice to thymus-independent antigens like bacterial lipopolysaccharide (Man-

ning, Reed and Jutila, 1972) or polymeric flagellin (Feldmann, Wagner, Basten and Holmes, 1972). The antibodies produced in response to these antigens are mainly of the IgM class. Consequently, nude mice have normal or increased serum levels of IgM, and reduced IgG and IgA levels (Pantelouris, 1978). Recently we found that this difference is less clear in aged nude mice (Mink, Radl, van den Berg, Haaijman and Benner, 1980).

Previously we have shown that the total number of plasmablasts and plasma cells as revealed by cytoplasmic fluorescence (C-Ig cells) do not differ greatly between nu/nu and nu/+ mice. In nu/nu mice most of these C-Ig cells produce IgM (Haaijman, Slingerland-Teunissen, Benner and van Oudenaren, 1979). Both in nu/nu and in nu/+ mice there is a shift of the majority of Ig-secreting cells from spleen to bone marrow as the animals get older. The nude mice seemed to be retarded, however, in this respect.

In mice receiving no intentional antigenic stimulation we observed a more or less transient appearance of C-Ig cells in the gut-associated lymphoid organs, i.e., the mesenteric lymph nodes and Peyer's patches (Haaijman and Hijmans, 1978). The number of C-Ig cells in these organs increases sharply around 5-weeks of age, is constant up to 6 months of age, and than declines to almost zero.

In this paper we describe the long-term effects of thymus transplantation in nu/nu mice on the number of C-Ig cells in various lymphoid organs and on the Ig class distribution of the C-Ig cells. These results were correlated with the effect of thymus transplantation upon the serum Ig levels of the same mice.

MATERIALS AND METHODS

Animals.

Nude mice on a B10.LP background and their heterozygous littermates were purchased from the Central Institute for the Breeding of Laboratory Animals, TNO, Zeist, The Netherlands. During the experiments the animals were kept in laminar flow hoods. Each experimental group consisted of 5-11 animals.

Thymus transplantation.

Single thymic lobes of neonatal B10.LP mice were transplanted under the kidney capsule of 8-week-old B10.LP nu/nu mice. The thymus donors were irradiated with 300 rad (Philips-Müller MG-300 X-ray machine) as described previously (Benner and van

Oudenaren, 1975). Control groups (nu/nu and nu/+ mice) received a sham-surgery.

Enumeration of C-Ig cells.
Cell suspensions of spleen, femoral bone marrow, mesenteric lymph nodes and Peyer's patches and cytocentrifuge slides were prepared as described previously (Haaijman et al., 1979). The total number of C-Ig cells was determined by immunofluorescence microscopy using a fluorescein labelled goat antiserum directed against mouse immunoglobulins (GAM/FITC, lot no. 2-873, Nordic Immunological Laboratories, Tilburg, The Netherlands).
From the number of positive cells per slide, the number of cells spun down onto the slide and the total cell yield of a given organ follows the total number of C-Ig cells per organ. For bone marrow it was assumed that 2 femurs contain 12.8% of the total bone marrow in mice (Benner and van Oudenaren, 1975). The immunoglobulin class distribution was determined according to Hijmans, Schuit and Klein (1969), using combinations of fluorescein and rhodamine labelled antisera specific for the heavy chains of IgM, IgG1, IgG2 and IgA. The fluorescent conjugates were generously supplied by Dr. J. Radl and Miss P. van den Berg of the Institute for Experimental Gerontology, TNO. The antisera met all the specificity criteria described earlier (Haaijman et al., 1979). The specificity of the reagents was corroborated by the fact that preparations of quite a number of nu/+ animals did not show any C-Ig cell containing Ig of two different (sub)classes, notably in Peyer's patches and mesenteric lymph nodes. The fluorescence microscope equipment has also been described in a previous paper (Haaijman et al., 1979).

Estimation of Ig levels in serum
Rocket electrophoresis according to Laurell (1972) was used to determine the absolute and relative amount of various Ig (sub) classes in sera. Details of the antisera used, the absolute standard for and sensitivity of the method have been described extensively in a previous paper (Mink et al., 1980). The levels of IgM, IgG1, IgG2b and IgA were related to a serum pool from normal CBA mice. In order to avoid possible influences of allotype specificities of the anti-IgG2a antiserum (Epstein and Gottlieb, 1977), the levels of IgG2a were determined relative to a serum pool from C57BL/KaLwRij mice. The IgG2a level in the C57BL/KaLwRij standard was determined by comparison with a purified C57BL IgG2a (κ) paraprotein (5T2), which was kindly supplied by Dr. J. Radl.

TABLE I. THE NUMBER OF C-Ig CELLS x 10^{-3} PER ORGAN IN nu/nu, nu/nu + TH AND nu/+ MICE OF VARIOUS AGES

Mice	Age(wks)	Spleen	Bone marrow	Mesenteric lymph nodes	Peyer's patches	Total
nu/nu	8	122(69) [1]	48(27)	4(2)	2(1)	176
nu/+	8	130(28)	292(62)	29(6)	18(4)	469
nu/nu	12	380(81)	63(14)	21(5)	3(<1)	447
nu/nu + TH [2]	12	818(83)	139(14)	22(2)	1(<1)	980
nu/+	12	257(39)	376(56)	22(3)	12(2)	667
nu/nu	16	464(84)	74(13)	12(2)	1(<1)	551
nu/nu + TH	16	466(59)	250(32)	62(8)	6(1)	784
nu/+	16	189(37)	308(60)	7(1)	9(2)	513
nu/nu	20	290(83)	51(15)	7(2)	1(<1)	349
nu/nu + TH	20	326(55)	221(38)	24(4)	17(3)	588
nu/+	20	172(25)	489(71)	19(3)	12(2)	692
nu/nu	40	140(33)	288(67)	2(<1)	1(<1)	431
nu/nu + TH	40	277(24)	805(69)	80(7)	9(1)	1171
nu/+	40	159(13)	1023(85)	19(2)	6(<1)	1207

1) Values in brackets indicate the percentage contributed by the different organs to the total number of C-Ig cells.
2) Thymus-lobes from 300 rad irradiated neonatal mice were transplanted under the kidney capsule of 8-week-old nude mice. Such mice are indicated as nu/nu + TH.

RESULTS

C-Ig cells in different lymphoid organs

The distribution of C-Ig cells over spleen, bone marrow, mesenteric lymph nodes and Peyer's patches is shown in Table I for nude mice with (nu/nu + TH group) and without (nu/nu group) thymus transplant, and their heterozygous littermates (nu/+ group) at various ages. The per cent contribution of the four lymphoid organs to the total number of C-Ig cells is indicated within brackets. The data confirm our earlier finding that at young age the majority of the C-Ig cells is localized in the spleen, while the bone marrow becomes the major site of C-Ig cells in adult animals (Haaijman and Hijmans, 1978; Haaijman et al., 1979). As shown previously (Haaijman et al., 1979), nude mice were retarded in the development of the full complement of C-Ig cells. This was evident especially for the bone marrow, which has in nude mice significantly less C-Ig cells up to an age of 40 weeks. At 12 and 16 weeks of age, the spleen of nude mice contained more C-Ig cells than the spleen of the normal littermates. The gut-associated lymphoid organs of nude mice were highly deficient in C-Ig cells. The mesenteric lymph nodes of nude mice did contain some C-Ig cells. Their number varied as a function of age in much the same way as was seen in the euthymic littermates.

Four weeks after transplantation of a thymic lobe under the kidney capsule the number of C-Ig cells in the spleen of nude mice had increased 2-3 fold as compared to normal nude mice and normal heterozygous littermates of the same age. By that time the C-Ig cell number in the bone marrow was also slightly increased. Eight weeks after transplantation the overshoot of splenic C-Ig cells already subsided, while the bone marrow C-Ig cell number further increased. An almost complete recovery of the C-Ig cell pattern as compared with euthymic littermates was observed 32 weeks posttransplantation.

Ig class distribution of the C-Ig cells

The Ig class distribution of C-Ig cells in spleen and bone marrow of nu/nu, nu/nu + TH and nu/+ animals at various ages is given in Tables II and III, respectively. The majority of splenic C-Ig cells in nude mice was of the IgM class. High numbers of C-Ig cells positive for both IgM and IgG were observed especially in young nude mice. Wherever the analysis was extended to the IgG subclasses 1 and 2, it appeared that the number of cells containing both IgM and IgG1 and the number of the cells containing both IgM and IgG2 were roughly equal in spleen but often markedly different in bone marrow. The number of doubly produ-

TABLE II. Ig CLASS DISTRIBUTION OF C-Ig CELLS IN THE SPLEEN OF nu/nu, nu/nu + TH AND nu/+ MICE

Mice	Age(wks)	IgM	IgG	IgG1	IgG2	IgA	M+G[1)]	M+G1	M+G2	M+A
nu/nu	8	67±7[2)]	2±0.1	nd[3)]	nd	2±0.2	20±4	nd	nd	7±2
nu/+	8	33±2	19±2	nd	nd	33±4	12±4	nd	nd	2±0.5
nu/nu	12	77±2	nd	2±2	1±1	6±0.3	nd	7±2	7±1	nd
nu/nu + TH[4)]	12	55±7	nd	8±3	15±5	10±2	nd	6±1	6±1	nd
nu/+	12	37±2	nd	4±1	13±2	41±4	nd	2±1	4±1	1±1
nu/nu	16	79±4	nd	0.3±0.2	2±1	3±1	nd	10±5	6±1	nd
nu/nu + TH	16	44±3	nd	6±1	18±2	22±3	nd	6±3	4±2	nd
nu/+	16	40±3	nd	6±3	10±1	39±5	nd	1±0.5	4±1	1±0.3
nu/nu	20	59±13	6±3	nd	nd	2±0.4	27±22	nd	nd	4±2
nu/nu + TH	20	47±1	22±6	nd	nd	19±4	10±1	nd	nd	2±1
nu/+	20	43±2	33±3	nd	nd	14±4	9±3	nd	nd	1±0.2
nu/nu	40	83±3	7±3	nd	nd	2±1	3±1	nd	nd	5±3
nu/nu + TH	40	26±3	32±8	nd	nd	25±11	11±2	nd	nd	6±3
nu/+	40	31±7	29±1	nd	nd	22±9	16±3	nd	nd	2±1

1) In the combinations M stands for IgM, G for IgG and A for IgA.
2) Average percentage ± standard error of the mean.
3) nd = not determined.
4) TH stands for thymus transplantation in nude mice at 8 weeks of age.

cing cells was somewhat higher in the nu/nu and nu/nu + TH groups than in the heterozygous littermates. An exception was the 40-week-old group in which the nude mice showed hardly any cells producing more than one Ig heavy chain.

The percentages of C-IgG and C-IgA cells were consistently low in nude mice of the ages studied. In the spleen of euthymic mice the Ig class distribution of the C-Ig cells was more or less constant from 8 to 40 weeks of age. The bone marrow contained a relatively high percentage of C-IgA cells next to C-IgG cells. Percentagewise the number of C-IgM cells was smaller than in the spleen.

Transplantation of a thymus in nude mice restored the Ig class distribution of the spleen and bone marrow in 8-12 weeks to a pattern characteristic for heterozygous littermates. The C-IgM cells gradually disappeared and were replaced by C-IgG and C-IgA cells.

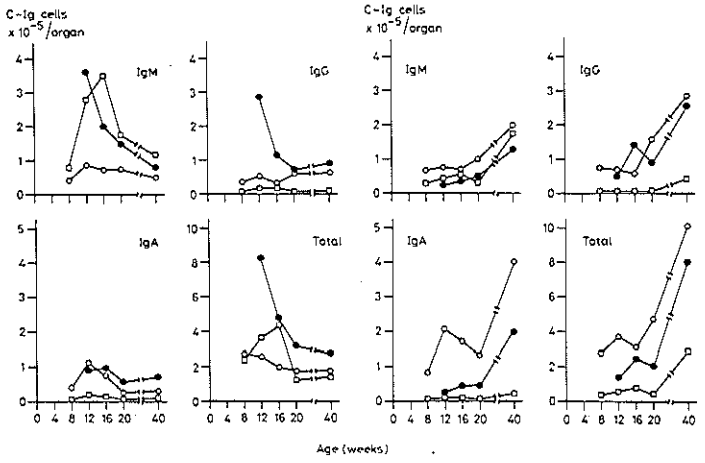

Figure 1. Cytoplasmic immunoglobulin containing cells (C-Ig cells) in the spleen (left) and bone marrow (right) of nu/nu (□), nu/nu + TH (●) and nu/+ (○) mice as a function of age. Calculation of C-Ig cell numbers in the total bone marrow was done on the assumption that two femurs contain 12.8% of the total marrow. One neonatal thymus lobe was transplanted under the kidney capsule of nu/nu mice at 8 weeks of age. Each group consisted of 5-11 animals.

Ig class distributions have been shown to be more or less independent of the total number of C-Ig cells (Haaijman and Hijmans, 1978). Therefore, the absolute number of C-IgM, C-IgG and C-IgA cells have been plotted as a function of time in Figs. 1 and 2 for spleen and bone marrow, and for Peyer's patches, respectively. The cells containing more than one Ig class have not been taken into account in the figures. For the sake of clarity the standard errors were omitted. Fig. 1 (left) shows that thy-

TABLE III. Ig CLASS DISTRIBUTION OF C-Ig CELLS IN THE BONE MARROW OF nu/nu, nu/nu + TH AND nu/+ MICE

Mice	Age(wks)	IgM	IgG	IgG1	IgG2	IgA	M+G[1)]	M+G1	M+G2	M+A
nu/nu	8	62±12	2±1	nd	nd	3±2	16±8	nd	nd	17±5
nu/+	8	25±5	26±5	nd	nd	28±7	15±2	nd	nd	6±2
nu/nu	12	53±12	nd	9±9	2±1	17±5	nd	1±1	18±7	nd
nu/nu + TH	12	43±8	nd	10±5	13±5	20±3	nd	3±1	12±3	nd
nu/+	12	20±2	nd	4±1	17±3	52±4	nd	1±0.1	4±1	2±1
nu/nu	16	71±5	nd	1±0.4	1±1	8±1	nd	4±2	15±7	nd
nu/nu + TH	16	19±3	nd	11±3	36±4	23±4	nd	2±1	9±2	nd
nu/+	16	24±5	nd	6±2	15±1	52±6	nd	1±0.1	2±1	2±1
nu/nu	20	65±8	4±2	nd	nd	2±1	22±7	nd	nd	6±4
nu/nu + TH	20	18±3	43±6	nd	nd	19±6	16±4	nd	nd	4±1
nu/+	20	21±1	34±4	nd	nd	28±6	14±3	nd	nd	4±1
nu/nu	40	69±4	16±5	nd	nd	6±2	1±0.4	nd	nd	9±4
nu/nu + TH	40	16±6	32±7	nd	nd	26±7	19±7	nd	nd	8±3
nu/+	40	20±7	28±11	nd	nd	39±18	12±7	nd	nd	1±1

1) Abbreviations as in Table II.

mus transplantation did not appreciably affect the number of C-IgM cells in the spleen. The number of C-IgG cells had already dramatically increased four weeks after transplantation. Their number returned to the nu/+ level by 12 weeks posttransplantation. Four weeks after receiving a thymus graft the number of C-IgA cells in the nu/nu + TH group was already comparable to that of nu/+ mice. No overshoot was observed for C-IgA cells in the spleen. Also in the bone marrow the C-IgM cell number was hardly influenced by the thymus transplantation (Fig. 1, right). However, the C-IgG and C-IgA cell number in the marrow of nude mice with a thymus transplant showed a clear age-related increase, just as in nu/+ mice.

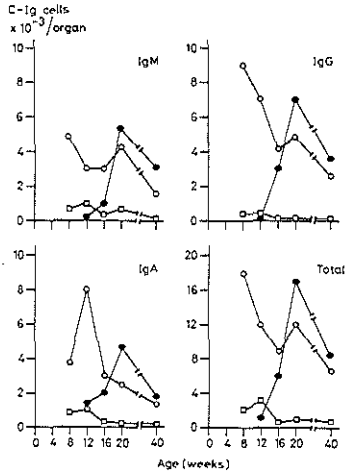

Figure 2. Cytoplasmic immunoglobulin containing cells (C-Ig cells) in the Peyer's patches of nu/nu (□), nu/nu + TH (●) and nu/+ (○) mice as a function of age. Note the difference in the ordinate scale (10^{-3} in stead of 10^{-5}) between this figure and figure 1. See for further details the legend to figure 1.

Thymus transplantation did tremendously increase the C-Ig cell number in the Peyer's patches. Peak numbers were observed 12 weeks posttransplantation (Fig. 2). However, it should be stressed that these absolute numbers were small as compared to spleen and bone marrow. The increase was almost equally distributed over the IgM, IgG and IgA C-Ig cell compartments. The C-IgG cell compartment was the first showing an increase around 8 weeks after transplantation. By forty weeks of age the C-Ig cell numbers in Peyer's patches of nude mice with a thymus transplant were no longer significantly different from those in the heterozygous littermates.

Serum Ig levels after thymus transplantation

The serum levels of IgM, IgG1, IgG2a, IgG2b and IgA in the nu/nu mice and the relevant control groups were measured 4 and 8 weeks after thymus transplantation. These sera were from the same mice

as used for C-Ig cell determinations (Figs. 1 and 2). In 12-week-old nude mice Ig of most (sub) classes were clearly present in all animals tested (Fig. 3). Only IgG1 was at the borderline of detectability in the sera of most nude mice. One animal showed an extremely high IgG1 level, evidenced by the large standard error of the mean in that group. In that particular mouse the C-IgG1 cell numbers in the various lymphoid organs tested were also increased, but by far not to the same extent. Serum levels of IgG1, IgG2a, IgG2b and IgA were lower in nude mice than in the heterozygous littermates ($p < 0.01$). Thymus transplantation at 8 weeks of age raised the serum level of IgG1 in nude mice significantly ($p < 0.05$) within 4 weeks. The increase was not significant for IgG2a and IgG2b, while for IgM and IgA even no indication for an increase of the serum level was obtained by that time. At 8 weeks after thymus transplantation the IgG1, IgG2a and IgG2b levels had further increased, while the IgA level of nude mice with a thymus transplant was now also higher than in the controls. The serum levels of the three subclasses of IgG tested had significantly surpassed the levels in nu/+ mice ($p < 0.02$). Due to the large variation between the individual Ig levels, the overshoot of the IgM and IgA levels in nude mice with a thymus transplant was not significant as compared to the heterozygous littermates.

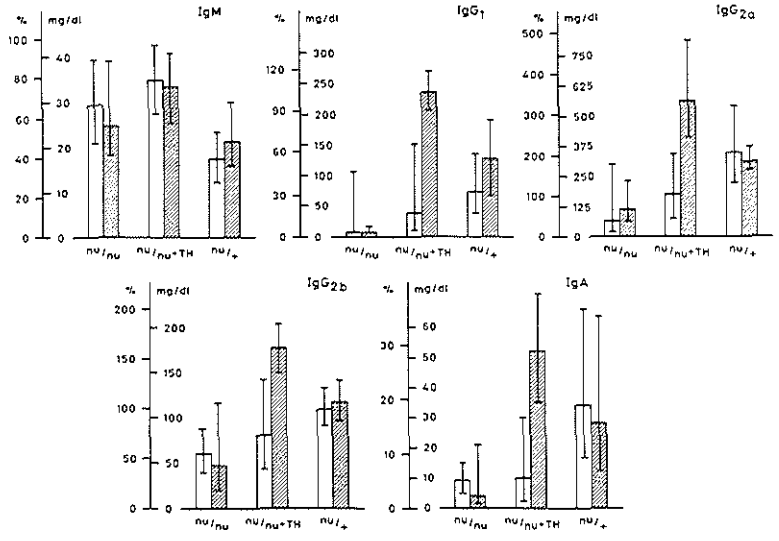

Figure 3. Serum Ig levels in nu/nu, nu/nu + TH and nu/+ mice at 12 (open columns) and 16 weeks (arced columns), respectively. The nu/nu + TH mice received a thymus graft at 8 weeks of age. The levels are expressed in both absolute (mg/dl) and relative values (percentage of a CBA (IgM, IgG1, IgG2b and IgA) or C57BL (IgG2a) reference serum). The bars indicate the 95 per cent confidence limits of the geometric mean.

DISCUSSION

Nude mice are deficient in the formation of antibodies against certain antigens which have been called henceforward T dependent antigens. This deficiency can be alleviated by thymus transplantation or infusion of thymus cell suspensions (Kindred, 1978). The restoration of the humoral immune responses against injected antigens have been monitored by enumerating plaque-forming cells (PFC) in spleen and by measuring serum antibody levels. When young adult nude mice are compared with their heterozygous littermates the C-Ig cell pattern of nude mice is characterized by a normal or increased incidence of C-IgM cells, a deficiency of C-IgG and C-IgA cells, and an increased incidence of C-Ig cells producing more than one Ig heavy chain (Haaijman et al., 1979). In view of these data it was of interest to follow the course of restoration of the C-Ig cell pattern in the various lymphoid organs of nude mice after thymus transplantation. The results presented in this paper clearly show that nude mice have a full B cell potential able to generate in the course of 8-12 weeks a normal C-Ig cell complement, provided T cell help is available. This holds for the spleen as well as the bone marrow and gut-associated lymphoid organs. In the spleen the reaction to the thymus transplantation is most swift for IgG: already 4 weeks after transplantation there is a 6-fold increase of the number of C-IgG cells (Fig. 1), with a predominance of C-IgG2 cells (Table 2). Expansion of the C-IgA cell population in the spleen also occurs already within 4 weeks after thymus transplantation. The influence of the thymus transplant on the C-IgM cells is difficult to assess because the decline with time is superimposed on the normal decline seen in nude mice during ageing.

The bone marrow becomes the major site of C-Ig cells around half a year of age (Haaijman, Schuit and Hijmans, 1977). This is especially true for IgA, suggesting that the marrow is the major source of serum IgA. There is no evidence so far that bone marrow-derived IgA contributes to IgA in secretions.

The large number of C-Ig cells in the bone marrow of advanced-aged mice is in concord with earlier data (Benner and Van Oudenaren, 1975), showing that the spleen is the major source of PFC and serum antibodies during primary responses, but the bone marrow for secondary responses. We have already shown (Haaijman et al., 1979) that the bone marrow in nude mice acquires C-Ig cells in comparable numbers to normal mice, but only in old age (around 2 years of age), and almost exclusively of the IgM class. The results presented here indicate that the

retarded appearance of marrow C-Ig cells is not due to a genetic defect of the bone marrow microenvironment but to lack of functional T cells.

While the lag time before the increase of the C-Ig cell numbers was already longer for bone marrow than for spleen, Peyer's patches reacted even more slowly upon thymus transplantation (Figs. 1 and 2). Between 4 and 12 weeks after thymus transplantation the number of C-IgM, C-IgG and C-IgA cells reach and even surpass the level in the heterozygous littermates (Fig. 2). Subsequently, the Peyer's patches of thymus-grafted nude mice follow the age-related decline characteristic for normal, thymus bearing, mice.

From the difference in timing of the C-Ig cell increase between Peyer's patches and spleen it is suggestive that most Peyer's patch B cells do not differentiate into mature immunoglobulin secreting cells *in situ*. This is in harmony with observations of Cooper, Halliday and Thonard (1967) who inoculated *Salmonella* and *Shigella* antigens into intestinal blind loops of rats, and found that PFC appeared first in the spleen, and subsequently in Peyer's patches. Initially this was interpreted as evidence against a significant share of the gut in the immune response against antigens of enteric origin. Recently, Kagnoff (1977) showed that the number of antigen specific B cells in the Peyer's patches decrease upon antigen feeding. Taken together, these results suggest that antigen-activated B cells from Peyer's patches are likely to migrate to extra-intestinal lymphoid tissues, proliferate there and differentiate into antibody-secreting cells. These cells eventually accumulate in the lamina propria, Peyer's patches and exocrine tissue, where they differentiate into mature plasma cells (Cebra, Gearhart, Kamat, Robertson and Tseng, 1977; Weisz-Carrington, Roux, McWilliams, Phillips-Quagliata and Lamm, 1979).

An alternative explanation for the late appearance of C-Ig cells in the Peyer's patches would be that Peyer's patch B cells have special requirements for T cells which either take long to develop or long to arrive in the gut environment. IgA specific T helper cells have been postulated by Elson, Heck and Strober (1979). We think this explanation to be less likely in view of the rapid IgA development in spleen (Fig. 1).

Up to 27% in spleen (nu/nu, 20-week-old) and 18% in bone marrow (nu/nu, 12-week-old) of the C-Ig cells contain Ig of more than one heavy chain (sub)class. The most frequently observed combination was IgM + IgG, and in cases in which a distinction was

made between IgG1 and IgG2, the combination IgM + IgG2. With exception of the 40-week-old group, the highest number of doubly producing cells was found in nude mice, thus implying a role of functional T cells in the heavy chain isotype switching process. The number of cells containing more than one heavy chain isotype was markedly lower in the gut-associated lymphoid organs (data not shown) than in spleen and bone marrow. This finding might be due to the antigenic load of the gut (Gearhart and Cebra, 1979). Experiments suggesting that the incidence of doubly producing cells is also dependent upon the antigenic load of the animal will be presented elsewhere.

The data on the serum Ig levels of the mice tested and the effect of thymus transplantation (Fig. 3) confirm the data of previous studies (Pantelouris, 1978; Mink et al., 1980). In general, nude mice have similar or increased IgM levels when compared with heterzygous littermates, whereas IgG and IgA levels are depressed. These differences are alleviated by thymus transplantation. The present study enables comparison of C-Ig cell numbers in the various lymphoid organs and serum Ig levels of the same mice. No correlation was found between the serum IgM levels and number of C-IgM cells, which may be explained by the short half life of IgM in serum. Such a correlation was found for the levels of IgG1, IgG2a and IgG2b and the numbers of C-IgG1 and C-IgG2 cells in spleen and bone marrow, indicating that these organs are the major source of serum IgG at the ages tested. The overshoot of C-IgG cells in spleen and bone marrow of nude mice with a thymus transplant 4 and 8 weeks postransplantation causes a higher serum level of the various IgG subclasses than present in the thymus-bearing littermates. The elevated levels of serum IgG in transplanted nude mice between 4 and 8 weeks posttransplantation in spite of the decrease of the total C-IgG cell number might be due to the relatively long half life of IgG (Spiegelberg, 1974).

As for IgM, no correlation could be found between the serum IgA levels and the C-IgA cell numbers in the various lymphoid organs tested. This might be due to a quantitatively important synthesis of IgA at other sites such as the lamina propria. Alternatively, one might argue that the serum IgA level is rather independent of the actual amount of IgA which is synthesized, since, at least in rats, a large proportion of serum IgA is excreted via the bile (Orlans, Peppard, Reynolds and Hall, 1978).

ACKNOWLEDGMENTS

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APPENDIX PUBLICATION V

Immunology 1979 37 889

Homogeneous immunoglobulins in the serum of irradiated and bone marrow reconstituted mice: the role of thymus and spleen

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Summary. The influence of thymectomy and splenectomy on the frequency and class distribution of homogeneous immunoglobulins (H-Ig) in serum was studied in lethally irradiated (DBA/2 × C57Bl/Rij)F_1 mice reconstituted with syngeneic bone marrow. During four follow-up periods in the first 9 months after transplantation, the sham-operated controls and splenectomized animals developed transient H-Ig in an average frequency of 14·2 and 15·7% respectively. There were no marked differences in the incidence of H-Ig within these two groups. In contrast, thymectomized mice and mice both thymectomized and splenectomized showed H-Ig in much higher frequencies (average percentages 31·6 and 36·5, respectively). The highest frequency of H-Ig was observed between 1·5 and 3·5 months after transplantation. H-Ig of the IgG1 and IgG2 subclasses were most frequent in all groups during the first 3·5 months. Later, H-Ig belonging to the IgM class also appeared in somewhat higher numbers. H-Ig of the IgA class was a very rare finding at any time. These results indicate that the presence of the thymus, but not necessarily of the spleen, is an important factor in the regulation of the immunoglobulin heterogeneity during the reconstitution of the immune system in lethally irradiated and bone marrow reconstituted mice.

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INTRODUCTION

Transient homogeneous immunoglobulins (H-Ig) have frequently been observed during the reconstitution process in the sera of recipients of bone marrow grafts in both clinical and experimental transplantations (reviewed by Radl, 1979). Children with severe combined immunodeficiency and patients with aplastic anaemia or leukaemia, who received pre-treatment with an immunosuppressive regimen, developed immunoglobulins of restricted heterogeneity and transient H-Ig (paraproteins) in their sera. In lethally irradiated and bone marrow reconstituted monkeys and mice, similar changes in immunoglobulin heterogeneity were observed. It was shown, that these H-Ig were specific antibodies which gradually became heterogeneous as the reconstitution proceeded (Van den Berg, Radl, Löwenberg & Swart, 1976).

As an explanation for this phenomenon, the presence of insufficient numbers of newly developing B-cell clones capable of responding to an antigenic stimulus was first suggested (Radl, Dooren, Eijsvoogel, Van Went & Hijmans, 1972). Later, imbalanced

T–B cell interactions due to T-cell deficiency as a result of a slower maturation of the T cells, were considered to be responsible for the restrictions in the antibody heterogeneity (Van Muiswinkel, Radl & Van der Wal, 1976). In the present study, the effect of thymectomy, splenectomy and both operations on the incidence and class distribution of H-Ig in sera of lethally irradiated and reconstituted mice was investigated. The presence of the thymus, but not the spleen, was shown to be an important factor in the regulation of the immunoglobulin heterogeneity during reconstitution of the immune system after lethal irradiation and bone marrow transplantation.

MATERIALS AND METHODS

Animals

(DBA/2 × C57Bl/Rij)F_1 male and female mice were used. They were obtained from the Medical Biological Laboratory TNO, Rijswijk, The Netherlands.

Surgery

Splenectomy (Sx), sham splenectomy (SSx), thymectomy (Tx) and sham thymectomy (STx) were performed when the mice were 6–7 weeks of age. The mice were anaesthetized by intraperitoneal injection of 0·5 ml of a 10% solution of Evipan (Bayer A.G., Leverkusen, G.F.R.) in saline containing 0·03% atropin sulphate (Bayer A.G.). For splenectomy, the splenic blood vessels were tied with a single suture, then cut and the spleen was removed. Thymectomy was performed as described by Miller (1960). When splenectomy and thymectomy were carried out in the same animal, both operations were performed within 1 h. Sham surgery was performed similarly, except that the spleen and thymus were not removed.

Irradiation

The recipient mice received 850 rad whole body irradiation, generated in a Philips Müller MG 300 machine. Animals were irradiated in well aerated circular perspex cages. Physical constants of the irradiation were: 250 or 300 kV (10 mA; 1·0 mm Cu filtration; dose rate: 32 or 75 rad/min). Maximal back scatter was achieved by placing the cage on a layer of 11 cm hardboard. During irradiation, the dose was measured with a Baldwin Ionex dosimeter. Irradiation was carried out 2–4 weeks after surgery.

Cell suspensions

After killing the mice with ether, lymphoid organs and/or femurs were isolated in a balanced salt solution (BSS) according to Tyrode (1910) or Mishell & Dutton (1967), supplemented with 5–8% newborn calf serum (NCS). Bone marrow was obtained by flushing the femurs with BSS solution containing NCS. Single cell suspensions of spleens, mesenteric lymph nodes and bone marrow were counted for viable nucleated cells in a haemocytometer, using 0·2% trypan blue in BSS solution as a diluent.

Bone marrow transplantation

Irradiated mice were reconstituted by intravenous (i.v.) injection of 3×10^6 viable bone marrow cells in 0·5 ml BSS within 4 h after irradiation. In one experiment, the bone marrow cells were treated with anti-Thy-1.2 antiserum and guinea-pig complement *in vitro* before i.v. injection. The anti-Thy-1.2 antiserum was raised in AKR mice and used for selective elimination of T cells as described previously (Van Muiswinkel & Van Soest, 1976).

Serum analysis for H-Ig

At various times after irradiation and reconstitution, the anaesthetized mice were bled from the retro-orbital sinus or by heart puncture. The sera were

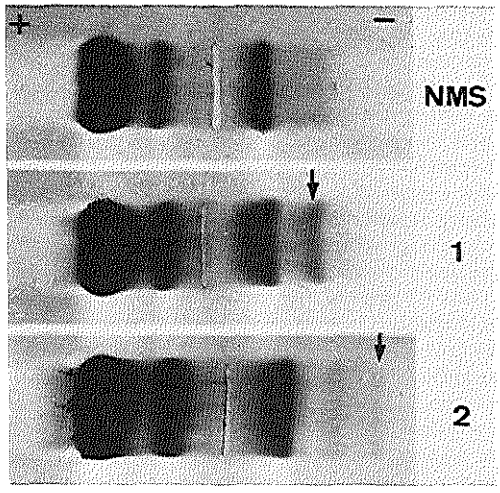

Figure 1. Wieme's agar electrophoresis of pooled normal (DBA/2 × C57Bl/Rij)F_1 mouse serum (NMS) and sera from irradiated and bone marrow reconstituted mice (1 and 2). Note the distinct homogeneous immunoglobulin component in serum 1 and the faint but still distinct band in serum 2, both indicated by arrows. The anode is to the left.

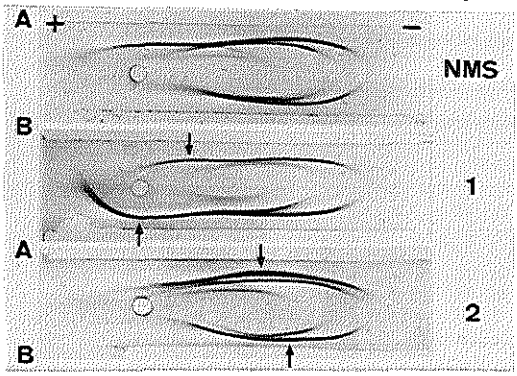

Figure 2. Immunoelectrophoresis of pooled normal mouse serum (NMS) and sera from irradiated and bone marrow-reconstituted mice (1 and 2). Rabbit antiserum to mouse immunoglobulins (A) and to mouse IgG2 (B) were placed in the troughs. Note the homogeneous component of different Ig subclasses in sera 1 and 2; they are indicated by arrows. The anode is to the left.

examined for the presence of H-Ig by agar electrophoresis according to Wieme (1959) and by immunoelectrophoresis according to Scheidegger (1955) using polyvalent and monospecific antisera to mouse immunoglobulin classes and subclasses (Nordic, Tilburg, The Netherlands and Meloy Laboratories Inc., Springfield, Virginia, U.S.A.). The criterion for the designation of H-Ig in a serum sample was the occurrence of a narrow, homogeneous extra band in the $\beta-\gamma$ region when tested by electrophoresis according to Wieme (Fig. 1), a symmetric deviation of an immunoglobulin precipitin line when examined by immunoelectrophoresis (Fig. 2). In some sera in which the presence of H-Ig could not be established with certainty, a technique of immunofixation according to Cejka & Kithier (1976) was applied.

RESULTS

The incidence of H-Ig

H-Ig were detected in a number of sera of splenectomized (Sx), thymectomized (Tx) and sham-operated mice during the entire observation period. The percentage of mice with H-Ig at various times after irradiation and reconstitution is given for each experimental group in Table 1. The sham-operated and Sx mice developed H-Ig in their sera in comparable average frequencies, i.e. 14·2 and 15·7%, respectively. There was no clear-cut indication for a peak incidence in any of the four follow-up periods in either of the two groups. In contrast, Tx and animals undergoing both Tx and Sx showed H-Ig in much higher frequencies (average percentages of 31·6 and 36·5, respectively). Here, the highest percentage of H-Ig was observed in the second period of observation, i.e. between 1·5 and 3·5 months after transplantation. During the follow-up of individual mice, it was noted that most of the H-Ig were transient in their appearance. In some animals, however, they persisted for a period of 2 or 3 months.

Class distribution of H-Ig

The class distribution of the H-Ig in the four experimental groups is shown in Table 2. In some animals, two components of H-Ig of different Ig classes were found at the same time. For that reason, the sum of the

Table 1. Percentage of mice with homogeneous immunoglobulins after irradiation and reconstitution with bone marrow cells

Exp.	Surgery* before irradiation	Months after reconstitution 0·5–1·0	1·5–3·5	4·0–5·5	6·0–9·5	Mean percentage of the total period
I	Sx	19 (91)†	8 (85)	22 (122)	14 (95)	15·7
II‡	Tx	35 (62)	36 (119)	n.t.§	24 (41)	31·6
III	Sx+Tx	29 (21)	48 (21)	43 (21)	26 (19)	36·5
Control	—	19 (28)	13 (90)	5 (75)	20 (87)	14·2

* Sx, splenectomy; Tx, thymectomy; Control, sham Sx and/or Tx. All mice were lethally irradiated and reconstituted with 3×10^6 syngeneic bone marrow cells.

† Number of mice tested in parentheses.

‡ In this particular experiment reconstitution was done with bone marrow cells, treated with Thy-1.2 antiserum and complement *in vitro* before transfer.

§ n.t., not tested.

Table 2. Class distribution of homogeneous immunoglobulins in irradiated and reconstituted mice*

Exp.	Surgery before irradiation	Months after reconstitution															
		0·5–1·0				1·5–3·5				4·0–5·5				6·0–9·5			
		μ	α	γ1	γ2†	μ	α	γ1	γ2	μ	α	γ1	γ2	μ	α	γ1	γ2
I	Sx	0	1	6	12	1	0	3	5	14	0	4	6	9	0	4	1
II	Tx	0	2	19	16	5	1	18	18	n.t.				10	2	7	7
III	Sx+Tx	0	0	19	14	0	0	24	24	5	0	14	29	0	0	5	21
Control	—	0	0	5	14	0	0	7	7	1	0	4	0	5	1	5	9

* Data were obtained from the same mice used to determine the incidence of H-Ig at various times after irradiation and reconstitution (Table 1). In some groups H-Ig of more than one class or subclass were found in a single mouse. That is why the sum of the percentages of H-Ig in some groups of Table 2 is larger than in the groups of Table 1. For abbreviations, see Table 1.

† Percentage of mice with H-Ig in this particular Ig class or subclass.

percentages of H-Ig in some groups of Table 2 is higher than in the corresponding group of Table 1. During the two first observation periods (0·5–3·5 months), H-Ig of the IgG1 and IgG2 subclasses predominated in all groups of mice. H-Ig of the IgM class were absent in control and both Tx and Sx animals or low in number in Tx and Sx mice. After 4·0–9·5 months, H-Ig of the IgM class also appeared in higher numbers, while the incidence of H-Ig of the IgG class decreased or remained approximately the same. H-Ig belonging to the IgA class were a very rare finding in any of the experimental groups.

DISCUSSION

Immunodeficient individuals who were successfully treated by bone marrow transplantation, frequently developed transient H-Ig in their sera during the reconstitution period (Radl, 1978). The mechanisms, responsible for the appearance of H-Ig are not yet fully understood. Some of the H-Ig appearing early in the reconstitution process, might reflect the immune responsiveness of single B-cell clones, which are available to respond to an antigenic stimulation in a similar way as observed in limiting dilution experiments (Kreth & Williamson, 1973) or by mechanisms, described under the term clonal dominance (Williamson, Zitron & McMichael, 1976). Other observations (Radl, 1978; Van Muiswinkel *et al.*, 1976), including those reported here, indicate that it is mainly a defect of the T immune system and not of the B immune system which leads to the restriction in the heterogeneity of immunoglobulins and to the appearance of H-Ig. During reconstitution, this kind of deficiency may occur as a result of a gradual and unequal development, where the T system matures more slowly than the B system. It has been shown, that B cells in mice reach their normal values 4–6 weeks after irradiation and reconstitution (Nossal & Pike, 1973; Rozing & Benner, 1976), whereas the T-cell system is still below its normal level at 30 weeks after reconstitution (Rozing & Benner, 1976). In accordance with this view are experiments which showed that repeated infusions of additional T cells to the reconstituted mice remarkably reduced the phenomena of restricted heterogeneity of the immunoglobulins during the recovery period (Van Muiswinkel *et al.*, 1976). The question of the role of T helper or suppressor cells or both in the regulation of the heterogeneity of the immune response, remains open and should be studied in similar experiments using separated T-cell subpopulations.

The influence of splenectomy on the heterogeneity of serum immunoglobulins during the reconstitution process was investigated, because of the known major role, which this organ plays in the immune response. Congenital absence of the spleen as well as neonatal and adult splenectomy affects the humoral immune response (reviewed by Auerbach, 1978). B and T lymphocytes generated by hereditarily asplenic mice and neonatally splenectomized mice have been reported to be deficient in their capacity to cooperate in adoptive antibody formation; later studies, however, did not confirm this observation. Our experiments were designed in order to answer the question, whether the absence of the spleen would be detrimental to the

function and retard the maturation of both T and B cells (as reflected in the Ig heterogeneity) during the reconstitution period. The results demonstrated no differences in the frequency of H-Ig between splenectomized and sham-operated controls. Splenectomy performed in addition to thymectomy also showed no clear-cut additive effect as far as the numbers of paraproteins were concerned. These results indicate, that other sites of the immune system can readily compensate the removal of the spleen as was also observed in normal splenectomized mice (Rozing, Brons & Benner, 1978).

The follow-up study showed that the serum pattern might be used as a sensitive indicator of the reconstitution process. Considering a heterogeneous immunoglobulin spectrum in the serum of all mice as a criterion for the completion of the reconstitution, none of the experimental groups achieved that during the observation period of 9·5 months. This indicates that normalization of the immune system after a lethal irradiation and bone marrow transplantation is a long-lasting process. In irradiated and reconstituted monkeys, a normal heterogeneous Ig spectrum was achieved only after 1–2 years following transplantation (Van den Berg *et al.*, 1976).

Under normal conditions, the (DBA/2 × C57Bl/-Rij)F_1 mice used in this study show no spontaneous H-Ig in their sera during the first 12 months of life; they develop H-Ig spontaneously in increasing frequencies, however, later during ageing (Radl, Hollander, Van den Berg & De Glopper, 1978). These H-Ig in aged animals are not transient and they reflect a different condition, the so-called idiopathic paraproteinaemia. Because of the fact that the H-Ig in mice in the present experiments were transient, it is unlikely that our figures were distorted by, e.g. premature ageing of the irradiated animals. Additional influence of the irradiation, thymectomy and splenectomy on the appearance of idiopathic paraproteinaemias in these animals during ageing was not studied.

The analysis of the class distribution of H-Ig showed a clear-cut predominance of the IgG class in all four experimental groups and during the entire observation period. H-Ig of the IgM class were absent or in very low numbers during the first three months after transplantation. Only later they appeared in somewhat higher numbers. H-Ig of the IgA class were found only sporadically in any of the four groups and during the whole experiment. This frequency distribution of H-Ig can perhaps be explained by the high thymus dependency of the B cells committed to the production of antibodies of the IgG class (Pritchard, Riddaway & Micklem, 1973; Van Muiswinkel & Van Soest, 1975). The high representation of H-Ig of this class, especially in the Tx and both Tx and Sx groups, may support this idea. Only investigations using specific T dependent and T independent antigen–antibody systems could clarify this point.

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APPENDIX PUBLICATION VI

INCREASED FREQUENCY OF HOMOGENEOUS IMMUNOGLOBULINS IN THE SERA OF NUDE ATHYMIC MICE WITH AGE

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SUMMARY

Sera from aging nude athymic mice, their heterozygous littermates and normal mice of the BALB/c and CBA background strains were investigated for the presence of homogeneous immunoglobulins (H-Ig) by agar electrophoresis, immunoelectrophoresis and immunofixation. While both the normal CBA and BALB/c mice showed only a very low incidence and a late onset of H-Ig, the barrier maintained and even more so the conventionalized nude mice developed H-Ig in very high frequencies with age. The incidence curve of H-Ig in the heterozygous mice occupied an intermediate position between that for the nude mice and the mice of their background strains. Follow-up studies of 50 barrier maintained nude mice demonstrated persisting H-Ig in 46% and transient H-Ig in 20% of the cases. The percentage distribution of individual Ig isotypes among 320 H-Ig components from the sera of nude mice was 1, 17, 43, 19, 8 and 12 for IgA, IgM, IgG1, IgG2a, IgG2b and IgG3, respectively. Of these H-Ig, 15% contained λ and 85% κ light chains. These findings stress the importance of the T immune system in the regulation of the heterogeneity of Ig and are compatible with the hypothesis on the crucial role of an impairment in the T system in the development of age-related Ig abnormalities, including idiopathic paraproteinemia.

INTRODUCTION

Changes occurring during aging in the spectrum of serum immunoglobulins (Ig) are characterized by the appearance of Ig of restricted heterogeneity, transient homogeneous immunoglobulins (H-Ig) and persistent H-Ig, the so called idiopathic paraproteinemia (IP). On the basis of results of investigations in humans and experimental laboratory animals (reviewed in 1) a hypothetical explanation of these changes has been offered (2), suggesting that all of the phenomena appear as

a consequence of an age-related deficiency of the T immune system. The resulting imbalance in the T-B immune system network leads to restricted (as far as the numbers of clones are concerned) but overshooting responses to antigenic stimulation (i.e., antibodies of restricted heterogeneity and transient H-Ig). Eventually a defect in cell regulation within one B cell clone can occur which then continues at its high rate of proliferation and H-Ig production even after the antigenic stimulus has disappeared (i.e, IP). The crucial role of a defect in the T immune system in the development of these age-related changes is indicated by previous studies in thymectomized C57BL and CBA mice (3) and further supported by the present investigation in congenitally athymic nude mice.

MATERIALS AND METHODS

Mice

Nude athymic (Nu) mice and their heterozygous littermates, normal BALB/c and CBA/BrARij mice were bred and maintained in the mouse colonies of the REP institutes TNO in Rijswijk. Nu mice and their heterozygous littermates were the second and third generations after cross breeding of male Nu mice on a CBA background and a female BALB/c mice (mice up to 2 years of age) or they were of a CBA background only (mice over 2 years of age). At the beginning of the experiments, all Nu mice and their heterozygous littermates were pathogen free and barrier maintained. A number of these mice was transferred to conventional facilities at the ages of 3, 10 and 12 months; they are referred to in the text as conventionalized. The other Nu and heterozygous mice were barrier maintained throughout the experiments. The normal BALB/c and CBA mice were bred and kept under conventional conditions. All barrier maintained mice received sterilized pelleted food (Hope Farms, Woerden, The Netherlands) and sterilized water. Conventionally kept mice received similar, but unsterilized food and acidified water (pH 3-4). Food and water were available *ad libitum*. Small blood samples were taken at intervals varying from 1 to 3 months and the serum was investigated within 24 hours or stored frozen at -20°C for later use. In addition to cross-sectional studies on Nu mice of different ages, two groups of barrier maintained Nu mice were followed-up monthly for periods of more than 6 months in order to determine the frequency of transient and persistent H-Ig in their sera. Prolonged follow-up of the conventionalized Nu mice was not possible because of their short survival time (2 to 3 months) under conventional conditions. Complete necropsies and histo-

logical examinations were performed on animals which were killed and on those dying spontaneously if not severely autolyzed. The necropsies were done according to a standard protocol.

Detection of homogeneous immunoglobulins
All sera were investigated for the presence of H-Ig by agar electrophoresis according to Wieme (4) and by immunoelectrophoresis using specific antisera to individual mouse Ig isotypes. In doubtful cases and in the follow-up studies, the technique of immunofixation (5) performed on Wieme's agar plates (4) was used. Preparation and specificity testing of antisera and methods of evaluation of the Ig spectra have been reported in detail elsewhere (1,6,7).

RESULTS

Histopathology
Histological examination was carried out on 40 barrier maintained Nu mice killed at approximately 15 months of age, and on 30 conventionalized Nu mice dying between 12 and 15 months of age. In addition, 14 barrier maintained heterozygous mice killed at approximately 20 months of age were examined histologically. Careful examination of the anterior mediastinal region revealed the presence of thin-walled epithelial-lined multiloculated cysts in the majority of the Nu mice, and partially atrophic thymic lobes in the heterozygous mice.

Lesions consistent with mouse hepatitis virus infection were found in 28 of the 30 conventionalized Nu mice. These lesions included mild to severe multifocal hepatic necrosis, syncytial giant cell formation in mucosal epithelium of cecum and colon, in endothelium of meningeal blood vessels, and in lymph nodes, spleen and bone marrow. In addition, a number of these mice showed multifocal interstitial pneumonia associated with syncytial giant cells in bronchial mucosa and free in alveolar lumens.

Lymphoreticular neoplasms were found in 5 of the 40 barrier maintained Nu mice. Three of these were lymphoblastic malignant lymphomas, and 2 were compatible with reticulum cell sarcoma type B. None of the mice with malignant lymphomas had persistent H-Ig. An intestinal malignant lymphoma was found in one of the 30 conventionalized Nu mice; it was not accompanied by H-Ig. No lymphoreticular tumors were identified in the barrier maintained heterozygous mice. The reason for the higher incidence of lymphoreticular tumors in barrier maintained Nu

mice as compared with mice from the other two groups is not clear at present.

Other neoplasms found in the barrier maintained Nu mice included pulmonary alveologenic carcinoma (1 case), adrenal cortical adenoma (2 cases), ureter transitional cell carcinoma (1 case), and Harderian gland adenoma (1 case). The only non-lymphoreticular neoplasm found in the group of conventionalized Nu mice was a pulmonary alveologenic carcinoma. Seven of the 14 barrier maintained heterozygous littermates had alveologenic carcinomas. Other tumors in this group included one adrenal cortical adenoma, and one Harderian gland adenoma.

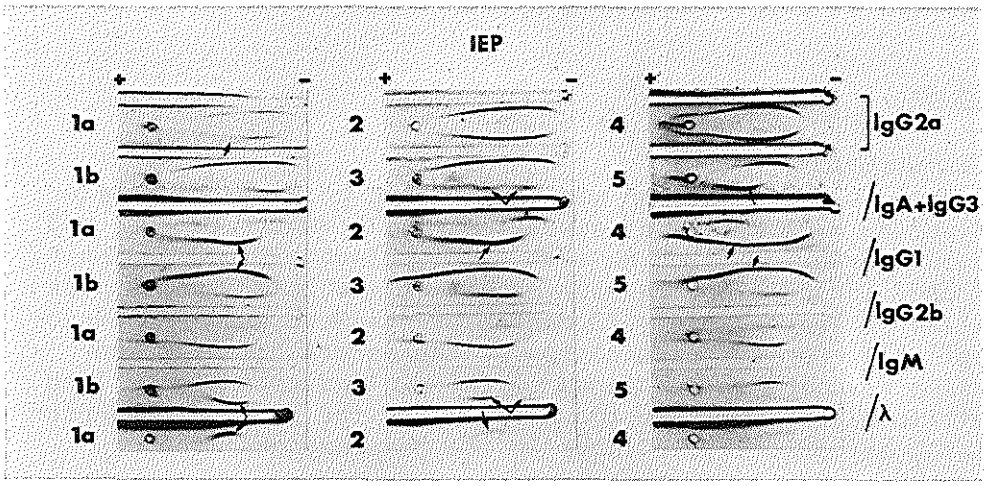

Figure 1. Demonstration of Ig abnormalities in the sera of five conventionalized nude athymic mice by means of immunoelectrophoresis. Individual serum samples are indicated by numbers (left side), the antisera used (specific for individual Ig classes and subclasses) are indicated next to each corresponding trough (right side). Note the symmetric deviations of the precipitin lines (arrows) indicating the presence of H-Ig of the IgG2a (1a), IgG3 (3 - two paraproteins, both of the λ light chain type - and 5), and IgG1 (1a and 1b - the same mouse at age of 10 and 12 months, respectively, paraprotein of λ light chain type - and samples 2 (λ), 4 and 5) subclass. Note also the restrictions in the IgG2a (5), IgM (5) and the IgG2b (2,4) lines and the absence of the IgA (5), IgG3 (4) and the IgG2b (1a) precipitin lines.

Analysis of serum Ig

The serum Ig of the Nu mice generally showed a distinctly abnormal pattern. A decreased level or deficiency in Ig of some of the classes or subclasses (usually of the IgA and IgG2b isotypes) was often accompanied by the appearance of Ig of restricted heterogeneity and/or of H-Ig components of the other isotypes (Fig. 1). These H-Ig were usually rather low in concentration (below 2mg.ml^{-1}) in the barrier maintained Nu mice; therefore, they were usually detectable only by immunoelectrophoresis and immunofixation but not by agar electrophoresis. In

contrast, the level of H-Ig in the conventionalized Nu mice was often higher; consequently, these components could also be seen on the agar electrophoresis (Fig. 2). With increasing age, multiple H-Ig components were often found in individual Nu mice of both groups. Such abnormalities in the serum Ig were far less pronounced in the heterozygous mice. Even though some of the aging individuals in this group also showed H-Ig in their sera, multiple forms were never found in a single mouse.

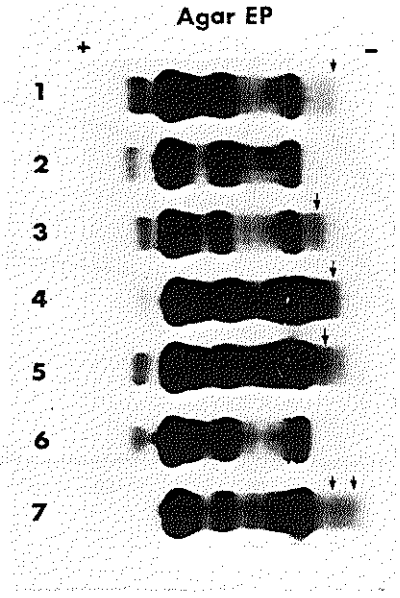

Figure 2. Comparison of the agar electrophoresis pattern of seven different sera from conventionalized 1 year old nude mice. Note the homogeneous components (arrows) in samples 1, 3, 4, 5 and 7, the "empty" (2,6) or restricted pattern (3,4) of the γ-globulin region.

The increase in frequency of H-Ig with age in the sera of both barrier maintained and conventionalized nude mice and in sera of heterozygous mice is demonstrated in Fig. 3. For comparison, data on the incidence of H-Ig in the sera of the background strains are also given. While both the normal aging CBA and BALB/c mice showed only a very low incidence and a late onset of H-Ig, the barrier maintained Nu mice, and even more so the conventionalized Nu mice, developed H-Ig in their sera with age in very high frequencies. The incidence curve for H-Ig in the heterozygous mice occupied an intermediate position between that for the Nu mice and mice of their background strains.

Follow-up studies of 50 barrier maintained Nu mice for periods of more than 6 months demonstrated persisting H-Ig in 46% of the mice and a transient appearance of H-Ig in 20%. However,

about 50% of the mice with the persistent paraproteinemia also showed additional transient H-Ig in their sera during the observation period. An example of such a follow-up examination by immunofixation is shown in Fig. 4. Histopathological analysis performed in 40 of these 50 mice excluded the possibility that the persistent H-Ig were products of a lymphoreticular malignancy.

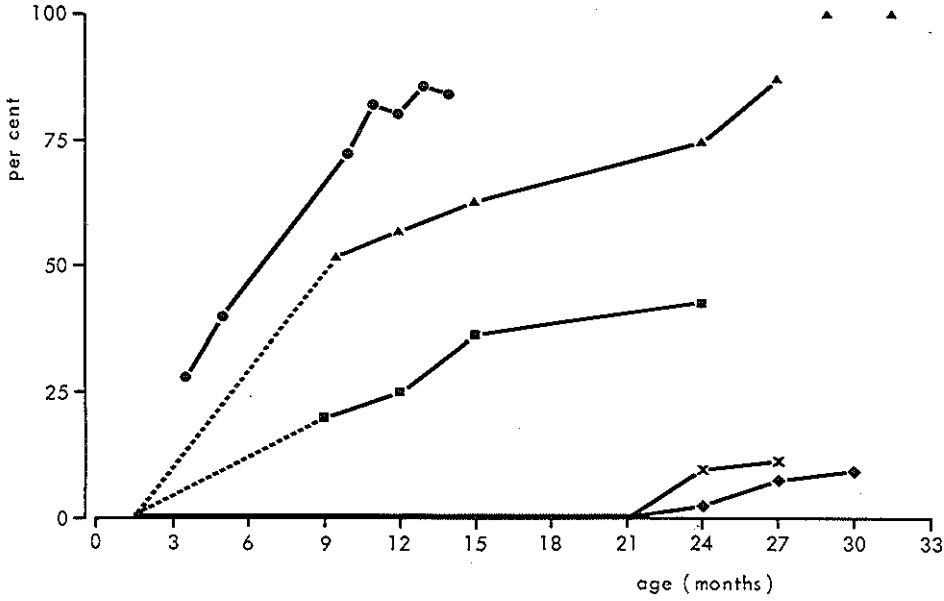

Figure 3. Frequency of homogeneous Ig in the sera of nude athymic mice, their heterozygous littermates and control background CBA and BALB/c mouse strains during aging. (●—●) conventionalized nude mice (n = 169); (▲—▲) barrier maintained nude mice - the last two values from 7 mice only (n = 190); (■—■) barrier maintained heterozygous mice (n = 59); (×—×) conventional control BALB/c mice (n = 327); (◆—◆) conventional control CBA mice (n = 359).

The percentage distribution of individual Ig classes and subclasses among 320 H-Ig components from the sera of Nu mice was 1, 17, 43, 19, 8 and 12 for IgA, IgM, IgG1, IgG2a, IgG2b and IgG3 respectively. Typing of the light chains of the H-Ig revealed that 15% of these belonged to the λ and 85% to the κ type. Too few of H-Ig components were available for a comparable classification in the heterozygous and control mice.

DISCUSSION

The results of this study are in agreement with those of our previous investigations (3,8,9,10), showing that mice with a severe impairment in the T immune system are able to produce immunoglobulins. However, these are often of restricted hetero-

geneity or are homogeneous, reflecting deficiencies in some and excess proliferation in other B cell clones. With aging, these abnormalities increase, are more pronounced and appear earlier in animals with a higher antigenic load (i.e., in the conventionalized Nu mice). The majority of the dead Nu mice from the conventionalized groups examined histologically were shown to suffer from a hepatitis virus infection. Considering the reports of Tamura et al. (11) and Okudaira et al. (12) on the stimulating influence of the mouse hepatitis virus on the immune system in Nu mice, it would be interesting to know to what extent this specific infection was responsible for the increased frequency of H-Ig in our Nu mice.

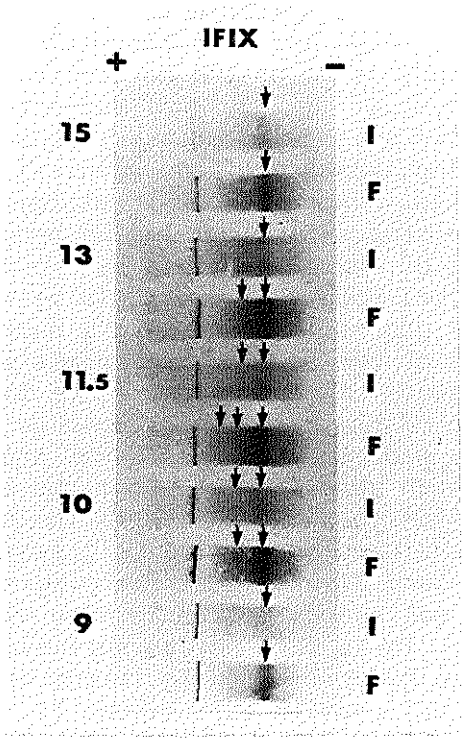

Figure 4. Example of the follow-up investigation of H-Ig in the (1:4 diluted) serum of one barrier maintained nude mouse by immunofixation. Symbols: age in months - left side; antisera used for the fixation of the serum Ig (I = rabbit antiserum to all Ig classes and subclasses, F = rabbit antiserum to the Fab fragment of heterogeneous IgG) - right side. H-Ig are indicated by arrows.

Compared with the Nu mice, the heterozygous littermates distinctly showed fewer abnormalities in their serum Ig spectrum with age. However, in comparison with the mice of the background strains, the frequency of H-Ig was much higher and the onset markedly earlier in the heterozygous mice. This may indicate that the T immune system in mice heterozygous for the

Nu gene is not entirely normal. If proved correct, such a conclusion would have consequences for the results of experiments on the immune functions where abnormal Nu mice are compared with their "normal" littermates. As especially indicated by reconstitution experiments (reviewed in 13), it seems that the appearance of Ig with restricted heterogeneity and of transient H-Ig in serum may be considered as very sensitive indicators of an impairment in the T immune system and a consequent imbalance of the T-B immune system network.

The question of how many of the H-Ig in the sera of Nu mice represent permanent idiopathic paraproteinemia is difficult to answer with certainty at present. According to our previous studies in aging C57BL mice (7), the criteria for IP can be briefly summarized as follows: a long lasting paraproteinemia (at least 6 months) with a steady level of the paraprotein not exceeding a concentration of 4 $mg.ml^{-1}$; levels of the other, normal Ig within normal range or only slightly decreased; absence of Bence Jones protein in the urine; absence of any clinical signs of malignancy in the animals. A more recent investigation (14) demonstrated that an IP-producing clone can be propagated in young healthy recipients of the same strain by a bone marrow or spleen cell transfer; however, this could be maintained for only three to four generations and with a decreasing "take" frequency. Because of the technical problems involved in the handling and follow-up of larger numbers of barrier maintained Nu mice, only some of the parameters could be checked. Nearly one half of the Nu mice from the follow-up study showed a paraproteinemia which persisted at about the same concentration (below 4 $mg.ml^{-1}$) for the entire observation period of more than 6 months. There were no clinical signs of a malignancy in any of the mice and also no histopathological findings of a lymphoreticular malignancy in the Nu mice with persistent H-Ig in this group. These data may be sufficient to make the assumption that the majority of H-Ig in the aging Nu mice were not products of a malignant B cell clone. It is likely that many of the H-Ig reflected an impairment in B cell regulation due to the inherited defect in the T immune sytem and that many of the B cell clones which proliferated in excess also became targets for an - up to now unknown - event leading to the development of IP, most probably a benign neoplasm of a B cell line (2). Further studies, e.g., on the transplantability of these H-Ig producing clones, are warranted in order to prove whether or not this assumption is correct.

The percentage distribution of individual Ig classes and subclasses among the H-Ig components in the sera of Nu mice showed

the most prevalent to be the IgG1 and the least to be the IgA and the IgG2b isotypes. In fact, this distribution of H-Ig in the sera of Nu mice from this study roughly correlates with that of the individual Ig isotypes according to their average serum concentration as determined in Nu mice in our previous investigation (10). However, this percentage distribution of the Ig isotypes among the paraproteins in the Nu mice differs from that found in both normal and thymectomized C57BL mice (the most prevalent being the IgG2a isotype) as well as in thymectomized CBA mice (the H-Ig occurring most frequently were of the IgM isotype) (3). These differences may reflect genetic influences on the repertoire of specific antibodies within individual isotypes; however, they may also indicate that B cells committed to the production of a certain isotype might have a genetically determined increased susceptibility to develop an intrinsic cellular defect in the control of cell proliferation and H-Ig production.

In conclusion, the findings of immunoglobulins with restricted heterogeneity and of transient and persisting homgeneous Ig components in high frequencies in the sera of the aging nude athymic mice add new evidence for the notion on the important role of the T immune system in the regulation of the heterogeneity of immunoglobulins, i.e., antibodies. They are also compatible with the hypothesis (2) on the crucial role of an impairment in the T immune system for the development of age-related Ig abnormalities, including the benign proliferative B cell disorder designated as idiopathic paraproteinemia or benign monoclonal gammapathy.

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