PATHOGENETIC ASPECTS OF BENIGN MONOCLONAL GAMMAPATHY

- STUDIES IN AN EXPERIMENTAL MODEL -

PATHOGENETISCHE ASPECTEN VAN BENIGNE MONOCLONALE GAMMAPATHIE

- STUDIES IN EEN EXPERIMENTEEL MODEL -

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
Prof. Dr. A.H.G. Rinnooy Kan
en volgens besluit van het College van Dekanen.
De openbare verdediging zal plaatsvinden op
woensdag 16 december 1987 om 14.00 uur

door

THEODOOR WILHELM VAN DEN AKKER

geboren te Alblasserdam

1987
Offsetdrukkerij Kanters B.V.,
Alblasserdam

PROMOTIECOMMISSIE

Promotoren:	Prof. Dr. R. Benner Prof. Dr. O. Vos
Overige leden:	Prof. Dr. J. Abels Prof. Dr. R.O. van der Heul

Dit proefschrift werd bewerkt binnen het instituut Immunologie van de vakgroep Celbiologie en Genetica van de Erasmus Universiteit te Rotterdam, in samenwerking met het TNO Instituut voor Experimentele Gerontologie te Rijswijk.
Het onderzoek werd mede mogelijk gemaakt door financiële steun van het Koningin Wilhelmina Fonds.

... uit Hem en door Hem en tot Hem zijn alle dingen.

(Rom. 11:36)

Aan Greetje

CONTENTS Page

Chapters III-VIII represent the appendix papers of this thesis.

CHAPTER I

GENERAL INTRODUCTION

I.1. INTRODUCTION TO MONOCLONAL GAMMAPATHIES

1. Definition

Monoclonal gammapathy (MG) can be defined as a monoclonal B-cell proliferation characterized by the production of a component of homogeneous immunoglobulins (H-Ig), that can usually be detected in the serum. Synonymous with H-Ig component, the terms M-component ('M' from monoclonal) and paraprotein are common. In this thesis, the term H-Ig component will be used. The above definition of MG implies that the indicated monoclonal B-cell proliferation involves eventually those cells of the B-cell lineage that are mature enough to secrete immunoglobulins (Ig) or an Ig fragment in special situations.

2. Techniques

In 1939, Arne Tiselius was the first to report the use of electrophoresis for the detection of H-Ig (Tiselius and Kabat, 1939). His free boundary electrophoresis for the analysis of sera with H-Ig of high concentration was of great importance in the diagnosis of multiple myeloma (MM) and Waldenström's macroglobulinemia (MW). The development of electrophoresis on paper and later on agar (Wieme, 1959) and membrane carriers increased the detection sensitivity of this diagnostic tool. Furthermore,

this type of diagnostic electrophoresis became accessible even to small hospital laboratories. A consequence of this was the occasional report of distinct H-Ig components in the serum of patients without an apparent plasma cell malignancy. The technical improvements of the detection of H-Ig continued and nowadays the techniques of immunoelectrophoresis (Grabar and Williams, 1953; Scheidegger, 1955), immunoselection (Radl, 1970), isoelectric focusing (Williamson, 1973), immunofixation (Cejka and Kithier, 1976) and immunoblotting (since 1985) provide a firm basis for detailed investigations of the sera of patients with a MG. The sensitivity of some routine techniques is represented in Table I (Radl, 1985a).

TABLE I
LOWER LIMITS IN THE DETECTION SENSITIVITY FOR HOMOGENEOUS IMMUNOGLOBULIN COMPONENTS IN THE SERUM IN SOME ROUTINE TECHNIQUES

Technique	H-Ig concentration in $\mu g/ml$	Remarks
Electrophoresis on paper and other carriers	2000-5000	some H-Ig in $\alpha 2$-$\beta 2$ region can be missed
High voltage, zone electrophoresis (agar, membranes)	100	some H-Ig in $\alpha 2$-$\beta 2$ region can be missed
Immunofixation	50	
Immunoblotting	0.5-1	

(From Radl, 1985a)

The present technical possibilities have resulted in the discovery of a broad array of conditions accompanied by a MG. It became clear that especially elderly persons were prone to develop H-Ig in their sera. These H-Ig turned out to be far more often the result of benign than malignant monoclonal proliferations (frequency ratio about 200 : 1).

3. Classification of monoclonal gammapathies

Because of the heterogeneity among MG as indicated above, several investigators tried to classify MG. Since the information on the etiology and pathogenesis of the MG was mostly absent or at best scarce, the different attempts at classification of MG were usually of a descriptive nature. Such classifications were published by several investigators, for instance, Zawadski and Edwards (1972), Waldenström (1973), Kyle and Bayrd (1976), Stone (1982) and Radl (1982). More recent investigations substantially improved our understanding of the development of the different forms of MG. Three factors greatly contributed to this progress: 1) the development and continuous improvement of laboratory techniques for the detection of H-Ig, as mentioned above; 2) long-term clinical follow-up studies; and especial-

ly, 3) the discovery of experimental animal models of the human disorders with MG. On the basis of the results of these recent clinical and experimental investigations, which our studies as described in this thesis form part of, Radl lately proposed that most MG can be classified into one of four major categories (Radl, 1985b): 1) B-cell malignancies; 2) B-cell benign neoplasias; 3) immunodeficiencies characterized by a T < B immune system imbalance; and 4) homogeneous antibody response due to a particular antigenic stimulation. This classification in four categories is represented in Table II, together with examples of clinical conditions, animal models and some characteristics of the accompanying H-Ig components.

4. Differential diagnostic problems

For the patient's sake, it is most important to be able to discriminate malignant from benign MG. On the one hand, incorrect interpretation of the clinical and laboratory findings may result in an unnecessary and even harmful treatment with cytostatics in patients with benign monoclonal gammapathy (BMG) or immunodeficiency. On the other hand, a timely diagnosis and application of appropriate therapy of a B-cell malignancy can be missed if a faulty evaluation of the patient's data leads to the discharge of the patient from the regular controls (Radl, 1982).

In the majority of cases, modern clinical, hematological and immunological investigations will lead to the correct diagnosis. However, problems arise, when all these data are within normal ranges and a small H-Ig component of low concentration (H-Ig concentration < 10 g/l) is present in the patient's serum. Since there is a considerable overlap of the values of the H-Ig concentrations in the four categories in some cases, this patient may suffer from a beginning malignant MG, a BMG or an immunodeficiency, while a homogeneous antibody response due to a particular antigenic stimulation may also be the cause of the small H-Ig component. Then, the most reliable parameter in differential diagnosis is the behaviour of the MG in time. Most malignant MG (category 1) show a clear-cut progression within months to a few years at the most (H-Ig concentration increasing above 20 g/l). The H-Ig component of BMG (category 2) is characterized by the pronounced appearance and the constant serum concentration with time (< 20 g/l). In exception, 'slow' or 'smoldering' myeloma with a seemingly benign course for 10 to 20 years followed by a rather abrupt development into clearcut MM should be mentioned (Kyle and Greipp, 1980; Rödjer, Swolin and Westin, 1983; Greipp and Kyle, 1983). This condition, which concerns a very small minority of all MG poses major problems in differential diagnosis from BMG. In practice however, both 'slow' or 'smoldering' myeloma and BMG do not require any treatment, but should remain under regular control. In the near future, progress in molecular biology concerning the function of different oncogenes may provide new tests for better discrimination between benign and malignant MG.

The H-Ig components of the third and fourth category are usually transient and of low concentration (< 10 g/l). They result from a dysregulation due to a deficiency in the T-immune system (third category) or from a situation where particular antigenic stimulation elicits an excessive monoclonal antibody production (fourth category). The persistence of the H-Ig components in the serum of patients with a MG of category 3 or 4 is variable. It largely depends on the underlying disorder and the duration of the antigenic stimulation. However, with time, the H-Ig component of MG category 3 or 4 usually disappears.

TABLE II

M O N O C L O N A L

Category	Condition Human	Animal model	Monoclonal Ig occurrence
1	B-cell malignancies		
	a) Multiple myeloma Waldenström's macroglobulinemia	dog,cat,cow, mouse (C57BL);	obligatory
	b) Plasmacytoma (solitary, extramedullary)	induced plasma-cytoma:mouse,rat	facultative
	Lymphoma, CLL (LCHD + amyloidosis, HCHD - some forms)	mouse	
2	B-cell benign neoplasias Benign monoclonal gammapathy (Idiopathic paraproteinemia, benign immunocytoma)	mouse (C57BL)	increase with age
3	Immunodeficiency with T < B immune system imbalance		
	A) primary: Wiskott-Aldrich,DiGeorge, Nezelof syndromes, SCID?	nude athymic mouse,rat	frequent
	B) secondary:		
	a: due to aging	aging mouse	increase with age
	b: immunosuppressive treatment (recipients of organ transplant)	irradiated mouse and monkey	frequent, age dependent
	c: malignancies of immune system other than B	mouse	occasionally
	d: acquired, idiopathic		
	C) Reconstitution of the immune system after bone marrow transplant (SCID,aplastic anemia)	mouse,monkey	often beween 2nd and 12th month after transpl.
	D) Early ontogenesis with excessive antigenic stimulation (intra-uterine infection)		infrequent
4	Homogeneous antibody response due to a particular antigenic stimulation		
	a) excess stimulation with poly-saccharides (bacterial), haptens	rabbit,mouse	
	b) autoimmune disorders (some forms, e.g. chronic cold agglutinin disease)	mouse (NZB)	
	c) subacute sclerosing pan-encephalitis, multiple sclerosis	EAE?	frequent in CSF occasionally in serum
	d) papular mucinosis (lichen myxedematosus)		

Abbreviations: SCID = severe combined immunodeficiency; LCHD = light chain disease; HCHD = heavy chain disease; CSF = cerebrospinal fluid; EAE = experimental allergic encephalomyelitis

G A M M A P A T H I E S

component in serum			Bence Jones	Remark
concentr. g/l	duration	Ig isotype	urine/serum	
>20 progressive	persistent	G>A>D>E M	frequent	Bence Jones type low frequency,'nonsecretory' type rare
<20	persistent	M>G	infrequent	(light chains,incomplete heavy chains, respectively)
<20 constant	persistent	G>A=M (D,E?)	rare exceptions	probably as a consequence of age-related immunodeficiency
<10	transient	mainly G M	-	often multiple H-Ig
<10	transient	mainly G M	-	clonal dominance,genetically determined
<20	persistent or transient	mainly M G	-	some may belong to categories 1, 2 or 3
<10	transient	mainly G	-	
<20		mainly IgG	-	activity to dermal mucopolysaccharides postulated

(From Radl, 1985a)

5. Purpose of this study

When after a period of observation it is concluded that an individual suffers from BMG, several matters of practical urgency have to be considered: For instance, what is the relationship between BMG and malignant MG? Does BMG represent a premyeloma stage? Is there any relationship between BMG and other nonmalignant MG? In fact, very few data concerning the etiology, pathogenesis and significance of BMG are available. Because of this lack of data, experimental studies in an animal model have been started. The experiments described in this thesis form part of these studies and are aimed at increasing insight into the pathogenesis of BMG. Special attention was paid to the effects of aging, the T-immune system, antigenic stimulation and genetic influences in the development of BMG.

Before going further into this matter, the major features of BMG in humans will be presented in the next paragraph.

I.2. BENIGN MONOCLONAL GAMMAPATHY IN HUMANS

BMG was first defined as a pathological entity by Waldenström in 1961. Several hundreds of case reports and clinical and laboratory studies on BMG have been published and repeatedly reviewed, some of which are quoted here (Hällén, 1966; Zawadski and Edwards, 1972; Waldenström, 1973; Ritzmann et al., 1975; Kyle and Bayrd, 1976; Axelsson, 1977; Van Camp, 1980; Radl, 1982; Kyle, 1984; Waldenström, 1984; Axelsson, 1986). The condition has been reported under several names (rev. by Ritzmann et al., 1975). Initially, Waldenström used the term benign essential monoclonal gammapathy (1961). Nowadays, the names used most frequently are BMG or idiopathic paraproteinemia. On the basis of the data from the above literature references, the major features of BMG are summarized in the following paragraphs.

1. Characteristics

1.1. Serology

BMG is characterized by a distinct H-Ig component in the serum, which persists at a generally constant concentration over many years, usually until the death of the individual. Mostly, the concentration of the H-Ig remains below 20 g/l. Some well documented cases with a somewhat higher level have been reported (Waldenström, 1984). Most H-Ig in BMG belong to the IgG isotype (60%), while the IgM and IgA classes are equally distributed among the remaining H-Ig (both 20%). BMG with H-Ig of the IgD or IgE class have not yet been convincingly reported in humans. The serum levels of the immunoglobulin classes and subclasses other than that of the paraprotein may show various changes. Never, these changes are so serious as in MM or MW. Bence Jones proteinemia or proteinuria is very rare in BMG (Radl, 1982).

1.2. Histology

Criteria for the diagnosis of BMG include the presence of < 5% plasma cells in the bone marrow (Kyle, 1985). In bone marrow studies using immunofluorescence it has been shown that the responsible plasma cell clone is equally distributed in the bone marrow. An increased monoclonal cell proliferation can be observed. However, these cells are morphologically 'normal'

and are not as dominant as in plasma cell malignancies. Further, a large number of lymphoid cells producing immunoglobulins of other classes and subtypes are usually present (Hijmans et al., 1971; Turesson, 1979). The benign character of the plasma cells in BMG is also reflected in the absence of osteolytic lesions of the skeleton as detected by X-ray examination. Using ^{3}H-thymidine or a monoclonal antibody reactive with 5-bromo-2-deoxy-uridine, the DNA synthesis of the plasma cells could be studied. High percentages of plasma cells actively synthesizing DNA were found in untreated MM and low percentages in smoldering MM and BMG (Kyle, 1985).

1.3. **Delineation**

In a recent report Kyle (1985) delineated BMG or monoclonal gammapathy of undetermined significance (MGUS) from MM and 'smoldering' MM.

Multiple myeloma. Minimal criteria for the diagnosis of MM include the presence of at least 10% abnormal, immature and atypical plasma cells in the bone marrow or histologic proof of an extramedullary plasmacytoma, the usual clinical features of MM (symptoms of anemia, weight loss, frequent infections or bone pains) and at least one of the following abnormalities: H-Ig in the serum (usually > 30 g/l), monoclonal protein in the urine or osteolytic lesions.

Benign monoclonal gammapathy or MGUS. This condition is characterized by a H-Ig component level of < 30 g/l in the serum, < 5% plasma cells in the bone marrow, no anemia or osteolytic bone lesions, normal serum albumin, no or small amounts of the H-Ig component in the urine, and no evidence of progression.

Smoldering multiple myeloma. Here, the patient has a H-Ig component level > 30 g/l in the serum and > 10% atypical plasma cells in the bone marrow. In addition, the patient frequently has a small amount of H-Ig component in the urine and a reduction of uninvolved immunoglobulins in the serum. However, anemia, renal insufficiency and skeleton lesions do not develop and the patient remains stable.

2. Incidence

BMG is seen far more frequently than MM, which has an incidence of 2-3 per 100.000 inhabitants in North and West Europe (Blattner, 1980). The time trend pointed out an increasing MM incidence between 1947 and 1971. The occurrence of BMG is clearly age-related: starting in the fourth decade, the frequency increases up to almost 20% in the tenth decade of life. The ratio of the incidence of malignant versus nonmalignant MG can be estimated as about 1 : 200. Within the non-malignant MG, the BMG may represent about 50% of the cases. The other half concerns MG of the other categories, i.e., MG due to a T < B immune system dysbalance (category 3) and antigen driven MG (category 4) (Radl, 1986). A slight male predominance has been found (Blattner, 1980).

3. Course

The opinions regarding the true nature of BMG diverge considerably. Several authors (Kyle and Bayrd, 1966; Kyle and Greipp, 1980; Lindström and Dahlström, 1978) regard BMG as more or less identical with early MM. For

instance, Kyle (1984) studied 241 hospitalized patients with the original diagnosis of MGUS. After a follow-up period of more than 10 years, 19% of them developed MM, MW or amyloidosis. In addition, 5% showed a 50% increase in their H-Ig-component concentration. Further, Kyle et al. described slow or 'smoldering' myeloma, a very slowly progressing disease that ends up as typical myeloma (Kyle and Greipp, 1980).

Others believe that BMG and MM are separate conditions and that the H-Ig component increases rapidly or slowly, but steadily in MM, whereas it is constant in BMG (Derycke, Fine and Boffa, 1965; Zawadski and Edwards, 1972; Radl, 1982; Waldenström, 1984). Data from Axelsson (1986) support the latter notion. His study involved 64 persons with H-Ig components in the serum, who were found in a health survey of 6995 subjects in 1964. A total observation time of 950 patient years was reported. Malignant disease was detected three times during the initial period. During the 20-year follow-up study of the individuals the M-components remained rather stable and no development into malignancy was seen. Radl (1986) also indicates that starting with BMG, the risk of the development of a malignancy is extremely low.

4. Pathogenetic aspects

A large body of clinical and laboratory data on many aspects of BMG has been collected during the last 25 years. From the abundance of data on BMG, several factors emerged as possibly pathogenetic. Since the occurrence of BMG is clearly age-related, aging was implicated as being important in the pathogenesis of BMG. A genetic predisposition for BMG had to be considered as probable because of suggestions of an increased incidence of BMG within the families of individuals with BMG, MM or MW (Blattner, 1980). Further, the occurrence of H-Ig in the sera of patients with immunodeficient conditions suggested a possible role of immunodeficiency in the pathogenesis of BMG (Zawadski and Edwards, 1972). Radl pointed out that the occurrence of H-Ig was related to immunodeficient conditions characterized by an impaired T-cell function (Radl, 1979a). The finding of H-Ig components reactive with well defined antigens raised questions with regard to the significance of antigenic stimulation in the pathogenesis of BMG (Seligmann and Brouet, 1973).

However, essentially, the etiology, pathogenesis and significance of BMG remained obscure. Together with the questions posed in paragraph 1.5, all of these aspects are of daily clinical relevance for making the correct diagnosis and applying the right therapy. The need for a solution of these problems several years ago led to the search for a suitable animal model for studies of the etiology and pathogenesis of BMG (Radl, 1978).

I.3. MOUSE MODEL OF BENIGN MONOCLONAL GAMMAPATHY

The investigation of sera from mice of different inbred strains resulted in the detection of an age-related increase of H-Ig in the sera of all mouse strains studied (Radl et al., 1978). Different frequencies and times of onset of H-Ig were found in different mouse strains during aging. These data emphasize the role of aging and genetic factors in the development of BMG. C57BL/KaLwRij mice showed the highest frequencies of H-Ig already starting in early adulthood, whereas BALB/c and CBA mice developed

H-Ig in low frequencies and in the last period of their life. Because of the very high frequency of MG in C57BL/KaLwRij mice, this strain was chosen for further studies. MG due to MM and MW-like lymphoma were found in about 0.5% of mice older than two years of age. In about 10% of the cases the MG was transient. The vast majority of the remaining MG closely resembled human BMG (reviewed by Radl, 1981). The major features of mouse BMG as compared to human BMG are depicted in table III. Except for some quantitative differences, these features are essentially the same in both species.

A crucial problem, first investigated in the mouse model, was whether factors intrinsic or extrinsic to the proliferating H-Ig producing cell clone were responsible for the development of mouse BMG. Successful transplantation of different individual H-Ig producing clones from old to young healthy syngeneic recipients by bone marrow or spleen cell transplantation indicated that BMG represents an intrinsic B-cell abnormality expressed as an autonomic monoclonal proliferation (Radl et al., 1979). These transplanted BMG clones maintained their nonprogressive character in the recipients. However, the take frequency of BMG in the recipient mice gradually decreased and propagation of a given BMG clone for more than 4 generations never succeeded. In sharp contrast, transplantation of C57BL B lymphoma cells, MM cells and even 'smoldering' MM cells could be performed continuously, with a take frequency of approximately 100%, with progressive development of the paraproteinemia and with shortened survival times of the recipient mice. In conclusion, BMG can be regarded as an autonomous proliferative disorder, characterized by a limited life-span of the clonal B cells.

Further studies on factors which may contribute to the development of BMG demonstrated the crucial role of a T-immune system defect. Thymectomy performed in young adult C57BL mice (ATx), and even more clearly, thymectomy in neonatal mice (NTx), substantially increased the frequency of transient MG as well as of persistent BMG which appeared during aging (Radl et al., 1980a). A correlation between the frequency of H-Ig and the gradation of the T-immune system defect was found (NTx > ATx > sham-thymectomized mice). Similar results were obtained in CBA mice. However, the numbers of transplantable MG, were very low. Comparing the results in CBA mice with those from C57BL mice, these data stress the importance of genetic factors in the pathogenesis of BMG. In accordance with the thymectomy experiments, high frequencies of H-Ig during aging were also found in athymic BALB/c nude mice, whereas the BALB/c background strain shows only a few H-Ig with a late onset in the sera during aging under normal conditions. Nude mice with a higher antigenic load showed significantly more H-Ig in their sera than barrier maintained nude mice (Radl et al., 1980b; Mink, 1980) This finding suggested a possible role for antigenic stimulation in the pathogenesis of BMG.

On the basis of the available clinical and laboratory indications on the one hand, and the results of the initial experiments performed in the mouse model on the other hand, a 'three-stage' hypothesis on the development of BMG was forwarded by Radl in 1979 (Radl, 1979b).

TABLE III
COMPARISON BETWEEN BMG IN MAN AND C57BL MOUSE

BMG	HUMAN	C57BL/KaLwRij
Persistent H-Ig component in serum	yes	yes
Without progressive increase in concentration	yes	yes
Usually during rest of life	yes	yes
Frequency increases with age	up to 19% in 10th decade	up to 60% at 30 months (male)
Essentially 'benign'	yes	yes
Frequency ratio BMG/malignant MG	± 100/1	probably > 100/1
Serum H-Ig concentration	usually < 20 g/l	usually < 4 g/l
Criteria for monoclonal Ig fulfilled	yes	yes
Number of H-Ig components	usually 1	often > 1
Class distribution of H-Ig components	IgG >> IgA = IgM > IgD	IgG >> IgM > IgD > IgA
Class, subclass, type frequency reflects roughly normal Ig distribution in serum	yes	yes (except for IgA)
Excess production of Bence Jones proteins	exceptional	not yet found
Level of other Ig	normal or slightly decreased	normal or slightly decreased
Bone marrow		
- moderate monoclonal proliferation	yes	yes
- without morphological abnormalities	yes	yes
Bone destruction on X-rays	no	no
Antibody activity of BMG known	exceptionally	exceptionally
Genetic predisposition	probably	most likely

(According to Radl et al., 1978)

I.4. THREE-STAGE HYPOTHESIS ON THE DEVELOPMENT OF BENIGN MONOCLONAL GAMMAPATHY

The hypothesis on the development of BMG as forwarded by Radl (1979b) suggests that BMG develops as a consequence of an age-related immunodeficiency in three successive stages:

Stage 1 : During aging, involution of the thymus is followed by a genetically determined selective decrease in certain T-cell populations leading to an impairment of the regulatory T-cell function. The onset, extent and progress of this dysfunction can be influenced by some extrinsic factors such as environment, chronic antigenic stimulation and virus infection.

Stage 2 : As a consequence of improper helper and control T-cell functions, an impairment of the B-cell activities will occur. The resulting imbalance in the T < B immune system network becomes expressed in restriction of the heterogeneity of the antibody response and in excessive B-cell clonal proliferations with an overshoot production of H-Ig. This second stage is still reversible and the MG are of transient character.

Stage 3 : On a susceptible genetic background, the repeated mono- or oligoclonal expansions result in a higher probability for a mutation of the regulatory genes within a given B-cell clone. If the mutation involves sequences which exert a negative control on cell proliferation, the clone will continue to proliferate and secrete its immunoglobulin product even after the original antigenic stimulation has disappeared. This third stage is irreversible. This intrinsic defect in cell regulation in BMG is different from that in B-cell malignancies.

It is possible that BMG and malignant B-cell neoplasias have some similar steps in their development (e.g. previous clonal expansions or genetic predisposition). However, in its final stage, BMG is clearly different from B-cell neoplasias, which implicates that the defect in intrinsic cell regulation occurs at a different subcellular level in BMG and B-cell malignancies.

REFERENCES

Axelsson U. (1977) An eleven-year follow-up on 64 subjects with M-components. Acta Med. Scand. 201, 173.

Axelsson U. (1986) A twenty-year follow-up study of 64 subjects with M-components. Acta Med. Scand. 219, 519.

Blattner, W.A. (1980) Epidemiology of multiple myeloma and related plasma cell disorders: An analytical review. In Progress in myeloma (ed. by M. Potter) pp. 1-65. Elsevier North Holland Inc., The Netherlands.

Cejka J. and Kithier K. (1976) A simple method for the classification and typing of monoclonal immunoglobulins. Immunochemistry 13, 629.

Derycke C., Fine J.M. and Boffa G.A. (1965) Dysglobulinémies 'essentielles' chez les gens âgés. Nouv. Rev. Franç. Hémat. 5, 729.

Grabar P. and Williams C.A. (1953) Méthode permettant l'étude conjugée des propriétés électrophorétique et immunochimiques d'un mélange de protéines; application au sérum sanguin. Biochim. Biophys. Acta 10, 193.

Greipp P.R. and Kyle R.A. (1983) Clinical, morphological and cell kinetic differences among multiple myeloma, monoclonal gammopathy of undetermined significance, and smoldering multiple myeloma. Blood 62, 166.

Hällén J. (1966) Discrete gammaglobulin (M-)components in serum. Acta Med. Scand. (Suppl.) 462, 1.

Hijmans W., Schuit H.R.E. and Hulsing-Hesselink E. (1971) An immunofluorescence study on intracellular immunoglobulins in human bone marrow cells. Ann. N.Y. Acad. Sci. USA 177, 290.

Kyle R.A. (1984) 'Benign' monoclonal gammopathy. A misnomer? J. Am. Med. Assoc. 251, 1849.

Kyle R.A. (1985) Malignant B-cell monoclonal gammopathies. In MG-clinical

significance and basic mechanisms (ed. by J. Radl, W. Hijmans and B. van Camp) Vol. 5 pp. 15-23. Eurage, Rijswijk, The Netherlands.

Kyle R.A. and Bayrd E.D. (1966) 'Benign' monoclonal gammopathy: a potentially malignant condition? Am. J. Med. 40, 426.

Kyle R.A. and Bayrd E.D. (1976) The monoclonal gammopathies, multiple myeloma and related plasma cell disorders. In Benign monoclonal gammopathy (ed. by I.N. Kugelmass) pp. 284-368. C.C. Thomas, Springfield, Illinois.

Kyle R.A. and Greipp P.R. (1980) Smoldering multiple myeloma. N. Engl. J. Med. 302, 1347.

Lindström F.D. and Dahlström U. (1978) Multiple myeloma or benign monoclonal gammopathy? A study of differential diagnostic criteria in 44 cases. Clin. Immunol. Immunopathol. 10, 168.

Mink J.G. (1980) Serum immunoglobulin levels and immunoglobulin heterogeneity in the mouse. Dissertation, Erasmus University Rotterdam, The Netherlands.

Radl J. (1970) Light chain typing of immunoglobulins in small samples of biological material. Immunology 19, 137.

Radl J. (1979a) The influence of the T immune system on the appearance of homogeneous immunoglobulins in man and experimental animals. In Humoral immunity in neurological diseases (ed. by D. Karcher, A. Lowenthal and A.D. Strosberg) pp. 517-522. Plenum Press, New York.

Radl J. (1979b) Idiopathic paraproteinemia. A consequence of an age-related deficiency in the T immune system. Three-stage development. A hypothesis. Clin. Immunol. Immunopathol. 14, 251.

Radl J. (1981) Animal model of human disease. Benign monoclonal gammopathy (Idiopathic paraproteinemia). Am. J. Pathol. 105, 91.

Radl J. (1982) Effects of aging on immunoglobulins. In Protein abnormalities, Vol. 2. Pathology of immunoglobulins - diagnostic and clinical aspects (ed. by S.E. Ritzmann) pp. 55-69. Alan R. Liss Inc., New York.

Radl J. (1985a) Four major categories of monoclonal gammapathies. Introductory remarks. In MG-clinical significance and basic mechanisms (ed. by J. Radl, W. Hijmans and B. van Camp) Vol. 5 pp. 3-8. Eurage, Rijswijk, The Netherlands.

Radl J. (1985b) Monoclonal gammapathies. An attempt at a new classification. Neth. J. Med. 28, 134.

Radl J. (1986) Benign monoclonal gammapathy (BMG). Curr. Top. Microbiol. Immunol. 132, 221.

Radl J., De Glopper E., Schuit H.R.E. and Zurcher C. (1979) Idiopathic paraproteinemia. II. Transplantation of the paraprotein-producing clone from old to young C57BL/KaLwRij Mice. J. Immunol. 122, 609.

Radl J., De Glopper E., Van den Berg P. and Van Zwieten M.J. (1980a) Idiopathic paraproteinemia. III. Increased frequency of paraproteinemia in thymectomized aging C57BL/KaLwRij and CBA/BrARij mice. J. Immunol. 125, 31.

Radl J., Hollander C.F., Van den Berg P. and De Glopper E. (1978) Idiopathic paraproteinaemia. I. Studies in an animal model - the ageing C57BL/KaLwRij mouse. Clin. exp. Immunol. 33, 395.

Radl J., Mink J.G., Van den Berg P., Van Zwieten M.J. and Benner R. (1980b) Increased frequency of homogeneous immunoglobulins in the sera of nude athymic mice with aging. Clin. Immunol. Immunopathol. 17, 469.

Ritzmann S.E., Loukas D., Sakai H., Daniels J.C. and Levin W.C (1975) Idiopathic (Asymptomatic) monoclonal gammopathies. Arch. Intern. Med. 135, 95.

Rödjer S., Swolin B. and Westin J. (1983) Monoclonal gammapathy - a diagnostic challenge. Acta Med. Scand. 214, 325.
Scheidegger J.J. (1955) Une micro-méthode de l'immunoélectrophorèse. Int. Arch. Allergy 7, 103.
Seligmann M. and Brouet J.-C. (1973) Antibody activity of human myeloma globulins. Semin. Hematol. 10, 163.
Stone M.J. (1982) Monoclonal gammopathies: clinical aspects. In Protein Abnormalities, Vol. 2. Pathology of immunoglobulins - diagnostic and clinical aspects (ed. by S.E. Ritzmann) pp. 161-236. Alan R. Liss Inc., New York.
Tiselius A. and Kabat E.A. (1939) An electrophoretic study of immune sera and purified antibody preparations. J. Exp. Med. 69, 119.
Turesson I. (1979) Studies on multiple myeloma and related disorders with special reference to lymphoid cell markers. Dissertation, University of Lund, Sweden.
Van Camp B. (1980) Clinical and experimental studies on the nature of monoclonal gammopathies. Dissertation, Free University of Brussels, Belgium.
Waldenström J.G. (1961) Studies on conditions associated with disturbed gammaglobulin formation (gammopathies). Harvey Lect. 56, 211.
Waldenström J.G. (1973) Benign monoclonal gammapathy. In Multiple myeloma and related disorders (ed. by H.A. Azar and M. Potter) Vol. 1 pp. 247-286. Harper and Row, New York.
Waldenström J.G. (1984) Benign monoclonal gammapathy. Acta Med. Scand. 216, 435.
Wieme R.J. (1959) Studies on agar gel electrophoresis. Techniques - Applications. Arscia, Brussels.
Williamson A.R. (1973) Isoelectric focusing of immunoglobulins. In Handbook of Experimental Immunology (ed. by D.M. Weir) Vol. 1 pp. 8-18. Blackwell Scientific, Oxford.
Zawadski Z.A. and Edwards G.A. (1972) Nonmyelomatous monoclonal immunoglobulinemia. In Progress in clinical immunology (ed. by R.S. Schwartz) Vol. 1 pp. 105-156. Grune and Stratton Inc., New York.

CHAPTER II

INTRODUCTION TO THE EXPERIMENTAL WORK

Since the formulation of the hypothesis on the development of BMG as described in paragraph I.4 (Radl, 1979), experiments have been devised and performed to test the hypothesis. The investigations described in this thesis form part of these studies.

In stage 1 and 2 of the 'three-stage' model, the emphasis is laid on the impairment of the T-immune system during aging resulting in an imbalance in the T-B immune system network; this would lead to a restriction of the heterogeneity of the immune response and to the production of transient H-Ig. On the basis of the hypothesis, it was predicted that 1) athymic nude mice with a C57BL background should show even higher incidences of H-Ig than their high H-Ig frequency background strain, and 2) T-cell supplementation, i.e. the infusion of immunocompetent T cells into nude mice showing a high frequency of H-Ig (Radl et al., 1980), should lead to the correction of the imbalance in the T-B immune system network and consequently, to the diminished appearance of the transient H-Ig and therefore to a decrease in the total incidence of H-Ig during aging. These aspects were studied in the experiments described in Chapter III.

The imbalance in the T-B immune system network leading to the appearance of transient H-Ig results from an impaired cooperation with and control of B cells by the T lymphocytes (stage 2 of the 'three-stage' hypothesis). Interference with the control function of the T cells can be achieved by the administration of 2'-deoxyguanosine (dGuo). Administration of dGuo inhibits the proliferation dependent induction of suppressor T (Ts) cells but not that of helper T cells (Dosch et al., 1980; Bril et al., 1984). In Chapter IV, a system with rapidly proliferating hemopoietic cells and a resulting T-B immune system imbalance with production of transient H-Ig was created by lethal irradiation and reconstitution of C57BL/KaLwRij mice. On the basis of the 'three-stage' hypothesis, a further increase of the T-B immune system imbalance by the dGuo mediated inhibition of the generation of Ts-cell activity should result in a higher frequency of transient H-Ig in the sera of dGuo treated mice.

In stage 1 of the 'three-stage' hypothesis, it is stated that the selectivity and extent of the age-related decrease in T-cell functions and T-cell subpopulations is largely genetically determined, but may also be influenced by some extrinsic factors, among which chronic antigenic stimulation. In Chapter V, the influence of long lasting antigenic stimulation on the development of BMG is described. At 3, 4, 5, 6, 7, 8, 10 and 12 months of age, C57BL/KaLwRij mice were immunized with dinitrophenylated human serum albumin (DNP-HSA), ovalbumin (OVA) and pneumococcal polysaccharide (PPS). The frequency and antibody reactivity of the H-Ig detected in the sera of these mice were investigated during aging.

Genetic factors are proposed to exert their influence in all three stages of the hypothesis: in the first stage, genetic factors may influence the selectivity and extent of the decrease in T-cell functions and T-cell

subpopulations; in the second stage, genetic factors may affect the cooperation with and control of B cells by the T immune system; in the third stage, genetic factors might influence the occurrence of a mutation of the regulatory genes within a given B-cell clone. Considering the genes which could be candidates for possible pathogenetic factors in the development of MG, the major histocompatibility complex (MHC) (Svejgaard, Platz and Ryder, 1983) and the immunoglobulin heavy chain locus (Igh) (Van Loghem, 1984) appeared among the most obvious. Since the control of the contacts among various cells involved in immune responses is a basic biological function of the MHC, one might suspect an association between the MHC and an imbalanced T-B lymphocyte cooperation during aging to be a contributing factor in the development of BMG. In Chapter VI, mice of six congenic strains of C57BL and BALB origin expressing the H-2^b, H-2^d or H-2^k haplotype were studied for the onset, occurrence, multiplicity, persistence and isotype of the H-Ig detected in their sera.

Successful transplantation of bone marrow or spleen cells from old mice with BMG into young, healthy recipients (Radl et al., 1979) indicated that this monoclonal proliferative disorder is ultimately the result of a defect within a single affected B-cell clone and not due to some specific age related extrinsic factors. These experiments contributed to the formulation of the 'three-stage' hypothesis. Further support for this view and evidence that genetically determined intrinsic cellular factors are responsible for the development of BMG were obtained by the results of transplantation experiments in which B cells from C57BL/Ka mice with a high BMG frequency led to the development of BMG in radiation chimeras of the CBA strain with a low frequency but not vice versa (Radl et al., 1984). In Chapter VII, this observation is extended by the study of MG which develop within the F1 generation of the low BMG frequency strain CBA and the high BMG frequency strain C57BL. Ighl and Igh5 allotypes were used as markers for the parental type origin of H-Ig, which appeared in the sera of the F1 hybrid mice during aging. If the allotype of the H-Ig developing in the F1 mice would be mainly that of the high BMG frequency parental strain, this would be an indication for the involvement of genetic factors associated with the Ig heavy chain loci (Igh) in the development of BMG.

On the basis of the results of the experiments described in Chapter VII, the significance of genetic factors associated with the Igh in the development of BMG was studied. Therefore the Igh congenic mouse strains C57BL/6, C57BL/6.Ig^a, CB-20, BALB.Ig^b, BAB-14 and BALB/c were investigated for the onset, occurrence, multiplicity, persistence and isotype of MG appearing with aging in these strains. The results from these studies are described in Chapter VIII.

Finally, in Chapter IX the data emerging from these Chapters III - VIII are discussed in the context of current thinking about the pathogenesis of BMG.

REFERENCES

Bril H., Van den Akker Th.W., Molendijk-Lok B.D., Bianchi A.T.J. and Benner R. (1984) Influence of 2'-deoxyguanosine upon the development of DTH effector T cells and suppressor T cells in vivo. J. Immunol. 132, 599.

Dosch H.M., Mansour A., Cohen A., Shore A. and Gelfand E.W. (1980) Inhibition of suppressor T cell development following deoxyguanosine administration. Nature 285, 494.

Radl J. (1979) Idiopathic paraproteinemia. A consequence of an age-related deficiency in the T immune system. Three-stage development. A hypothesis. Clin. Immunol. Immunopathol. 14, 251.
Radl J., De Glopper E., Schuit H.R.E. and Zurcher C. (1979) Idiopathic paraproteinemia. II. Transplantation of the paraprotein-producing clone from old to young C57BL/KaLwRij mice. J. Immunol. 122, 609.
Radl J., De Glopper E., Van den Berg P. and Van Zwieten M.J. (1980) Idiopathic paraproteinemia. III. Increased frequency of paraproteinemia in thymectomized aging C57BL/KaLwRij and CBA/BrARij mice. J. Immunol. 125, 31.
Radl J., Heidt P.J., Knaan-Shanzer S. and Van Zwieten M.J. (1984) Idiopathic paraproteinaemia. IV. The role of genetic factors in the development of monoclonal B cell proliferative disorders - a study in the ageing C57BL/KaLwRij and CBA/BrARij mouse radiation chimeras. Clin. exp. Immunol. 57, 213.
Svejgaard A., Platz P. and Ryder L.P. (1983) HLA and disease 1982 - a survey. Immunol. Rev. 70, 193.
Van Loghem E. (1984) The immunoglobulin genes: genetics, biological and clinical significance. Clin. in Immunology and Allergy 4, 607.

CHAPTER III

THE INFLUENCE OF T CELLS ON HOMOGENEOUS IMMUNOGLOBULINS IN SERA OF ATHYMIC NUDE MICE DURING AGING

Th.W. van den Akker, A.P. Tio-Gillen, H.A. Solleveld*, R. Benner and J. Radl*

Department of Cell Biology, Immunology and Genetics, Erasmus University Rotterdam and *TNO Institute for Experimental Gerontology, Rijswijk, The Netherlands

Submitted for publication.

SUMMARY

In this study, results are presented which are in agreement with predictions made on basis of the 'Three Stage Hypothesis' on the development of benign monoclonal gammapathy (BMG). Using a T-cell depletion model, C57BL/Ka nude mice were shown to develop single and multiple homogeneous immunoglobulins (H-Ig) during aging in the highest frequencies known so far. Ninety percent of the C57BL/Ka nude mice displayed one or more H-Ig at 12 months of age. Using a T-cell supplementation model, infusion of corticosteroid resistant T cells into 9-months-old BALB/c nude mice resulted in a decrease in the frequency of H-Ig from 43% at 9 months down to 20% at 15 months of age. In contrast, the frequency of H-Ig in the control group increased from 40% at 9 months up to 68% at 12 months of age.

The results show that normally functioning T cells are essential for the generation of a normal heterogeneous Ig spectrum; they further support the validity of the 'Three Stage Hypothesis' with regard to the role of an impairment of the T-immune system in the pathogenesis of BMG.

INTRODUCTION

Genetic influences and impairment of the T-immune system are thought to be major causative factors in the development of transient and persistent homogeneous immunoglobulins (H-Ig) during aging (rev. in 1 and 2). So far, high frequencies of H-Ig were observed in aging C57BL mice, whereas BALB/c and CBA mice developed H-Ig in low frequencies during aging (3). Secondary T-cell deficient, neonatally (NTx) and adult (ATx) thymectomized C57BL and CBA mice showed substantially higher frequencies of transient and persistent H-Ig during aging than their sham-thymectomized (STx) controls (4). A correlation between the frequency of H-Ig and the grade of the T-immune system defect was found (NTx > ATx > STx) (4). Primary T-cell deficient athymic nude mice with a BALB/c or CBA background developed H-Ig in high frequencies with age (5), substantiating the major role of the T-immune system in the development of H-Ig. However, all results obtained so far about the role of T cells in the development of H-Ig originated from T-cell depletion models.

The above experiments support the so called 'Three Stage Hypothesis' (6) on the development of benign monoclonal gammapathy (BMG) suggesting that T-cell dysfunction plays an important role in the first two stages of this condition: An age-related involution of the thymus followed by a genetically determined selective decrease in certain T-cell subpopulations and an impairment of the regulatory T-cell function (stage 1) results in an imbalance in the T-B immune system network; this leads to restriction of the heterogeneity of the antibody response and to excessive B-cell clonal proliferations of a reversible, transient character (stage 2). On basis of this hypothesis the following results of experiments in athymic nude mice were predicted:

- Athymic nude mice with a C57BL background should show higher incidences of H-Ig than their high H-Ig frequency background strain.
- T-cell supplementation, i.e., the infusion of immunocompetent T cells into nude mice showing a high frequency of H-Ig (5), should lead to the correction of the imbalance in the T-B immune system network and, consequently, to the diminished appearance of the transient H-Ig and, therefore, to a decrease in the total incidence of H-Ig during aging.

This study confirms these predictions. First, C57BL/Ka nude mice were shown to develop H-Ig during aging in the highest frequencies from all mouse strains tested so far. Second, T-cell infused 9-months-old BALB/c nude mice showed a 54% decrease in the frequency of H-Ig, whereas the control group showed a 70% increase during aging.

MATERIALS AND METHODS

Mice

Barrier maintained athymic nude BALB/c, athymic nude C57BL/KaLwRij, C57BL/KaLwRij mice (further C57BL/Ka) and conventional BALB/c female mice were obtained from the REP Institutes TNO in Rijswijk. The BALB/c nude, C57BL/Ka nude and C57BL/Ka control mice remained barrier maintained throughout the experimental period. All barrier maintained mice received autoclaved pelleted food (Hope Farms, Woerden, The Netherlands) and water. Food and water were available ad libitum. Blood samples of the C57BL/Ka and C57BL/Ka nude mice were taken from birth onwards at 3 months intervals. BALB/c nude mice were bled at 9, 10, 12, 15 and 20 months of age. The sera

were stored frozen below -20°C for later examination. Complete necropsies and histologic examinations of 59% of the dead BALB/c nude mice were performed according to a standard protocol (7).

Detection of homogeneous Ig components

Sera were investigated for the presence of H-Ig by agar electrophoresis according to Wieme and by immunofixation performed on Wieme's agar plates (8) using a sheep antiserum against Fab fragments of mouse IgG. Sera with a H-Ig component were further investigated by immunoelectrophoresis using goat or sheep antisera specific for mouse IgM, IgG1, IgG2a, IgG2b, IgG3, IgA and lambda light chains (8). In addition, sera obtained 3 months before the death of each mouse were tested for the presence of IgD in a high concentration by a double radial immunodiffusion technique (9). Positive sera were further investigated by immunofixation using a rabbit antiserum specific for mouse IgD in order to detect H-Ig of the IgD isotype. All polyclonal sheep, goat and rabbit antisera were prepared at the Institute for Experimental Gerontology, TNO, Rijswijk, The Netherlands as described previously (8).

Mice were considered to be positive for H-Ig, when serum analysis by combination of agar electrophoresis, immunoelectrophoresis and immunofixation revealed a H-Ig component in the immunoglobulin spectrum.

Preparation of corticosteroid resistant thymocytes (CRT)

One intraperitoneal (i.p.) injection of dexamethasone (30 mg/kg) was given to 6-weeks-old female BALB/c mice. These mice were killed two days later. Their thymi were removed and used for cell suspensions. Thymocytes prepared in this way are known to reflect the immunocompetent T-lymphocyte fraction (10) and are referred to below as corticosteroid resistant thymocytes (CRT).

Infusion of T lymphocytes

Nine-months-old BALB/c nude mice received three i.p. infusions of 1 x 10^7 CRT in two-day-intervals, whereas control mice received phosphate buffered saline (PBS) at these intervals.

RESULTS

H-Ig in athymic nude C57BL/Ka mice

The sera of 41 female C57BL/Ka nude and 40 control female C57BL/Ka mice were examined for the occurrence of H-Ig at regular intervals throughout their life. The respective 90%, 50% and 10% survival times were 19, 27 and 30 months for the C57BL/Ka nude and 15, 25 and 28 months for the C57BL/Ka control mice. No significant difference in survival was observed between the two groups of mice. The age-related increase of H-Ig in the sera of C57BL/Ka nude and control mice is shown in Fig. 1. The H-Ig frequency in the C57BL/Ka nude mice was found to be significantly higher than in the controls. Often, more than one H-Ig component was detected, particularly in the sera of the nude mice. The number of mice with multiple H-Ig also increased with age (Fig. 2); Up to 5 H-Ig were found in serum samples of some C57BL/Ka nude mice.

Categorization of the monoclonal gammapathies (MG) (11) on the basis of individual follow-up investigations of the mice was performed in 83% and 78% of the nude and control C57BL/Ka mice, respectively (Table 1). Only

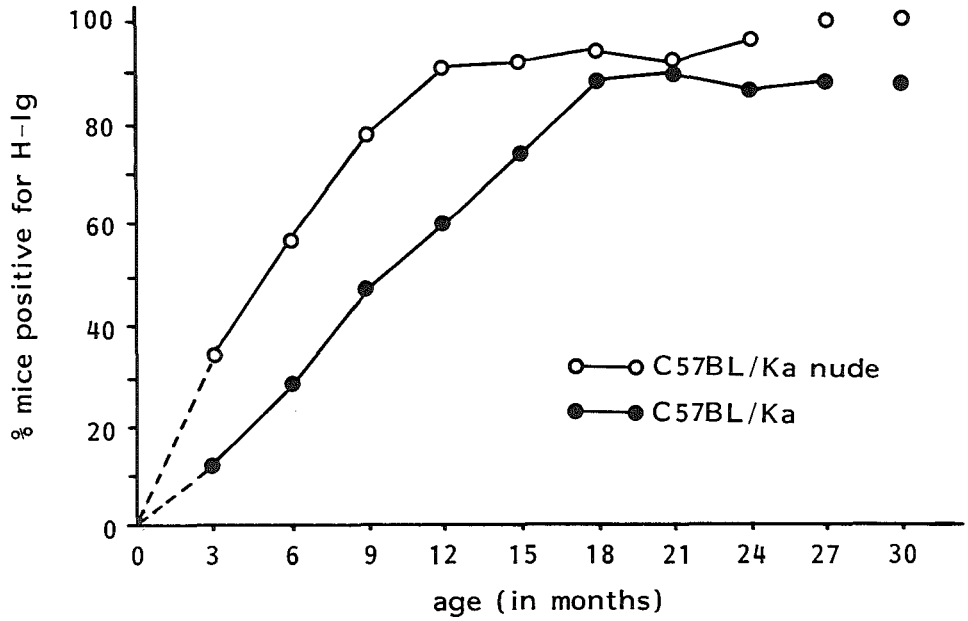

Figure 1. Frequency of H-Ig in the sera of C57BL/Ka athymic nude (o) and control mice (●) during aging.

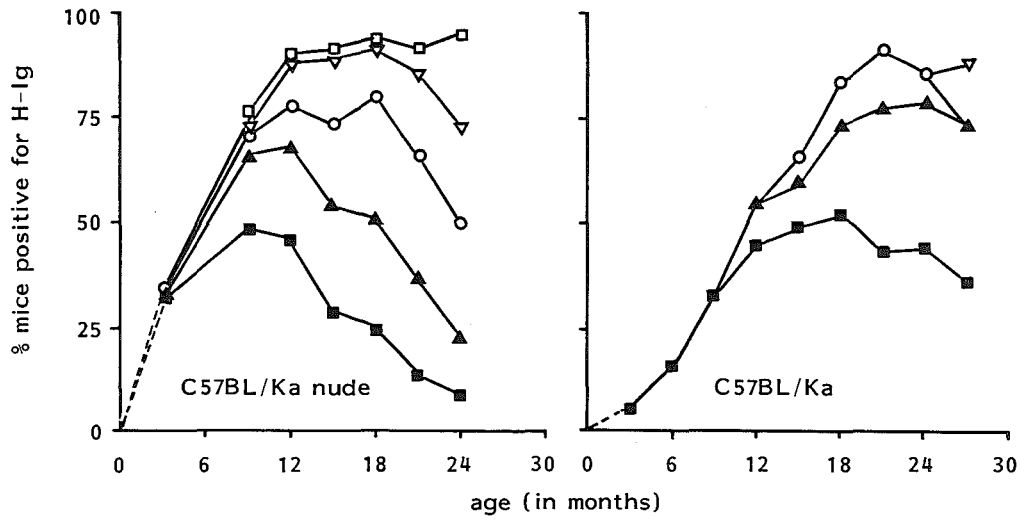

Figure 2. Cumulative representation of single and multiple H-Ig components in the sera of C57BL/Ka nude and control mice during aging. ■ , 1 H-Ig; ▲ , 1-2 H-Ig; o , 1-3 H-Ig; ▽ , 1-4 H-Ig; □ , 1-5 H-Ig.

TABLE 1

DISTRIBUTION (%) OF NON-MALIGNANT PERSISTENT (POSSIBLY BMG) AND TRANSIENT H-Ig COMPONENTS DETECTED IN THE SERA OF AGING NUDE MICE AND CALCULATED ON THE BASIS OF INDIVIDUAL FOLLOW-UP CASE HISTORIES

Strain	Mice (n)	Possibly BMG	Transient	Unclassifiable	Absent
C57BL/Ka nude	41	81	2	12	5
C57BL/Ka	40	71	7	15	7
BALB/c nude	89	31	15	33	21
BALB/c nude, CRT injected	82	10	21	27	42

minor differences between the C57BL/Ka athymic nude and euthymic mice were found. A single transient monoclonal B-cell proliferation was found in 2% and 7% of the C57BL/Ka nude and control mice, respectively. In 81% and 71% of the C57BL/Ka nude and control mice, respectively, the H-Ig persisted for longer than 6 months, usually until the death of the animals and without the development of any sign of plasmacellular malignancy. Therefore, these long-lasting H-Ig were considered to fulfill the criteria for BMG as known in man (6). No malignant monoclonal B-cell proliferations were found in these groups. The persistency of the MG in the remaining 12% and 15% of the C57BL nude and control mice, respectively, could not be determined, because the H-Ig appeared in old animals in which death precluded a sufficiently long follow-up.

The heavy and light chain isotype distribution of the H-Ig detected in the sera of all experimental and control mice is shown in Table 2. The most frequent heavy chain isotypes of the H-Ig were IgM and IgG2a for the C57BL/Ka nude and control mice, respectively. H-Ig of the IgG1 subclass were very uncommon in the C57BL/Ka mice. Since the higher frequency of multiple H-Ig in the C57BL/Ka nude mice as compared to control mice might have influenced the heavy and light chain isotype distribution, recalculations were made using only the first appearing H-Ig in the serum and omitting the additionally appearing H-Ig. Then, in C57BL/Ka nude mice, a change to the IgG3 subclass as the most frequent isotype was observed. In C57BL/Ka control mice, the IgG2a subclass was found to be the most frequent isotype using both the corrected and uncorrected data.

Influence of T-cell supplementation on H-Ig in athymic nude BALB/c mice.

For investigating the influence of T-cell supplementation on H-Ig, athymic nude BALB/c mice were chosen because they and their normal BALB/c background mice offer a good system for measuring the influence of T-cell addition on the H-Ig incidence: BALB/c athymic nude mice develop H-Ig with an early onset, in high frequencies and often of transient appearance during aging, whereas euthymic BALB/c mice show H-Ig with a late onset and in low frequencies (5). The survival times of the 82 CRT injected and 89 control BALB/c nude mice were only slightly different. A significant diffe-

TABLE 2

HEAVY AND LIGHT CHAIN ISOTYPE DISTRIBUTION (%) AMONG H-Ig DETECTED IN THE SERA OF AGING C57BL/Ka NUDE, CRT INJECTED BALB/c NUDE AND CONTROL MICE (CORRECTED DATA FOR FIRST H-Ig BETWEEN BRACKETS)

Strain	No of mice	No of H-Ig	IgM	IgG1	IgG2a	IgG2b	IgG3	IgA	IgD	lambda
C57BL/Ka nude	41	106 (39)	33 (23)	12 (13)	15 (20)	11 (8)	26 (36)	3 (0)	0 (0)	19 (3)
C57BL/Ka	40	75 (37)	14 (5)	1 (6)	31 (51)	17 (11)	23 (24)	11 (3)	3 (0)	23 (12)
BALB/c nude	89	104 (50)	23 (22)	35 (33)	26 (33)	4 (3)	11 (7)	1 (2)	0 (0)	17 (13)
BALB/c nude, CRT injected	82	52 (17)	17 (15)	42 (59)	31 (20)	6 (6)	4 (0)	0 (0)	0 (0)	13 (9)

rence was only found at 11 months after infusion: At 1, 3, 6 and 11 months after infusion, 95%, 91%, 83% and 70% of the CRT infused BALB/c nude mice were alive, versus 93%, 83%, 79%, and 50% of the control BALB/c nude mice, respectively. The frequency of H-Ig of BALB/c nude control mice increased from 40% at 9 months of age to 68% at 12 months of age and remained relatively constant (63-68%) throughout the rest of the observation period, i.e., up to 20 months of age (Fig. 3). In contrast, the experimental CRT infused group showed a gradual decrease in the frequency of H-Ig from 43% at 9 months of age down to 20% at the age of 15 months. Throughout the observation period from 9 to 20 months of age, the frequency of mice with multiple H-Ig was relatively constant within, but somewhat different between the groups under investigation, namely 5 to 10% and 14 to 23% of the mice in the CRT infused and control groups, respectively. However, these frequencies are much lower than those found for C57BL/Ka and C57BL/Ka nude mice (Fig. 2).

Categorization of the MG (11) was performed in 31% of the CRT injected BALB/c nude mice and in 46% of the control BALB/c nude mice. Following CRT infusion into the BALB/c nude mice, a shift in the character of the H-Ig was observed: only 10% of the CRT infused BALB/c nude mice against 31% of the control mice showed persistent H-Ig which was regarded as possible BMG (Table 1). In contrast, 21% and 15% of the CRT infused and control groups, respectively, showed a single transient monoclonal B-cell proliferation, whereas 42% and 21% of the CRT infused and control mice, respectively, did not show any H-Ig. Malignant monoclonal B-cell proliferations were not found in these two groups. The H-Ig of the remaining 27% and 33% of the CRT infused and control BALB/c nude mice, respectively, could not be evaluated, because the restricted length of the observation period (11 months) or

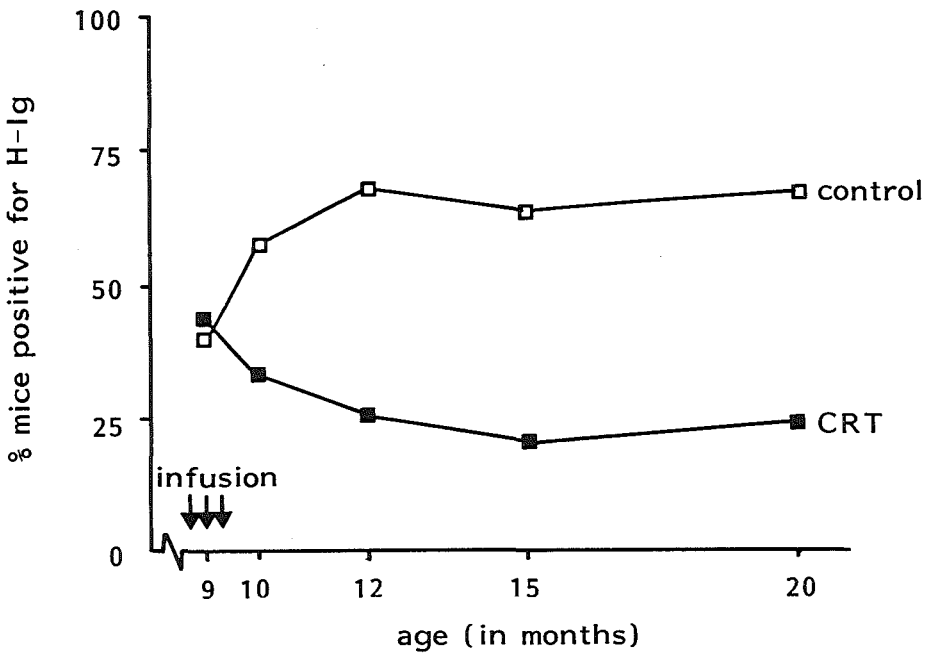

Figure 3. Age-related frequency of H-Ig in the sera of CRT infused (■; n=82) and control (□; n=89) athymic nude mice on BALB/c background.

TABLE 3

INCIDENCE (%) OF HISTOLOGICAL LESIONS OF THE LYMPHORETICULAR SYSTEM IN 58 CRT INFUSED AND 42 CONTROL ATHYMIC BALB/c NUDE MICE

Histological finding	CRT infused group	Control group
Cystic thymus	100	100
T-cell depletion (spleen,lymph nodes)	50	54
Germinal center formation* (spleen,lymph nodes)	55	22
Lymphoid hyperplasia (spleen,lymph nodes)	47	34
Malignant lymphoma*	12	29
Histiocytic sarcoma	2	2

* Significantly different using two-tailed Fischer's exact test.

death of the mice precluded a sufficiently long follow-up.

The heavy and light chain isotype distribution of the H-Ig is shown in Table 2. In both CRT infused and control BALB/c nude mice, the IgG1 subclass was found to be the most frequent isotype. Correction of the data by including only the first appearing H-Ig in the serum after CRT or control infusion and deleting the additionally appearing H-Ig left 17 CRT infused and 50 control BALB/c nude mice for analysis of isotypes. Most (59%) of these CRT infused BALB/c nude mice showed the IgG1 subclass to be the most frequent isotype, whereas both the IgG1 and IgG2a subclasses were the most frequent isotypes (both 33%) among the H-Ig of the control mice.

In order to investigate whether the differences in H-Ig frequencies between the CRT infused and control BALB/c nude mice were related to morphological changes, histopathological examination was performed on 58 CRT infused and 41 control BALB/c nude mice dying spontaneously between 9 and 20 months of age (17% and 21% of the total number, respectively) or killed at 20 months of age (83% and 79%, respectively). Special attention was paid to changes in the lymphoreticular tissues (Table 3). A significant difference in incidence was found for germinal center formation and malignant lymphomas. Germinal centers were seen more frequently and were larger in individual CRT infused BALB/c nude mice than in the concurrent controls, whereas lymphoreticular neoplasms occurred more frequently in the concurrent controls than in the CRT infused animals. Both changes were not correlated with the occurrence or isotype of H-Ig components. Lesions other than those seen in the lymphoreticular organs did not differ appreciably in incidence between CRT infused and control nude mice.

DISCUSSION

The present results are in agreement with the predictions made on basis of the 'Three Stage Hypothesis' on the development of BMG (6). Firstly, using a T-cell depletion model, C57BL/Ka nude mice were found to show the highest H-Ig frequencies of all mouse strains tested so far. Secondly, using a T-cell supplementary model, CRT infusion into 9-months-old BALB/c nude mice resulted in a 54% decrease in the frequency of H-Ig, whereas the control group showed a 70% increase during aging.

The frequencies of H-Ig found in C57BL/Ka nude mice were higher than those found in neonatally and adult thymectomized C57BL/Ka mice (4). However, the results of the two studies are not completely comparable, because in this study an immunofixation technique was applied in addition to agar electrophoresis and immunoelectrophoresis; this combination of techniques was shown to reveal up to 20-30% more H-Ig due to an increased detection sensitivity (11). The H-Ig frequencies of the C57BL/Ka nude mice were also higher than those of the barrier maintained and conventionalized BALB/c or CBA nude mice as reported previously (5). Apparently, genetic background and antigenic load, which are both of importance in the development of BMG according to the hypothesis, have an additive effect on T-cell depletion with regard to the magnitude of the H-Ig frequency. The PBS infused barrier maintained control BALB/c nude mice showed frequencies of H-Ig during the observation period comparable with those reported earlier for barrier maintained BALB/c or CBA nude mice (5). No further increase of H-Ig frequency was detected after 12 months of age. Selective mortality of mice with H-Ig was not responsible for this finding. The spontaneous increase of T cells in athymic nude mice during aging (12,13), although qualitatively and quantitatively profoundly impaired, might have some influence on the control of H-Ig producing B-cell clones. Chen et al. (13) found a minority of the 2% subpopulation of athymic CBA mouse spleen cells with mature T-cell markers displaying normal functional activity. In C57BL/Ka nude mice, the matter is more complex. Here, an increase of the frequency of mice with multiple H-Ig in the serum was found after 12 months of age (Fig. 2).

MG persisting longer than 6 months, usually until the death of the animal without showing clinical or histopathological signs of a B-cell malignancy were designated as possible BMG. Evidence that persistent H-Ig are true BMG should be confirmed by transplantation studies. Persistent H-Ig from C57BL mice were transplantable up to three to four times into young, healthy syngeneic recipients by bone marrow grafting (14) indicating an intrinsic defect but a limited life span of the B-cell clone affected in BMG. No data on transplantation of bone marrow cells from BALB/c and nude mice with spontaneously appearing persistent H-Ig are available. So far, no properly performed transplantation experiments with a sufficiently long follow-up have been performed. In the T-cell supplementary experiment, the CRT infusion contributed to the revelation of the nature and origin of the H-Ig detected in the sera of the 9-months-old BALB/c nude mice. On basis of the 'Three Stage Hypothesis' (6), transient MG (stage 2; see Introduction section) are the result of a principally reversible dysbalance in T-B lymphocyte cooperation and, thus, prone to correction by T-cell regulatory signals. BMG, however, showing an intrinsic B-cell defect (stage 3), is insensitive to T-cell reconstitution. At 9 months of age, 14 days prior to CRT infusion 35 out of 82 (43%) BALB/c nude mice showed H-Ig. Eight CRT infused BALB/c nude mice remained positive for the same H-Ig throughout the observation period of 11 months. Thus, maximally 22% (8 out of 35) of the

BALB/c nude mice with a H-Ig at 9 months of age have a MG insensitivity to CRT infusion, probably a BMG. Of the CRT-infused BALB/c nude mice with H-Ig at 9 months of age, in 43% of the cases (15 out of 35) the H-Ig disappeared and, therefore, were regarded to be transient MG. The MG of the remaining 35% (12 out of 35) of the CRT infused BALB/c nude mice with H-Ig at 9 months of age were unclassifiable because death precluded a sufficiently long follow-up. The categorization of the MG in these 9 months-old BALB/c nude mice showed the same ratio BMG/transient MG as that determined on basis of the entire observation period (Table 1). The distribution of malignant MG, possible BMG and transient MG as found in C57BL/Ka and BALB/c nude mice in our study was readily comparable with that of previous studies (3,4,5). Concordant with one of these investigations (4), the T-cell deficient groups of mice showed higher incidences of possible BMG, somewhat lower incidences of transient MG and fewer normal conditions than the groups with a properly functioning T-immune system. An apparent shift from normal via dysregulated to intrinsically defective B-cell clones can consistently be seen in some T-immune system deficiencies. However, no increase in plasma cell malignancies was found in relation to T-cell deficiency both in the previous (4) and present studies.

The significantly higher incidence of malignant lymphomas (5) and lower incidence of germinal center formation (15) in the control nude mice as compared to the CRT infused nude mice was also observed in previous studies. This finding is in agreement with the notion that lymphoreticular neoplasms occur more frequently in immunodeficiency (16, 17). Since germinal center formation is dependent on both antigenic load and T-cell function (15), it can be readily understood that CRT infused nude mice showed significantly more and larger germinal centers in spleen and lymph nodes than the concurrent controls.

In conclusion, a properly functioning T-immune system was shown to be a prerequisite for normal B-cell function as reflected in a normal heterogeneous Ig spectrum. The results obtained support the validity of the 'Three Stage Hypothesis' with regard to the T-immune system impairment playing a crucial role in the development of BMG. The reversibility of the monoclonal B-cell proliferations in 9-months-old BALB/c nude mice after CRT infusion indicates that most H-Ig in BALB/c nude mice of this age reflect MG of the immunodeficiency category rather than benign or malignant B-cell neoplasms (11).

ACKNOWLEDGMENTS

We gratefully acknowledge Ms. J. de Goey-van Dooren for typing the manuscript and the Dutch Cancer Foundation 'Koningin Wilhelmina Fonds' for its financial support.

REFERENCES

1. Van den Akker, Th.W., Van den Enden-Vieveen, M.H.M., Tio-Gillen, A.P. and Radl, J., In "Monoclonal Gammapathies. Clinical Significance and Basic Mechanisms" (J. Radl, W. Hijmans and B. Van Camp, Eds.) pp. 209-211, Eurage, Rijswijk, The Netherlands, 1985.

2. Benner, R., Van den Akker, Th.W., and Radl, J., In "Monoclonal Gammapathies. Clinical Significance and Basic Mechanisms" (J. Radl, W. Hijmans and B. Van Camp, Eds.) pp. 97-102, Eurage, Rijswijk, The Netherlands, 1985.

3. Radl, J., Hollander, C.F., Van den Berg, P., and De Glopper, E., Clin. exp. Immunol., 33, 395, 1978.

4. Radl, J., De Glopper, E., Van den Berg, P., and Van Zwieten, M.J., J. Immunol. 125, 31, 1980.

5. Radl, J., Mink, J.G., Van den Berg, P., Van Zwieten, M.J., and Benner, R., Clin. Immunol. Immunopathol., 17, 469, 1980.

6. Radl, J., Clin. Immunol. Immunopathol. 14, 251, 1979.

7. Zurcher, C., Van Zwieten, M.J., Solleveld, H.A., and Hollander, C.F., In "The Mouse in Biomedical Research. Vol. 4" (H.L. Foster, J.D. Small and J.G. Fox, Eds.), pp. 11-35, Academic Press, New York, 1982.

8. Radl, J., In "Immunological Techniques Applied to Aging Research" (W.H. Adler and A.A. Nordin), pp. 121-139, CRC Press, Boca Raton, Florida, USA, 1981.

9. Radl, J., Van den Berg, P., and Jol-Van der Zijde, C.M., J. Immunol., 124, 2513, 1980.

10. Raff, M.C., and Cantor, H., In "Progress in Immunology". (B. Amos, Ed.) pp. 83-93, Academic Press, New York and London, 1971.

11. Radl, J., In "Monoclonal Gammapathies. Clinical Significance and Basic Mechanisms." (J. Radl, W. Hijmans and B. Van Camp, Eds.) pp. 3-8, Eurage, Rijswijk, The Netherlands, 1985.

12. MacDonald, H.R., Blanc, C., Lees, R.K., and Sordat, B., J. Immunol. 136, 4337, 1986.

13. Chen, W.-F., Scollay, R., Shortman, K., Skinner, M., and Marbrook, J., Am. J. Anat. 170, 339, 1984.

14. Radl, J., De Glopper, E., Schuit, H.R.E., and Zurcher, C., J. Immunol. 122, 609, 1979.

15. Nieuwenhuis, P., and Opstelten, D., Am. J. Anat. 170, 421, 1984.

16. Berard, C.W., Greene, M.H., Jaffe, E.S., Magrath, I., and Ziegler, J., Ann. Intern. Med. 94, 218, 1980.

17. Sharkey, F.E., In "The Nude Mouse in Experimental and Clinical Research." (J. Fogh and B.C. Giovanella, Eds.) pp. 75-93, Academic Press, New York, San Francisco and London, 1978.

CHAPTER IV

INCREASED INCIDENCE OF TRANSIENT HOMOGENEOUS IMMUNOGLOBULINS IN IRRADIATED AND RECONSTITUTED C57BL/KaLwRij MICE TREATED WITH 2'-DEOXYGUANOSINE

This chapter has been published in Clinical experimental Immunology 54, 411-417 (1983).

Clin. exp. Immunol. (1983) **54**, 411–417.

Increased incidence of transient homogeneous immunoglobulins in irradiated and reconstituted C57BL/KaLwRij mice treated with 2′-deoxyguanosine

TH.W. VAN DEN AKKER, A. P. GILLEN, H. BRIL, R. BENNER & J. RADL*
*Department of Cell Biology and Genetics, Erasmus University, Rotterdam and *Institute for Experimental Gerontology TNO, Rijswijk, The Netherlands*

(Accepted for publication 4 July 1983)

SUMMARY

Prolonged administration of micromolar amounts of 2′-deoxyguanosine (dGuo) to lethally irradiated and reconstituted mice led to an increased incidence of transient homogeneous immunoglobulins (H-Ig) in their sera. Analysis of the heavy and light chain isotype distribution among the H-Ig revealed more of the IgG2b and IgG3 isotypes and less of the IgM isotype and the lambda light chain containing H-Ig in the dGuo-treated group as compared to the control group. The increased incidence of H-Ig was preceded by a decreased suppressor T cell generation and activation in the dGuo treated group. These data indicate that deficient suppressor T cell activity plays an important role in the development of transient H-Ig.

Keywords transient homogeneous immunoglobulins 2′-deoxyguanosine suppressor T cells

INTRODUCTION

A homogeneous immunoglobulin component which appears in the serum under certain conditions (reviewed by Radl, 1982) is the result of excessive immunoglobulin (Ig) production by a single B cell clone. Homogeneous immunoglobulins (H-Ig) of the transient type appear typically in the serum of experimental mice after lethal irradiation and reconstitution (Van Muiswinkel, Radl & Van der Wal, 1976). Explanation for this phenomenon could be sought in an immune system imbalance which develops due to the rapid recovery of the B cell compartment and the relatively slow recovery of the T cell compartment under these conditions (Rozing & Benner, 1976). The delayed recovery of of T cells might lead to poorly controlled clonal expansions of B cells (Van Muiswinkel *et al.*, 1976).

Similar T–B immune system imbalances are believed to be responsible for transient paraproteinaemias in some primary and secondary immune deficiencies in both man and experimental animals (Radl, 1979). Several data obtained from animal experiments and from clinical observations indicate that the appearance of Ig with restricted heterogeneity and of transient H-Ig are very sensitive indicators of an impairment in the T immune system and a consequent imbalance in the T–B immune system network (reviewed by Radl, 1981). Up to now, no information has been available on the involvement of individual T cell subsets in the development of the transient H-Ig in these clinical and experimental situations.

Data have recently been presented (Gelfand, Lee & Dosch, 1979) which indicate that antigen-induced human suppressor T cell activity can be abrogated by micromolar concentrations of 2′-deoxyguanosine (dGuo) *in vitro*. In contrast, non-proliferative helper T cell activity and the

Correspondence: Dr Th. W. van den Akker, Department of Cell Biology & Genetics, Erasmus University, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands.

differentiation and proliferation of precursor B lymphocytes into IgM producing antibody forming cells *in vitro* have a more than 1,000-fold higher resistance to dGuo. The *in vivo* development of murine suppressor T cell activity involved in antibody formation can also be inhibited by dGuo (Dosch *et al.*, 1980).

In this study we investigated whether dGuo treatment would further increase the imbalance between the various lymphocyte subpopulations in mice during recovery after irradiation and reconstitution by inhibiting the generation of suppressor T cell activity. If so, this should result in a higher frequency of H-Ig in the sera of dGuo treated mice. Our results support this assumption and indicate an influence of dGuo on the heavy and light chain isotype distribution among the H-Ig.

MATERIALS AND METHODS

Mice. Female C57BL/KaLwRij mice, 10 to 12 weeks of age and pregnant C57BL/KaLwRij mice were purchased from the Radiobiological Institute TNO, Rijswijk, The Netherlands. Female BALB/c mice, 12–20 weeks of age, were bred at the Laboratory Animals Centre of the Erasmus University, Rotterdam, The Netherlands.

Preparation of cell suspensions. Sixteen or seventeen day pregnant C57BL mice were killed by CO_2. Immediately afterwards, the fetuses were dissected from the uterus and their livers isolated. Cell suspensions of fetal livers were prepared in a balanced salt solution (BSS) and adjusted to a concentration of 2×10^7 cells per ml. Spleen and lymph nodes were prepared for single cell suspensions in BSS as described previously (Wolters & Benner, 1978). Nucleated cells were counted with a model B Coulter counter.

Irradiation and reconstitution. C57BL and BALB/c mice were irradiated with 800 and 750 rad of whole body X-irradiation, respectively. The irradiation was generated in a Philips Müller MG 300 X-ray machine as described in detail previously (Wolters & Benner, 1978). Reconstitution was performed within 4 h after irradiation by intravenous (i.v.) injection of either 1×10^7 C57BL fetal liver cells or 2×10^7 C57BL spleen cells which were suspended in a volume of 0·5 ml BSS.

For induction of suppressor T cells, the BALB/c spleen cells to be injected were irradiated *in vitro* with 2,000 rad in the same way as described above.

Analysis of serum immunoglobulins. Serum samples were investigated for the presence of H-Ig at 0 and 17 days and at 1, 2, 3 and 3·5 months after irradiation and reconstitution by agar electrophoresis, immunoelectrophoresis and immunofixation as described previously (Radl, 1981). The antisera against mouse IgM, IgG1, IgG2a, IgG3 and IgA isotypes used for immunoelectrophoresis were of goat origin. The antisera against mouse IgG2b and lambda light chains were raised in sheep. The antisera used for immunofixation were prepared by immunization of sheep with Fab fragments of heterogeneous mouse IgG. The antiserum used for immunofixation of lambda light chains was the same as used for immunoelectrophoresis. Preparation and specificity testing of these antisera have been reported elsewhere (Radl, 1981).

Mice were considered as positive and therefore included in the calculation of the positive incidence of homogeneous Ig, when serum analysis by combination of agar electrophoresis, immunoelectrophoresis and immunofixation revealed an H-Ig component in the immunoglobulin spectrum.

Suppression. Seventeen days after irradiation and fetal liver reconstitution, suppressor T cells were induced in C57BL mice by i.v. injection of 5×10^7 irradiated (2,000 rad) BALB/c spleen cells. Non-suppressed C57BL mice were injected i.v. with 5×10^7 irradiated (2,000 rad) syngeneic spleen cells (Bril & Benner, 1982). Spleen cells of suppressed and non-suppressed mice were tested for GvH reactivity at 4 days after the i.v. injection of irradiated spleen cells.

Acute graft versus host reactions. Acute graft versus host (GvH) reactions were elicited by i.v. injection of 2×10^7 C57BL spleen cells into lethally irradiated BALB/c mice. Five days later, spleen and lymph node cells from the recipient mice were passively transferred to naive C57BL mice. At that time, the spleen and lymph node cells display maximal anti-BALB/c delayed type hypersensitivity (DTH), as was shown in a previous report (Wolters & Benner, 1978).

DTH assay. DTH reactions were assessed by determining the difference in thickness of the hind

feet of the secondary recipients at 24 and 48 h after subcutaneous (s.c.) injection of 2×10^7 BALB/c spleen cells into the dorsum of the right hind foot. Details of this procedure have been described elsewhere (Wolters & Benner, 1978). A control group consisting of nonimmune mice challenged with the same number of BALB/c spleen cells as the mice to be tested was always included. The relative increase in foot thickness was calculated as:

$$\frac{\text{thickness right foot} - \text{thickness left foot}}{\text{thickness left foot}} \times 100\%.$$

The specific increase in foot thickness was calculated as the relative increase in foot thickness of the immune mice minus the relative increase in foot thickness of the control mice. The relative increase in foot thickness of the control mice ranged between 16 and 23%.

2'-Deoxyguanosine. The dGuo used (No. D-9125, grade II) was obtained from Sigma Chemical Company, St Louis, Missouri, USA. Experimental C57BL mice received a daily intraperitoneal (i.p.) injection of 1 mg dGuo in 0·5 ml BSS. Control mice received a similar injection of BSS only. This was done in three independent experiments during 17 days, 1 month and 3·5 months, respectively.

Statistical analysis. A chi-square test for a 2×2 table was applied to the data concerning the frequency of mice positive for H-Ig. Student's *t*-test was performed on the results dealing with the influence of dGuo on the generation and activation of suppressor T cells.

RESULTS

The incidence of H-Ig in irradiated, reconstituted C57BL mice after prolonged treatment with dGuo

In the first experiment, the H-Ig were tested for only at day 17 after irradiation and reconstitution. In the dGuo treated group 19% (four out of 21) and in the control group 14% (three out of 22) of the mice showed H-Ig in their sera. In the following experiments 59% of the mice in the dGuo group and 34% of the control mice were positive at 1 month after irradiation and reconstitution. Thus, we could conclude that the major proportion of all H-Ig became detectable only after 17 days after irradiation and reconstitution, in both the dGuo treated and the control group (Table 1).

The difference in the frequency of the H-Ig between the dGuo treated and control groups was significant at 1 month after irradiation and reconstitution. One group of mice was further followed-up for a period of 3·5 months in order to determine the behaviour of the H-Ig in the time. During that period, the incidences of H-Ig and the differences between the two groups gradually decreased (data not shown). However, the frequency of H-Ig in this entire 3·5 month period was significantly different: 76% in the dGuo treated compared with 47% in the control group (Table 1).

Table 1. Effect of daily administration of dGuo on the frequency of C57BL mice positive for H-Ig

Irradiation and reconstitution	Interval (in months)*	dGuo‡	Control‡	*P* value§
+	1	59% (34/58)	34% (20/58)	$0{\cdot}01 < P < 0{\cdot}05$
+	1, 2, 3, 3·5†	76% (25/33)	47% (16/34)	$0{\cdot}01 < P < 0{\cdot}05$
−	1, 2, 3, 3·5	15% (3/20)	16% (3/19)	$P > 0{\cdot}80$

* Interval between irradiation and reconstitution and the time of collection of the blood samples.

† All mice with an H-Ig in the serum at one or more of the indicated time points were considered as positive.

‡ Percentage (absolute frequencies of mice positive for H-Ig/total number of mice).

§ For each row a chi-square test for a 2×2 table was performed on the absolute numbers of mice.

The number of H-Ig components per positive mouse was the same: 1·6 in both the dGuo treated and the control group.

No effect of dGuo treatment was observed on the incidence of H-Ig during a 3·5 month period of daily administration of 1 mg dGuo to non-irradiated normal mice (Table 1). In this control experiment the dGuo treated and the BSS treated groups showed only three out of 20 and three out of 19 mice positive for H-Ig, respectively.

Heavy and light chain isotype distribution of H-Ig

The heavy and light chain isotype distribution of the H-Ig in the sera of the irradiated and reconstituted mice treated with dGuo or BSS was analysed in 60 and 36 cases, respectively.

Results given in Table 2 show that less H-Ig of the IgM isotype and more H-Ig of the IgG2b and IgG3 isotypes were found in mice treated with dGuo. No difference between the dGuo treated group and the control group was seen with regard to H-Ig of the IgG1 and IgG2a isotypes. Only one H-Ig of the IgA isotype was determined in the dGuo treated group and none in the control group. In the latter, one H-Ig could not be identified.

The light chain distribution of the H-Ig, as determined by immunoelectrophoresis and immunofixation showed a decreased incidence of lambda positive H-Ig in the dGuo treated as compared to the control group (Table 2).

Table 2. Influence of dGuo on the heavy and light chain isotype distribution of the H-Ig in the sera of irradiated and reconstituted mice

Number of mice	Treatment	Number of H-Ig analysed	IgM	IgG1	IgG2a	IgG2b	IgG3	IgA	Not identified	Lambda
58	dGuo	60	8%(5)*	30%(18)	30%(18)	12%(7)	18%(11)	2%(1)	0%	20%(12)
58	BSS	36	19%(7)	33%(12)	28%(10)	6%(2)	11%(4)	0%	3%(1)	39%(14)

* Absolute numbers of H-Ig of that particular isotype in parentheses.

Influence of dGuo on the generation and activation of suppressor T cells

The increased incidence of H-Ig in dGuo treated irradiated and reconstituted mice might have been due to a decreased capacity to generate suppressor T cell activity. Therefore we studied the effect of dGuo treatment on the suppressor T cell compartment.

Fig. 1 (upper part) shows that daily administration of 1 mg dGuo to the C57BL donor mice during the first 17 days after irradiation and reconstitution with fetal liver cells reduced the T cell-mediated suppression of the GvH related DTH. Presumably, the generation of suppressor T cell precursors in the reconstituted C57BL mice was partly affected by dGuo. As dGuo treated non-suppressed mice displayed a similar GvH related DTH reactivity as BSS treated non-suppressed mice, the generation of precursors of DTH related effector T cells is not affected by dGuo. When the C57BL donor mice were treated with BSS during the first 17 days after irradiation and reconstitution with fetal liver cells and with 1 mg dGuo per day during the interval of 4 days after the suppressive injection, normal anti-host DTH reactivity was found. Apparently, dGuo treatment during the antigen-induced generation of suppressor T cell activity can completely prevent the T cell-mediated suppression (Fig. 1, lower part).

These experiments show that daily administration of 1 mg dGuo partly inhibits the generation of suppressor T cell precursors in donor mice after irradiation and reconstitution with fetal liver cells and completely inhibits the activation of suppressor T cells after appropriate antigenic stimulation. DTH related T cells and their precursors, on the other hand, are not affected by dGuo treatment.

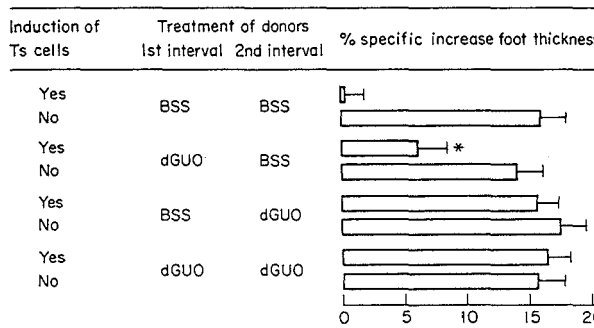

Fig. 1. Influence of dGuo on the generation and activation of suppressor T cells. C57BL mice were used as donors (graft) for reconstitution of lethally irradiated BALB/c mice (host) to elicit a GvH reaction. Twenty-one days before the use of the C57BL mice in the GvH reaction, these donors were irradiated and reconstituted with fetal liver cells. During the first 17 days after irradiation and reconstitution ('first interval') the C57BL donors were treated with 1 mg dGuo or BSS. After these 17 days the C57BL donors were either 'suppressed', i.e. i.v. injected with 5×10^7 irradiated BALB/c spleen cells, or 'non-suppressed', i.e. similarly injected with the same number of irradiated syngeneic C57BL spleen cells. During the following 4 days ('second interval': 18–22 days after irradiation and reconstitution), all mice were treated with either 1 mg dGuo in BSS or BSS only. After this period the ability to mediate a GvH reaction was tested and therefore 2×10^7 spleen cells from the C57BL donors were used as a graft to reconstitute lethally irradiated BALB/c hosts. Five days later, the spleen and lymph node cells of the BALB/c hosts were transferred i.v. into naive C57BL mice in order to measure the GvH related DTH reactivity. Therefore, these secondary C57BL recipients were challenged with 2×10^7 naive BALB/c spleen cells into the dorsum of the right hind foot. DTH responses were measured 24 h later. Values represent the arithmetic mean ± 1 s.e.m. ($n = 12$). The asterix indicates that that particular response was significantly different from the responses by all other groups of mice ($P < 0{\cdot}001$).

DISCUSSION

Immunodeficient patients successfully treated by bone marrow transplantation frequently develop transient H-Ig in their sera during the reconstitution period (reviewed by 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 a few available B cell clones. These might respond to exogenous or endogenous antigenic stimulation in a similar way as in limiting dilution experiments (Kreth & Williamson, 1973) or under conditions leading to the so called clonal dominance. Previous observations (Radl, 1979; Van Muiswinkel *et al.*, 1976; Mink *et al.*, 1979) indicate, however, that it is mainly a defect of the T immune system and not of the B immune system which leads to restricted heterogeneity of Igs and the appearance of H-Ig. During reconstitution, this kind of deficiency is most likely due to an unbalanced recovery, where the T system matures more slowly than the B system. It has been shown that B cells in mice reach their normal values at 4–6 weeks after irradiation and reconstitution (Nossal & Pike, 1973; Rozing and Benner, 1976), whereas the T cell system is still below its normal level at 30 weeks after reconstitution (Rozing & Benner, 1976). In accord with this view, additional T cell infusions into the irradiated and reconstituted mice markedly reduced the incidence of Igs of restricted heterogeneity and of H-Ig during the recovery period (Van Muiswinkel *et al.*, 1976). Little is known about the mechanisms involved and the influence of individual T cell subsets on the development of H-Ig during the reconstitution process. This study indicates that deficient suppressor T cell activity plays an important role in this phenomenon. When analysing our experimental data, the influence of the treatment with dGuo on the frequency of H-Ig seems to be obvious. The difference was most clear at 1 month after irradiation and reconstitution. Later, the difference between the dGuo treated and the control group gradually decreased.

The incidence of H-Ig in the BSS treated control group may deserve some more attention. It was found to be 34% at 1 month after irradiation and reconstitution, which is substantially higher than the 19% reported by Mink *et al.* (1979) and the 9% and 16% reported by Van Muiswinkel *et al.* (1976). These differences can most likely be explained by the different mouse strains used and the procedure of analysis of the serum samples. Mink *et al.* (1976) and Van Muiswinkel *et al.* (1976) used (DBA/2 × C57BL/Rij) F_1 mice, while we used C57BL/Ka mice which, in contrast to the F_1 mice, are known to have a higher incidence of spontaneously developing H-Ig from their young adult age onwards (Radl *et al.*, 1978). Furthermore, in contrast to the previous studies, we applied an immunofixation technique in addition to agar electrophoresis and immunoelectrophoresis. With this combination, about 20–30% more H-Ig can be revealed than with agar electrophoresis according to Wieme and immunoelectrophoresis alone.

As far as the isotype distribution of H-Ig is concerned, most of those detected in our experiments were found to be of the IgG1 or IgG2a subclasses. The dGuo treated group showed more H-Ig of the IgG2b and IgG3 subclasses and less H-Ig of the IgM class as compared to the BSS treated control group. Just as did Mink *et al.* (1979), we found an almost equal incidence of H-Ig of the IgG1 and IgG2 subclasses. Together, the H-Ig of these isotypes constituted about 70% of all those observed. In the experiments of Mink *et al.* (1979) this value was about 90%.

The analysis of the light chain distribution within the H-Ig showed 20% of the H-Ig to be of the lambda type in the dGuo treated mice. An unexpectedly high value of 39% of lambda H-Ig was found in the BSS treated mice. The frequency of H-Ig of the lambda type in normal untreated mice of the same strain varies from 2 to 10% (Radl *et al.*, 1978, 1980). We have no explanation for this relative preponderance of lambda H-Ig in this experiment. However, on the basis of findings of Haughton, Lanier & Babcock (1978), who reported a κ/λ light chain ratio among B cells to be about 1 during early ontogeny with a later shift to about 9, one may speculate that, during reconstitution, this high frequency of lambda H-Ig reflects an immature stage in B cell development. This possibility deserves further investigation, since it seems that the reconstitution process repeats steps of normal ontogeny in several aspects. On the other hand, this possibility must be very carefully considered because other investigators (Kessler, Jin Kim & Scher, 1981; McGuire & Vitetta, 1981) could not confirm the findings of Haughton *et al.* (1978).

The biochemical mechanism of the dGuo-mediated abrogation of suppressor T cell development has not yet been elucidated. It may quite possibly be related to the capacity of suppressor T cells to rapidly accumulate and maintain high levels of intracellular dGuo triphosphate (dGTP). High levels of dGTP can inhibit the enzyme ribonucleotide reductase and therefore DNA synthesis. As a result, dGuo might inhibit the proliferation of suppressor T cells via dGTP. Such a mechanism is consistent with findings of Gelfand *et al.* (1979).

Recently, Lelchuk, Cooke & Playfair (1982) reported the differential sensitivity to dGuo of antigen specific and non-specific suppressor T cells in delayed type hypersensitivity. Antigen non-specific suppressor T cells were found to be sensitive to dGuo, while antigen specific suppressor T cells appeared to be resistant. These authors used the same dose of dGuo as employed by us. Our data, on the other hand, indicate that dGuo can completely inhibit the generation of activated suppressor T cells. Furthermore, our data suggest that dGuo partly inhibits the generation of T cell precursors in lethally irradiated mice reconstituted with fetal liver cells. Most probably the discrepancy observed is due to a difference in requirement of proliferation. In our system, proliferation is required to induce specific suppressor T cell activity. In the experiments of Lelchuk *et al.* (1982), the proliferation requirements of the specific and non-specific suppression observed are unclear.

Hayward & Merrill (1981) have reported that with human cells dGuo interferes with both $OKT4^+$ and $OKT8^+$ lymphocytes *in vitro*. However, it must be stressed that they used a concentration of 50 μM in their culture medium. We administered 1 mg dGuo per mouse per day, as did Dosch *et al.* (1980). During 2–10 h after injection, we determined 0·2–0·3 μM dGuo in the plasma with high performance liquid chromatography (HPLC), with a peak value of 2 μM at 30 min after injection of the dGuo. Our *in vivo* experiments indicate that this concentration of dGuo has a differential influence on DTH reactive T cells and suppressor T cells. Since DTH reactivity and helper activity can be mediated by the same T cells (Bianchi *et al.*, 1981), we hypothesize that helper

and suppressor T cells also have a differential sensitivity for the inhibiting effects of dGuo. Studies on cloned or otherwise purified helper and suppressor T cells are required to test this hypothesis.

We gratefully acknowledge the technical assistance of Mr A. van Oudenaren and Mr G. Ziere. We are grateful to Professor O. Vos for his continuous support and to Mrs Cary Meijerink-Clerkx for typing the manuscript.

This investigation was financially supported by the Netherlands Cancer Foundation 'Koningin Wilhelmina Fonds'.

REFERENCES

BIANCHI, A.T.J., HOOIJKAAS, H., BENNER, R., TEES, R., NORDIN, A.A. & SCHREIER, M.H. (1981) Clones of helper T cells mediate antigen-specific, H-2 restricted DTH. *Nature*, **290**, 62.

BRIL, H. & BENNER, R. (1982) Specific suppression of anti-host immune reactivity in Graft-versus-Host reaction. *Adv. exp. Med. Biol.* **149**, 577.

DOSCH, H.M., MANSOUR, A., COHEN, A., SHORE, A. & GELFAND, E.W. (1980) Inhibition of suppressor T cell development following deoxyguanosine administration. *Nature*, **285**, 494.

GELFAND, E.W., LEE, J.J. & DOSCH, H.M. (1979) Selective toxicity of purine deoxynucleosides for human lymphocyte growth and function. *Proc. Natl. Acad. Sci. USA*. **76**, 1998.

HAUGHTON, G., LANIER, L.L. & BABCOCK, G.F. (1978) The murine kappa light chain shift. *Nature*, **275**, 154.

HAYWARD, A.R. & MERRILL, D. (1981) Requirement for $OKT8^+$ suppressor cell proliferation for suppression by human newborn T cells. *Clin. exp. Immunol.* **45**, 468.

KESSLER, S., JIN KIM, K. & SCHER, I. (1981) Surface membrane κ and λ light chain expression on spleen cells of neonatal and maturing normal and immune-defective CBA/N mice: the $\kappa:\lambda$ ratio is constant. *J. Immunol.* **127**, 1674.

KRETH, H.W. & WILLIAMSON, A.R. (1973) The extent of diversity of anti-hapten antibodies in inbred mice: anti-NIP (4-hydroxy-5-iodo-3-nitro-phenacetyl) antibodies in CBA/H mice. *Eur. J. Immunol.* **3**, 141.

LELCHUK, R., COOKE, A. & PLAYFAIR, J.H.L. (1982) Differential sensitivity to 2'-deoxyguanosine of antigen-specific and non-specific suppressor T cells in delayed hypersensitivity. *Cell. Immunol.* **72**, 202.

MCGUIRE, K.L. & VITETTA, E.S. (1981) κ/λ shifts do not occur during maturation of murine B cells. *J. Immunol.* **127**, 1670.

MINK, J., RADL, J., VAN DEN BERG, P., VAN MUISWINKEL, W.B. & VAN OOSTEROM, R. (1979) Homogeneous immunoglobulins in the serum of irradiated and bone marrow reconstituted mice: the role of thymus and spleen. *Immunology*, **37**, 889.

NOSSAL, G.J.V. & PIKE, B.L. (1973) Differentiation of B lymphocytes from stem cell precursors. Microenvironmental aspects of immunity. *Adv. exp. Med. Biol.* **29**, 11.

RADL, J. (1979) The influence of the T immune system on the appearance of homogeneous immunoglobulins in man and experimental animals. In *Humoral Immunity in Neurological Diseases* (ed. by D. Karcher, A. Lowenthal & A. D. Strosberg) p. 517. Plenum Press, New York.

RADL, J. (1981) Immunoglobulin levels and abnormalities in aging humans and mice. In *Immunological Techniques Applied to Aging Research* (ed. by W. H. Adler and A. A. Nordin) p. 121. CRC Press, Boca Raton, Florida.

RADL, J. (1982) Effects of aging on immunoglobulins. In *Protein Abnormalities. Vol. 2. Pathology of Immunoglobulins—Diagnostic and Clinical Aspects* (ed. by S.E. Ritzmann) p. 55. Alan R. Liss, Inc., New York.

RADL, J., DE GLOPPER, E., VAN DEN BERG, P. & VAN ZWIETEN, M.J. (1980) Idiopathic paraproteinemia. III. Increased frequency of paraproteinemia in thymectomized aging C57BL/KaLwRij and CBA/BrARij mice. *J. Immunol.* **125**, 31.

RADL, J., HOLLANDER, C.F., VAN DEN BERG, P. & DE GLOPPER, E. (1978) Idiopathic paraproteinaemia. I. Studies in an animal model—the aging C57BL/KaLwRij mouse. *Clin. exp. Immunol.* **33**, 395.

ROZING, J. & BENNER, R. (1976) The recovery of the B cell compartment in lethally irradiated and reconstituted mice. *Adv. exp. Med. Biol.* **66**, 203.

VAN MUISWINKEL, W.B., RADL, J. & VAN DER WAL, D.J. (1976) The regulatory influence of the thymus-dependent immune system on the heterogeneity of immunoglobulins in irradiated and reconstituted mice. *Adv. exp. Med. Biol.* **66**, 617.

WOLTERS, E.A.J. & BENNER, R. (1978) Immunobiology of the graft-versus-host reaction. I. Symptoms of graft-versus-host disease in mice are preceded by delayed-type hypersensitivity to host histocompatibility antigens. *Transplantation*, **26**, 40.

CHAPTER V

INFLUENCE OF LONG TERM ANTIGENIC STIMULATION STARTED IN YOUNG C57BL MICE ON THE DEVELOPMENT OF AGE-RELATED MONOCLONAL GAMMAPATHIES

Th.W. van den Akker, R. Brondijk* and J. Radl*

Department of Cell Biology, Immunology and Genetics, Erasmus University Rotterdam and *TNO Institute for Experimental Gerontology, Rijswijk, The Netherlands

Submitted for publication.

SUMMARY

Long term antigenic stimulation by multiple antigens (DNP conjugated to human serum albumin (DNP-HSA), ovalbumin and pneumococcal polysaccharide) without adjuvant in young C57BL mice resulted in the development of homogeneous immunoglobulins (H-Ig) during aging in frequencies higher than those in the control group. Our data showed that antigen specific B-cell clones were at least in part responsible for this increased incidence of age-related monoclonal gammapathies (MG). Using in situ adsorption performed on Wieme's agar plates and in immunoelectrophoresis, antibody activity to one of the immunizing agents (DNP-HSA) could be demonstrated within 10% of the in old mice appearing H-Ig components but in none of the H-Ig components of the aging control mice. The antigen specific MG belonged most likely to the category of benign monoclonal gammapathy. These findings indicate that long lasting antigenic stimulation contributes to the development of age-related B-cell proliferative disorders, namely of the benign monoclonal gammapathy.

INTRODUCTION

The role of antigenic stimulation in the development of B-cell proliferative disorders is suspected but not yet proven. That the past immunological history of an individual may be associated with the emergence of a malignant lymphoplasmacytic disorder has been suggested in previous reports, already reviewed by Seligmann and Brouet in 1973. For instance, multiple antigenic stimulation by bacterial antigens or horse serum was suspected to play a role in the development of malignant clones secreting monoclonal immunoglobulins with the corresponding antibody specificities. If the influence of antigenic stimulation on the development of B-cell proliferative disorders can be shown, it may substantially contribute to our understanding of the pathogenesis of these disorders.

In the present study, the influence of chronic, long term antigenic stimulation on the development of homogeneous immunoglobulins (H-Ig) was investigated in the C57BL mouse model of benign monoclonal gammapathy (BMG) (Radl et al., 1978). Intrinsic genetic factors, not linked to the H-2 major histocompatibility complex of the mouse (Radl et al., 1979; Van den Akker et al., 1987), and an age-related T-immune system deficiency are major causative factors in the development of BMG during aging (rev. in Radl, 1986 and Benner, Van den Akker and Radl, 1985). In addition, a possible contributing role of antigenic stimulation in the development of BMG was suggested in the so called 'three-stage hypothesis' (Radl, 1979) and indicated by the observation of a higher frequency of H-Ig in conventionalized versus barrier maintained athymic nude mice (Radl et al., 1980). Here, we report an increased incidence of H-Ig with aging in the repeatedly stimulated mice as compared to the control ones. Antibody activity to the immunizing agents could be demonstrated within 10% of the H-Ig of the aged experimental mice but not in H-Ig of the control mice.

MATERIALS AND METHODS

Mice

Female C57BL/KaLwRij mice were bred and maintained under conventional conditions in the mouse colonies of the REP Institutes TNO in Rijswijk. All mice received pelleted food (Hope Farms, Woerden, The Netherlands) and acidified water (pH 3-4) ad libitum. Small blood samples were taken at 3 monthly intervals and the serum was investigated within 24 hours or stored frozen at -20°C for later use.

Antigens

Human serum albumin (HSA), obtained from Nordic Immunological Laboratories, Tilburg, The Netherlands, was dinitrophenylated to a level of 23 DNP molecules per protein molecule according to the method of Eisen (1964). Ovalbumin (OVA) was purchased from Sigma Chemical Company, St. Louis, Missouri, USA. Pneumococcal polysaccharide (PPS) was bought from Wellcome Research Laboratories, Beckenham, England.

Immunization procedure

Eight intraperitoneal (i.p.) injections each containing 50 ug DNP-HSA, 50 ug OVA and 50 ug PPS in 0.1 ml phosphate buffered saline (PBS) or 0.1 ml PBS with 20 ug lipopolysaccharide (LPS from E.coli 055:B5 (Difco Laboratories, Detroit, Michigan, USA) and prepared according to the phenol-extrac-

tion method) were administered to C57BL mice at 3, 4, 5, 6, 7, 8, 10 and 12 months of age, respectively. Control mice received only 0.1 ml PBS i.p.

Detection of homogeneous Ig components

Sera were investigated for the presence of H-Ig by agar electrophoresis according to Wieme (Wieme, 1959). This technique has a detection sensitivity limit for H-Ig on heterogeneous background of approximately 100 µg/ml (Radl, 1985a).

Detection of specific antibody activity

Sera were tested for specific antibody activity to the antigens used for immunization by the double radial diffusion technique in Ouchterlony polyethylene glycol 6000 (PEG) plates (Radl, 1981). The results of the tests in Ouchterlony PEG plates were expressed semiquantitatively in + marks. The scale of + marks ranged from + (positive) to ++++ (very strongly positive) and was compared to the precipitin system using our own rabbit-anti-OVA or rabbit-anti-DNP-HSA antiserum. Specific antibody activity of the H-Ig which appeared in old mice, was tested by *in situ* adsorption performed on Wieme's agar plates. Three microliters of, respectively, 18 mg/ml DNP-HSA, 5 mg/ml DNP-OVA, 18 mg/ml HSA, 18 mg/ml OVA, 10 and 18 mg/ml PPS and PBS were applied to the slits made in the agar. After 30 minutes, serum samples were put into the same slits and an electrophoretic run was performed as usual (Radl, 1981). Disappearance of the H-Ig component, that was originally found in the serum without performing *in situ* adsorption, was considered as antibody activity of that H-Ig component to the given antigen. Sera with a H-Ig component showing antibody activity were further investigated in a similar way by immunoelectrophoresis using goat or sheep antisera specific for mouse IgM, IgA, IgG1, IgG2a, IgG2b, IgG3 and lambda light chains (Radl, 1981). A change of the precipitin line with a given isotype specific serum obtained after adsorption *in situ* revealed the isotype of the H-Ig.

RESULTS

Occurrence of H-Ig

The sera of 28 female C57BL mice immunized with DNP-HSA, OVA and PPS in PBS (Ag + PBS group), those of 28 female C57BL mice immunized with the same antigens in PBS with LPS (Ag + LPS group) and those of 24 control C57BL mice were examined for the occurrence of H-Ig at three monthly intervals. The life-spans of the mice from the experimental and control groups did not differ substantially. The 50% survival times of the Ag + PBS group, the Ag + LPS group and control group were 102, 114 and 108 weeks, respectively. The age-related increase of H-Ig in the sera of the experimental and control groups is shown in Fig. 1. From 9 months of age onwards, the H-Ig frequency in the experimental groups was higher than that in the control group. The H-Ig frequency of the Ag + PBS group was found to be slightly higher than that of the Ag + LPS group.

Antibody activity of H-Ig

Antibody activity to OVA was first tested semiquantitatively by the double diffusion technique in Ouchterlony PEG plates. Anti-OVA activity was maximal at the age of 9 to 12 months in both experimental groups, the titers being higher in the Ag + LPS group (+ to +++) than in the Ag + PBS

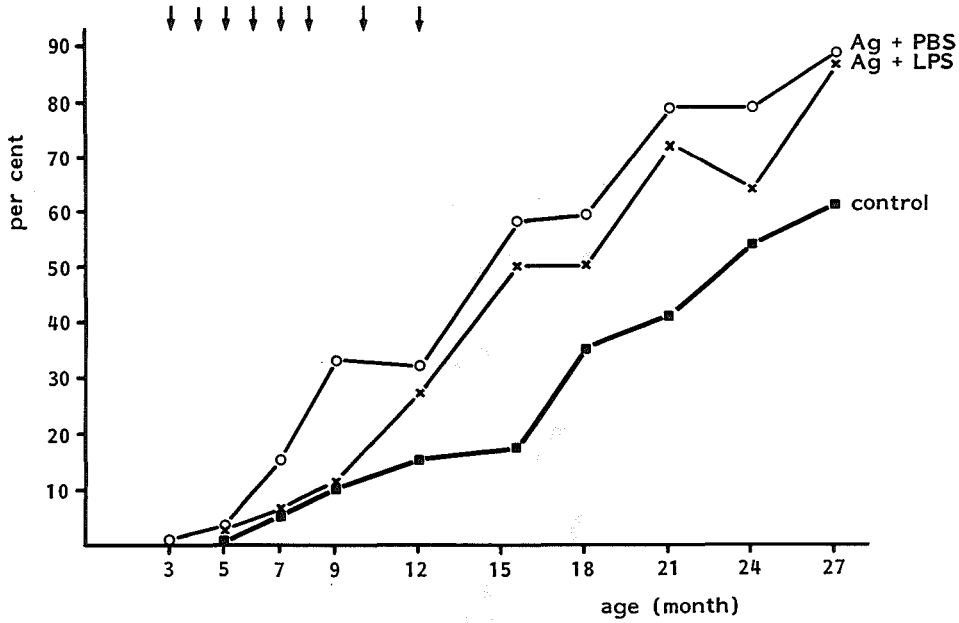

Figure 1. Frequency of homogeneous immunoglobulins (H-Ig) in the sera of aging C57BL mice after long lasting immunization.

group (- to ++). After 12 months of age, the anti-OVA titers gradually decreased to - at 27 months of age. Using in situ adsorption on Wieme's agar plates, no H-Ig showing anti-OVA activity could be demonstrated.

Antibody activity to DNP-HSA was also first tested semiquantitatively using Ouchterlony's double radial diffusion technique. Maximal anti-DNP activity was found at 12 months of age in the Ag + PBS group and at 12 to 15 months in the Ag + LPS group. The maximal anti-DNP titers (+) in both experimental groups were lower than the maximal anti-OVA titers. After the peak anti-DNP activity, the titers gradually declined to zero. However, sera of two mice became again positive and showed a clear-cut increase (+ to ++) of anti-DNP activity at 24 and 27 months of age, respectively.

Both sera were tested using in situ adsorption on Wieme's agar plates. One of these sera, belonging to a mouse of the Ag + LPS group was shown to contain a H-Ig component reactive with DNP-HSA, DNP-OVA, but not with HSA or OVA. Apparently, this emerging H-IgG1 component that could be detected from 24 to 34 months of age, was directed against the DNP hapten. The second serum was from a mouse of the Ag + PBS group (Fig. 2 and 3). At the age of 21 months of age a newly appearing H-Ig component of the IgG1 subclass was detected, that persisted until the death of the mouse at 27 months of age. This H-Ig component was reactive with DNP-HSA, but not with DNP-OVA, HSA or OVA. Here, the H-Ig component appeared to be directed against a new determinant constituted by the DNP to HSA transition area. Performing in situ adsorption of the sera of 25 experimental mice older than 24 months of age and 22 available control mice older than 18 months of

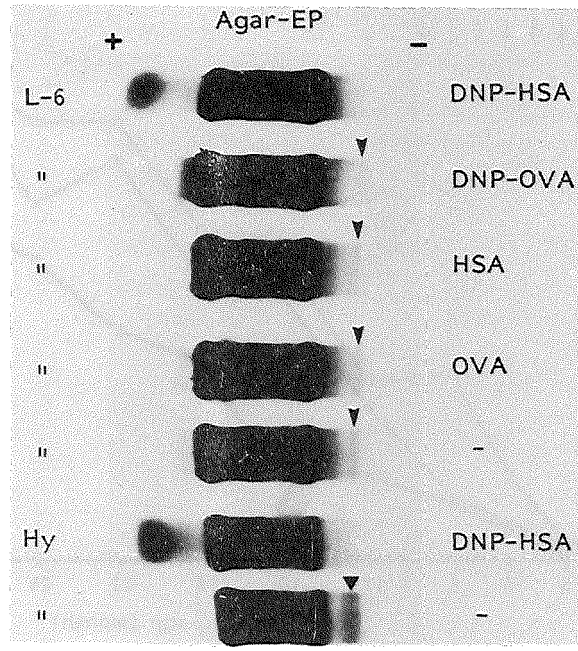

Figure 2. Example of an in situ adsorption test performed on Wieme's agar plates in serum sample of mouse L-6. The H-Ig component indicated by a small arrow disappeared after 30 minutes preincubation with DNP-HSA but not with DNP-OVA, HSA or OVA. Hy = DNP specific IgE secreting hybridoma (triangle), which served as positive control. The pre-albumin bands of the first and sixth lane from the top represent excess DNP-HSA.

age, a third serum with antigen reactivity was found in the Ag + PBS group. A small H-Ig component of the IgG1 subclass with slow electrophoretic mobility, that was not present before, had developed at 27 months of age. This H-Ig component, that could be also detected at 30 months of age when the mouse died, proved to be reactive with DNP-HSA, DNP-OVA, but not with HSA and OVA. Apparently this third H-Ig component consisted of non-precipitating anti-DNP antibodies.

Antibody activity to PPS could not be tested using Ouchterlony's double diffusion technique, because precipitation in agar with PPS did not occur. Using in situ adsorption on Wieme's agar plates, no H-Ig showing anti-PPS activity could be detected.

In order to test the possibility that H-Ig were directed to the idiotypes of the originally responding clones, sera from old individual mice were tested for reactivity to their own sera obtained at the twelfth month of age (the peak of the specific response) in Ouchterlony PEG plates and by in situ adsorption on Wieme's agar plates. All tests were negative.

No further investigations were possible because of the limited amount of the individual serum samples.

In conclusion, analysis of the sera of 25 experimental mice surviving

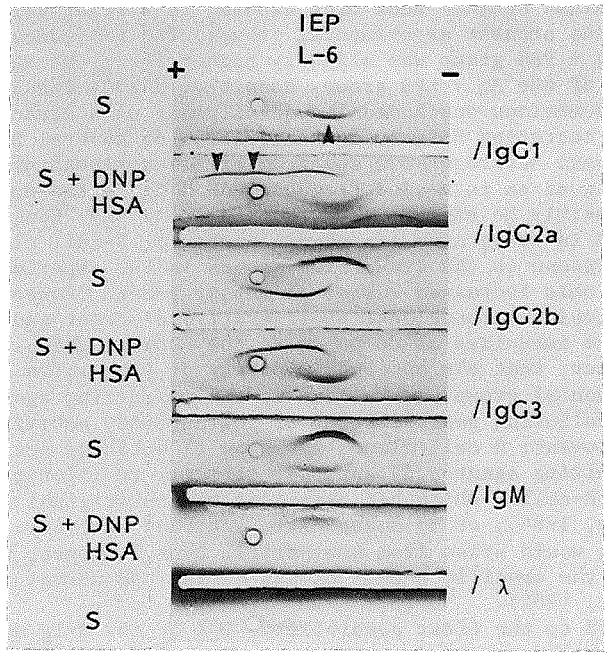

Figure 3. Demonstration of an in situ adsorption test performed in immunoelectrophoresis (IEP) in the same serum sample of mouse L-6 as in figure 2. The serum samples were applied to the wells directly (S) or 30 minutes after DNP-HSA (S + DNP-HSA). The antisera used, which were specific for individual classes, subclasses and light chain type, are indicated next to each corresponding through. A change of the precipitin line after adsorption in situ can be observed (small arrows) thereby revealing the IgG1 isotype of the H-Ig.

above 24 months of age using in situ adsorption on Wieme's agar plates revealed 22 mice with in total 30 H-Ig components. Three out of 30 H-Ig (10%) showed activity against the immunizing agent DNP-HSA. In the control group of 22 mice older than 18 months of age, 25 H-Ig components could be detected, none showing activity against the agents used for immunization of the experimental groups.

DISCUSSION

Eight monthly repeated immunizations with complex antigens in C57BL mice resulted in the development of H-Ig during aging in frequencies higher than in the control mice. Apparently, long lasting antigenic stimulation exerts an influence on the frequency of H-Ig during aging. This finding is in agreement with experiments performed in athymic nude mice (Radl et al., 1980). Conventionalized athymic nude mice exposed to a relatively high antigenic load showed an earlier onset and higher frequencies of H-Ig than

barrier maintained athymic nude mice exposed to a relatively low antigenic load. Within the present experimental groups, the frequency of H-Ig in the sera of the Ag + PBS group was slightly higher (from the age of 7 months on) than that of the Ag + LPS group. Possibly, a polyclonal B-cell stimulator like LPS sometimes precluded the development of a monoclonal B-cell proliferation secreting H-Ig by the activation of several B-cell clones with a consequent heterogeneous spectrum of immunoglobulins.

The finding of an increased frequency of H-Ig in the experimental groups can possibly be explained in the following way: Long lasting immunization started in young mice expanded a number of B-cell clones involved in the immune response to the immunizing agents in the experimental mice. During aging, this increased number of memory B-cell clones has been at risk for developing a monoclonal proliferative disorder and, thus, more H-Ig appeared in the immunized as compared to the control group. Consequently, one would expect, that the antibody activity of this extra number of H-Ig of the experimental group was related to the immunizing agents. In this respect, the following possibilities of the antibody activity of B-cell clones are relevant: B cell clones may show 1) antibody activity to the original immunizing agents, 2) antibody activity to idiotypes of specific antibodies of B-cells, which had responded to the original antigenic stimulations (Jerne, 1984), or 3) nonspecific antibody activity. In the last case, the H-Ig would arise from nonspecific B-cell clones, which responded together with the specific B-cell clones during the normal immune response (Benner et al., 1982).

With regard to the first possibility, 10% of the H-Ig detected in the experimental groups appeared to be directed against DNP-HSA, one of the immunizing agents. In contrast, none of the sera of the control group could be shown to be DNP-HSA reactive. An extension of the control group by including 300 sera with H-Ig that were discussed in a previous study (Radl et al., 1978) resulted in one serum with H-Ig showing anti-DNP reactivity (0.3%). This 10% of DNP-HSA reactive H-Ig detected in the experimental groups represents about one third to one half of the difference in H-Ig frequency between the experimental and control groups. Possibly, the detected percentage of H-Ig reactive with the immunizing agents might be underestimated. During an immune response degeneration of the antigen combining site may occur with a consequent loss of the original binding characteristic (Diamond and Scharff, 1984).

With regard to the second possibility, the extra H-Ig of the experimental groups might have developed from regulatory B-cell clones recognizing the idiotypes of specific antibodies of the B-cells which had responded to the original immunizations (Jerne, 1984). Analysis of such a possibility gave with the techniques used negative results in all cases. Considering that these tests would properly perform only in an optimal antigen-antibody balance, the negative results cannot exclude the possibility that such anti-idiotypic activities were present but were not detected; nor is a way available to test the specificities of other related idiotype anti-idiotype systems. These problems make a reliable estimation of the contribution of the nonspecific B-cell clones, the third possibility, to the extra number of H-Ig in the experimental groups impossible.

According to Radl's classification of monoclonal gammapathies (MG) (Radl, 1985a and 1985b), three groups of MG are age-related: 1) malignant MG 2) BMG and 3) transient MG due to an age-related decline in regulatory T-cell functions. About 90% of the MG detected in C57BL/KaLwRij mice during aging can be classified as BMG (Radl, 1986). Most likely, the majority of

H-Ig detected in our experimental and control groups can be also classified as BMG. Since definite proof that the 3 antigen-reactive H-Ig were true BMG, could only be obtained by transplantation studies (Radl et al., 1979), the diagnosis of BMG was based on remaining clinical and serological criteria. The H-Ig detected were of low and constant concentration and the levels of the other immunoglobulins were not depressed during the observation period, thereby excluding malignant MG. A sufficiently long follow-up of 6 and 10 months, respectively, was possible in 2 out of the 3 mice with antigen-reactive H-Ig. The observation period of the third mouse was only 3 months, because its death prevented a longer evaluation. A possiblity that the three MG were of transient nature originating from some kind of 'cross stimulation' by an antigen related to the original immunizing agent is unlikely. Cross stimulation would in most cases elicit an increase in immunoglobulin secretion by several B-cell clones resulting in a heterogeneous antibody spectrum and not of a H-Ig component. In addition, the concentration of transient MG is usually lower than that seen in the three experimental mice.

The question, why only B-cell clones responding to one of the three antigens used for immunization were found to participate in old-age MG, is difficult to answer. However, preliminary data from our other similar studies show that this phenomenon is not restricted to antigens of the DNP-conjugate type.

Larger and deeper studies are neccessary to elucidate in a more detail the mechanisms by which chronic diseases with a long lasting antigenic stimulation may contribute to the development of benign and possibly also malignant proliferative disorders of the immune system. The results of this first limited experimental study seem to be promissing enough to pursue the investigation along these lines in the C57BL mouse model.

ACKNOWLEDGEMENTS

We gratefully acknowledge Mrs. J. de Goey-van Dooren and Ms. G. de Korte for typing the manuscript.

REFERENCES

Benner R., Rijnbeek A.-M., Van Oudenaren A. and Coutinho A. (1982) Quantitative aspects of the nonspecific humoral immune response to sheep erythrocytes. Adv. Exp. Med. Biol. 149, 703.

Benner R., Van den Akker Th.W. and Radl, J. (1985) Monoclonal gammapathies in immunodeficient animals - a review. In: MG-Clinical Significance and Basic Mechanisms (eds J. Radl, W. Hijmans and B. van Camp), Vol. 5, p. 97, Eurage, Rijswijk, The Netherlands.

Diamond B., and Scharff M.D. (1984) Somatic mutation of the T15 heavy chain with autoantibody specificity. Proc. Natl. Acad. Sci. USA 81, 5841.

Eisen H.N. (1964) Preparation of purified anti-2,4-dinitrophenyl antibodies. In: Methods in Medical Research. Vol. 10 p. 94. Year Book Medical Publishers, Chicago.

Jerne N.K. (1984) Idiotype networks and other preconceived ideas. Immunol. Rev. 79, 5.

Radl J. (1979) Idiopathic paraproteinemia - a consequence of an age-related deficiency in the T immune system. Three-stage development - a hypothesis. Clin. Immunol. Immunopathol. 14, 251.

Radl J. (1981) Immunoglobulin levels and abnormalities in humans and mice. In: Immunological Techniques Applied to Aging Research (eds W.H. Adler and A.A. Nordin), p. 121. CRC Press, Boca Raton, Florida.

Radl J. (1985a) Four major categories of monoclonal gammapathies. Introductory remarks. In: MG-Clinical significance and Basic Mechanisms (eds J. Radl, W. Hijmans and B. van Camp), Vol. 5, p. 3. Eurage, Rijswijk, The Netherlands.

Radl J. (1985b) Monoclonal gammapathies - an attempt at a new classification. Neth. J. Med., 28, 134.

Radl J. (1986) Benign monoclonal gammapathy (BMG). Curr. Top. Microbiol. Immunol. 132, 221.

Radl J., De Glopper E., Schuit H.R.E. and Zurcher C. (1979) Idiopathic paraproteinemia. II. Transplantation of the paraprotein producing clone from old to young C57BL/KaLwRij mice. J. Immunol. 122, 609.

Radl J., Hollander C.F., Van den Berg P. and De Glopper E. (1978) Idiopathic paraproteinaemia. I. Studies in an animal model - the ageing C57BL/KaLwRij mouse. Clin. exp. Immunol. 33, 395.

Radl J., Mink J.G., Van den Berg P., Van Zwieten M.J. and Benner R. (1980) Increased frequency of homogeneous immunoglobulins in the sera of nude athymic mice with aging. Clin. Immunol. Immunopathol. 17, 469.

Seligmann M. and Brouet J.C. (1973) Antibody activity of human myeloma globulins. Sem. Hematol. 10, 163.

Van den Akker Th.W., Tio-Gillen A.P., Zurcher C., Benner R. and Radl J. (1987) The influence of H-2 genetic factors on the development of benign monoclonal gammapathy in ageing H-2 congenic C57BL and BALB mice. Immunology 61, 403.

Wieme R.J. (1959) Studies on agar gel electrophoresis. Techniques - Applications. Brussels, Arscia.

CHAPTER VI

THE INFLUENCE OF H-2 GENETIC FACTORS ON THE DEVELOPMENT OF BENIGN MONOCLONAL GAMMAPATHY IN AGING H-2 CONGENIC C57BL AND BALB MICE

This chapter has been published in Immunology 61, 403-408 (1987).

Immunology 1987 **61** 403–408

The influence of H-2 genetic factors on the development of benign monoclonal gammopathy in ageing H-2 congenic C57BL and BALB mice

T. W. VAN DEN AKKER, A. P. TIO-GILLEN, R. BENNER, C. ZURCHER* & J. RADL* *Department of Cell Biology, Immunology and Genetics, Erasmus University, Rotterdam, and * Institute for Experimental Gerontology TNO, Rijswijk, The Netherlands*

Accepted for publication 3 March 1987

SUMMARY

The role of H-2 genetic factors in the development of benign monoclonal gammopathy (BMG) was investigated in six H-2 congenic C57BL and BALB strains (C57BL/10.ScSn and BALB.B: H-2^b; B10.D2 and BALB/c: H-2^d; B10.BR and BALB.K: H-2^k) during ageing. The frequencies of homogeneous immunoglobulins (H-Ig), both single and multiple, in the three C57BL strains were higher than those in the corresponding three BALB strains. No relationship was found with a particular H-2 haplotype. The most frequent H-Ig isotype within the C57BL strains was IgG2a, within BALB.B and BALB.K mice IgG3 and in BALB/c mice IgG1. Categorization of the monoclonal gammopathies (MG) on the basis of their origin showed a single transient monoclonal B-cell proliferation in 2–5% and 3–9% of the C57BL and BALB mice positive for H-Ig, respectively. Multiple myeloma or B-cell lymphoma were found to be responsible for about 1% of the paraproteinaemias in all strains. Persistent, non-progressive MG, most likely BMG, was detected in 70–81% and 39–46% of the C57BL and BALB mice positive for H-Ig, respectively. The remaining 14–24% and 50–58% of the, respectively, C57BL and BALB mice positive for H-Ig could not be evaluated in time. The H-2 haplotypes under investigation were not associated with the onset, occurrence, multiplicity, persistence or isotype of the MG developing in these H-2 congenic C57BL and BALB strains during ageing.

INTRODUCTION

Genetic influences (Radl *et al.*, 1978, 1985), age-related T-immune system impairment (Mink *et al.*, 1979, 1980; Radl *et al.*, 1980a) and antigenic stimulation (Radl *et al.*, 1980b) have been indicated as being important factors in the development of benign monoclonal gammopathy (BMG)—a benign B-cell proliferative disorder (see 'three-stage hypothesis' by Radl, 1979). When investigating the genetic factors, the major histocompatibility complex (MHC) should be one of the first to be considered. Since the control of the contacts among various cells involved in immune responses is a basic biological function of the MHC, one might suspect an association between the MHC and an imbalanced T–B lymphocyte co-operation during ageing to be a contributing factor in the development of BMG. In the vast area of the search for HLA-related diseases in humans, some reports have dealt with monoclonal gammopathies (MG) (reviewed by Muylle *et al.*, 1982; Ludwig & Mayr, 1982; Leech *et al.*, 1983). Statistically significant correlation between HLA antigen distribution and benign or malignant MG has been observed by some authors. However, results of different studies were not consistent and some were even controversial. In order to evaluate the contribution of MHC genetic factors in the development of MG, we investigated this problem in a mouse model that was previously reported to develop BMG very similar to that in humans (Radl, 1981a). Strong indications for genetic influences in the development of BMG have been obtained by the finding of various frequencies of homogeneous immunoglobulins (H-Ig) in ageing mice of different mouse strains (Radl *et al.*, 1978). In that study C57BL mice showed a high frequency and early onset of BMG, in contrast to CBA and BALB/c mice in which a low frequency and late onset was observed. Further support for the influence of genetic factors on the pathogenesis of BMG was obtained in experiments using radiation chimeras and F_1 mice (Radl *et al.*, 1984, 1985).

In the present study, the influence of three different H-2 haplotypes on the development of MG was studied in ageing H-2 congenic C57BL and BALB strains. No correlation between the frequencies of MG and the presence of any given H-2 haplotype was found.

Correspondence: Dr T. W. van den Akker, Dept. of Cell Biology, Immunology and Genetics, Erasmus University, PO Box 1738, 3000 DR Rotterdam, The Netherlands.

MATERIALS AND METHODS

Mice

BALB/c, BALB.B, BALB.K, C57BL/10.ScSn, B10.D2 and B10.BR female mice were used. All mice, except the BALB/c

mice, were purchased from Olac Ltd, Bicester, Oxon, U.K. The BALB/c mice were obtained from the Laboratory Animals Centre of the Erasmus University, Rotterdam, The Netherlands. The BALB.B (H-2^b) and BALB.K (H-2^k) mice are H-2 congenic to the original BALB/c (H-2^d) strain, and B10.D2 (H-2^d) and B10.BR (H-2^k) mice are H-2 congenic to the original C57BL/10.ScSn (H-2^b) strain. Each group consisted of at least 98 mice. All mice were maintained under clean-conventional conditions in a separate room, where they were allowed to live out their life-spans. The mice received non-autoclaved diet AM II (Hope Farms, Woerden, The Netherlands) and acidified water (pH 3·5) *ad libitum*. Blood samples were taken at 3-monthly intervals and the serum was stored at −70° for later examination. Complete necropsies and histological examinations of 50–70% of the dead animals were performed according to a standard protocol (Van Zwieten *et al.*, 1981; Zurcher *et al.*, 1982).

Detection of homogeneous Ig components

Sera were investigated for the presence of H-Ig by agar electrophoresis according to Wieme and by immunofixation performed on Wieme's agar plates (Radl, 1981b) using a sheep antiserum against Fab fragments of mouse IgG. Sera with a H-Ig component were further investigated by immunoelectrophoresis using goat or sheep antisera specific for mouse IgM, IgG1, IgG2a, IgG2b, IgG3, IgA and lambda light-chain isotypes (Radl, 1981b). In addition, sera obtained 3 months before the death of each mouse were tested for the presence of IgD and IgE in a high concentration by a double radial immunodiffusion technique (Radl, Van den Berg & Jol-van der Zijde, 1980c). Positive sera were further investigated by immunofixation using a specific rabbit antiserum against IgD and goat antiserum against IgE in order to detect H-Ig of the IgD or IgE isotypes. All polyclonal sheep, goat and rabbit antisera were prepared at the Institute for Experimental Gerontology, TNO, Rijswijk, The Netherlands, as described earlier (Radl, 1981b). Mice were considered as positive for H-Ig, and therefore included in the calculation, when serum analysis by combination of agar electrophoresis, immunoelectrophoresis and immunofixation revealed an H-Ig component in the immunoglobulin spectrum.

RESULTS

Survival data

The survival times of the six different H-2 congenic strains are shown in Fig. 1. The life-span of the B10.D2 (H-2^d) mice was the shortest, and that of the B10.BR (H-2^k) mice was the longest. The 90%, 50% and 10% survival time values were 27, 71, 106 weeks and 65, 125, 138 weeks for B10.D2 and B10.BR mice, respectively. The values for the C57BL/10.ScSn and the BALB congenic strains took intermediate positions. No clear association between the survival and the H-2 complex of the congenic mice could be found. For instance, H-2^d BALB/c mice were relatively long survivors in contrast to H-2^d B10.D2 mice (Fig. 1). During the observation period no serious infections appeared that could have influenced the survival of the mice.

Frequency of H-Ig

The increasing frequencies of H-Ig in the sera of congenic C57BL and BALB strains with ageing are shown in Fig. 2. Mice of all three C57BL congenic strains developed H-Ig in a higher frequency and with an earlier onset than any of the three BALB congenic strains. Calculations were performed to correct these data for the different survival times of the H-2 haplotype compatible strains; this correction did not change the outcome of the original results (Fig. 3). In the sera of many C57BL and of some BALB congenic mice, more than one H-Ig component was detected (Fig. 4); the frequency of mice with multiple H-Ig also increased with ageing. Mice of the B10.BR and B10.ScSn strains showed a higher frequency of multiple H-Ig than B10.D2 mice; 49% and 48% versus 21%, respectively. BALB.K mice showed a higher frequency of multiple H-Ig than BALB/c and BALB.B mice, i.e. 26% versus 11% and 8%, respectively.

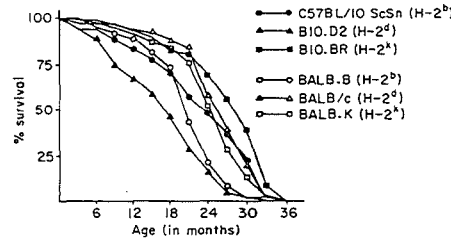

Figure 1. Survival of the H-2 congenic C57BL and BALB mice used: C57BL/10.ScSn (H-2^b); B10.D2 (H-2^d); B10.BR (H-2^k); BALB.B (H-2^b); BALB/c (H-2^d); BALB.K (H-2^k).

Persistence of H-Ig

Categorization of the MG (Radl, 1985a) on the basis of individual follow-up investigations of the mice was performed in, respectively, 81% and 48% of the 191 C57BL and 103 BALB congenic mice positive for H-Ig (Table 1). A single transient monoclonal B-cell proliferation was found in 2–5% and 3–9% of the C57BL and BALB mice positive for H-Ig, respectively. In 70–81% and 39–46% of the C57BL and BALB mice positive for H-Ig, respectively, the H-Ig persisted for longer than 6 months, usually until the death of the animals and without any sign of plasmacellular malignancy. Therefore, these long-lasting H-Ig were considered to fulfil the criteria for BMG as known in man (Radl *et al.*, 1978). Malignant monoclonal B-cell proliferations

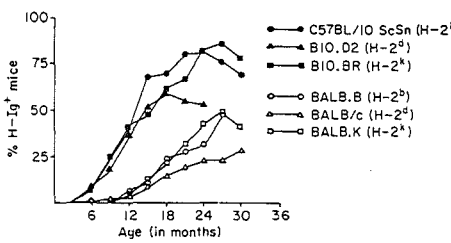

Figure 2. Frequency of homogeneous immunoglobulin components in the sera of H-2 congenic C57BL and BALB mice during ageing: C57BL/10.ScSn (H-2^b); B10.D2 (H-2^d); B10.BR (H-2^k); BALB.B (H-2^b); BALB c (H-2^d); BALB.K (H-2^k).

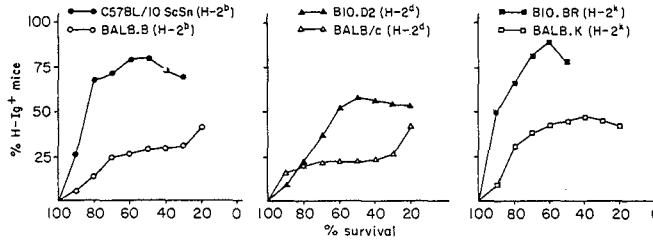

Figure 3. Frequency of homogeneous immunoglobulin components in the sera of H-2 congenic C57BL and BALB mice during ageing corrected for different survival times of the strains.

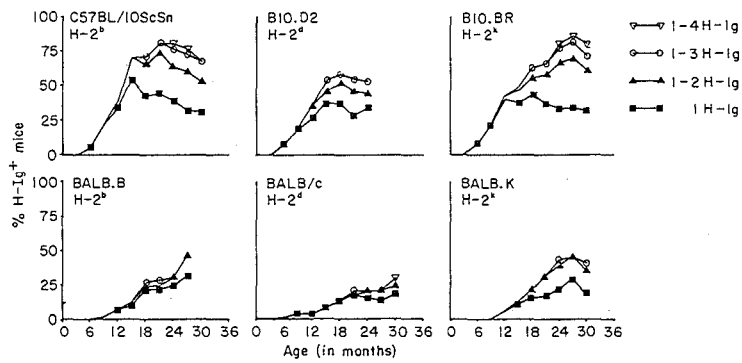

Figure 4. Cumulative representation of single and multiple H-Ig components in the sera of H-2 congenic C57BL and BALB mice during ageing.

Table 1. Distribution of malignant (MM), non-malignant persistent (possibly BMG) and transient H-Ig components detected in the sera of ageing H-2 congenic mice calculated on the basis of individual follow-up case histories

Strain	H-2	Mice (*n*)	H-Ig					Onset of persistent H-Ig before 12 months of age
			MM	Possibly BMG	Transient only	Unclassifiable	Absent	
C57BL/10.ScSn	b	104	1	55	1	15	32	26
B10.D2	d	101	1	29	1	10	60	22
B10.BR	k	103	0	63	4	11	25	34
BALB.B	b	98	0	12	1	13	72	5
BALB/c	d	105	0	12	1	18	74	3
BALB.K	k	103	1	18	4	23	57	2

were found or suspected in about 1% of the H-Ig in all mouse strains. The remaining 19% and 52% of the C57BL and BALB congenic mice positive for H-Ig, respectively, could not be evaluated in time because the H-Ig appeared in old animals in which death prevented a sufficiently long follow-up. The analyses of the H-Ig showed not only a higher incidence of persistent H-Ig in the C57BL congenic strains but also their appearance at an earlier age as compared with the BALB congenic mice.

H-Ig isotype distribution

The heavy- and light-chain isotype distribution of all H-Ig components detected in the sera of the 614 experimental mice is shown in Table 2. The most frequent heavy-chain isotype of the H-Ig was shown to be the IgG2a subclass for the C57BL congenic mice and the IgG3 or IgG1 subclass for the BALB congenic mice. H-Ig of the IgG2b subclass were almost absent in the BALB congenic strains, whereas in contrast the frequency of

Table 2. Heavy- and light-chain isotype distribution (%) among H-Ig detected in the sera of ageing H-2 congenic mice

Strain	H-2	Number of H-Ig	IgM	IgG1	IgG2a	IgG2b	IgG3	IgA	IgD	IgE	λ
C57BL/10.ScSn	b	129	15	17	38	13	13	2	2	0	13
B10.D2	d	68	21	18	26	22	7	3	3	0	4
B10.BR	k	140	11	30	31	21	6	0	1	0	3
BALB.B	b	36	22	19	28	0	31	0	0	0	6
BALB/c	d	41	22	27	22	2	17	10	0	0	6
BALB.K	k	75	24	15	25	4	28	1	0	3	7

H-Ig of the IgG3 subclass was considerably higher in BALB congenic mice as compared with C57BL congenic mice. No other consistent differences could be found among the strains under investigation. H-Ig of the IgD isotype were found at a low concentration twice in each of the C57BL congenic strains. No IgD H-Ig were found in the BALB congenic mice. Low concentration H-Ig of the IgE isotype were found in 3% of the H-Ig of the BALB.K mice. No other strains showed H-Ig of the IgE isotype. When taking all the H-Ig into consideration, the higher frequency of multiple H-Ig in the C57BL strains as compared with the BALB strains might have influenced the heavy- and light-chain isotype distribution. However, recalculations including only those H-Ig that initially appeared in the serum and omitting the additionally appearing H-Ig (see the composition of H-Ig in Table 2) did not change the isotype distribution of the H-Ig. In the sera of mice with multiple H-Ig, the combination of the IgM and the IgG3 isotypes in the BALB strains and that of the IgG2a and the IgG2b subclasses in the C57BL strains seemed to be more frequent than other combinations.

Histopathology

Autopsy with gross examinations was performed on about 60% of the mice; the histological findings were evaluated in about 35% of all mice. Since all the mice lived out their life-span and thus were of different ages when submitted to necropsy, a wide variety of histological lesions was observed. However, mice of C57BL and BALB/c background showed a different incidence pattern for specific lesions. For instance, lesions predominantly found in C57BL mice were brain ventricular dilatation and otitis media. Lesions characteristically observed in BALB strains were adrenal subcapsular spindle cell proliferation and degeneration of lumbar spinal roots. In order to investigate whether the high incidence of H-Ig in C57BL mice could be related to morphological changes reflecting a disordered immune system, special attention was paid to lesions or changes in the lymphoreticular tissues and to lesions with a presumptive immunological basis, such as periarteritis nodosa or amyloidosis. The incidence of such lesions is presented in Table 3. It appears that lymphoreticular tumours are common in all investigated strains, with a

Table 3. Incidence (%) of selected histological lesions in congenic strains of C57BL or BALB background

Congenic strain:	B10.ScSn*	B10.D2	B10.BR	BALB.B	BALB/c	BALB.K
H-2 Haplotype:	b	d	k	b	d	k
Histological diagnosis						
Lymphoreticular system						
Lymphoid hyperplasia	34	18	36	22	19	15
Plasmacellular increase only	45	18	52	10	3	3
Plasmacellular increase and pleimorphism	9	8	39	0	0	0
Malignant lymphoma						
Lymphoblastic	17	28	18	31	29	39
Follicle centre cell type (Pattengale & Taylor, 1983)	11	26	18	19	42	24
Histiocytic sarcoma	9	13	15	3	3	9
Periarteritis nodosa	20	20	21	34	39	21
Amyloidosis	0	0	0	0	0	0
Lumbar root degeneration	13	3	15	47	71	77
Brain ventricular dilatation	43	44	54	0	0	0
Adrenal subcapsular proliferation	9	0	3	65	55	78
Otitis media	35	43	42	0	13	10
No. of mice investigated	35	39	33	32	31	33

* C57BL 10.ScSn.

slight preponderance of lymphoblastic malignant lymphoma in BALB congenic mice. An increase of plasma cells in lymphoid tissues including the bone marrow was mainly observed in mice with a C57BL background. Of the C57BL mice with plasmacellular increase, 16% (C57BL/10.ScSn) to 43% (B10.BR) also showed plasmacellular pleomorphism, especially in femur, ilium and lumbar vertebrae. No clear-cut correlation between the histological observation of plasmacellular pleomorphism within the bone marrow and the serological finding of H-Ig of a high concentration, with a rapid development and decreased levels of other immunoglobulins (a typical pattern of developing multiple myeloma), could be demonstrated. In the mice submitted to histological investigation, BMG (serologically defined) in B10.BR mice was accompanied by plasmacellular increase in 91% (21 out of 23 mice with BMG); of these, 52% (13 out of 21) showed plasmacellular pleimorphism. BMG in all three C57BL strains together was accompanied by plasmacellular increase in 68% (41 out of 60 mice with BMG); of these, plasmacellular pleimorphism was found in 39% of the cases (16 out of 41). In the BALB strains BMG with these plasmacellular abnormalities was not seen.

DISCUSSION

Our data show that genetic factors other than those governed by H-2 loci influence the onset, occurrence, multiplicity, persistence or isotype of the BMG developing with ageing in the H-2 congenic C57BL and BALB strains investigated. All three C57BL congenic strains showed frequencies of H-Ig developing during ageing similar to those reported earlier for C57BL/KaLwRij mice (Radl *et al.*, 1978). The H-Ig incidences in females of the three congenic BALB strains were clearly higher than those in the male BALB/c mice investigated earlier. Male BALB/c mice were first found to be positive for H-Ig at 24 months of age (Radl *et al.*, 1978). In contrast, sera of the three female BALB strains in the present study showed H-Ig at 9 months of age, although at a very low frequency. The difference may be explained by the fact that female mice usually have substantially higher frequencies of H-Ig than male mice (Radl *et al.*, 1978, 1985). Furthermore, in contrast to the previous study (Radl *et al.*, 1978), an immunofixation technique was applied in addition to agar electrophoresis and immunoelectrophoresis. With this combination of techniques the detection sensitivity of H-Ig is highly increased, and consequently 20–30% more H-Ig can be revealed than with agar electrophoresis and immunoelectrophoresis alone (Radl, 1985b).

The frequencies of MG of transient nature and of those of malignant and benign neoplasias in the C57BL congenic mice correlate closely with those found in the ageing C57BL/KaLwRij mice (Radl *et al.*, 1978, 1980a). No comparable data concerning the origin of spontaneously appearing H-Ig in BALB mice are available. Evidence that the persistent H-Ig are true BMG should be confirmed by transplantation studies. Persistent H-Ig from C57BL mice could be transplanted up to three to four times into young, healthy syngeneic mice by bone marrow grafting (Radl *et al.*, 1979), indicating an intrinsic defect but a limited life-span of the B-cell clone affected in BMG. No data on transplantation of bone marrow cells from BALB/c mice with spontaneously appearing persistent H-Ig are available. Because of the limited supply of the congenic mice, no transplantation studies could be performed in this study; therefore, the diagnosis of BMG was based on other remaining criteria, such as the persistence of H-Ig and clinical and histopathological data.

With respect to the isotype distribution of H-Ig, the most frequent in each of the three C57BL strains was the IgG2a isotype; this finding is comparable with earlier data (Radl *et al.*, 1978, 1980a) for the C57BL/KaLwRij mouse. The BALB strain H-Ig isotype distribution showed the IgG3 or IgG1 subclass to be the most frequent among the H-Ig. No data on isotype distribution of BMG in BALB strains are available. In accordance with previous studies, the vast majority of H-Ig were found to be of κ light chain type (Radl *et al.*, 1978, 1980a, 1985).

When analysing the incidence and the duration of the H-Ig and the histology of the C57BL congenic mice, we found that B10.D2 mice showed a slightly different pattern than B10. BR and C57BL/10.ScSn mice. This difference may be explained by the shorter survival time of the B10.D2 mice as compared with the B10.BR and the C57BL/10.ScSn mice, which could have prevented the development of more and persistent H-Ig in time. However, considering all the data, these differences cannot be interpreted as an existing relationship between the H-2 complex and the occurrence of H-Ig.

In the histological study, an increase of plasma cells in lymphoid tissues, including the bone marrow, was mainly observed in mice with a C57BL background. In about half of these mice multifocal plasmacellular pleimorphism was found in the bone marrow. No direct correlation between this finding and the presence of BMG or MM could be demonstrated in individual cases. However, the difference between the high MG frequency strains (C57BL) and the low frequency strains (BALB) with respect to plasmacellular abnormalities in the bone marrow correlates well with all the other indications of a disordered B-cell immune system in C57BL mice. Amyloidosis was notably absent; this was in contrast to the high incidence of this lesion in ageing C57BL/KaLwRij mice (Zurcher *et al.*, 1982).

In conclusion, our data show that factors governed by the H-2 loci under investigation ($H\text{-}2^b$, $H\text{-}2^d$ and $H\text{-}2^k$) do not influence the onset, occurrence, multiplicity, persistence or isotype of the MG developing with ageing in these H-2 congenic C57BL and BALB strains. Apparently, other genetic factors inherent to the C57BL background are more important. Recent studies indicated that the development of BMG can be related to Igh-associated genetic material (Radl *et al.*, 1985). When the allotypes of our C57BL and BALB strains were checked, they were found to be Igh^b in all three C57BL strains and Igh^a in all three BALB strains in accordance with the data from Herzenberg & Herzenberg (1973). However, preliminary results from our studies in allotype congenic C57BL and BALB strains suggest that some other non-Igh-associated genetic factors still contribute to the development of mouse BMG.

ACKNOWLEDGMENTS

We gratefully acknowledge Mrs G. de Korte and Mrs J. de Goey-van Dooren for typing the manuscript. This investigation was supported by the Netherlands Cancer Foundation (Koningin Wilhelmina Fonds).

REFERENCES

HERZENBERG L.A. & HERZENBERG L.A. (1973) Mouse immunoglobulin allotypes: description and special methodology. In: *Handbook of*

Experimental Immunology (ed. D. M. Weir), p. 13.1. Blackwell Scientific, Oxford.

LEECH S.H., BRYAN C.F., ELSTON R.C., RAINEY J., BICKERS J.N. & PELIAS M.Z. (1983) Genetic studies in multiple myeloma. I. Association with HLA-Cw5. *Cancer*, **51**, 1408.

LUDWIG H. & MAYR W. (1982) Genetic aspects of susceptibility to multiple myeloma. *Blood*, **59**, 1286.

MINK J.G., RADL J., VAN DEN BERG P., HAAIJMAN J.J., VAN ZWIETEN M.J. & BENNER R. (1980) Serum immunoglobulins in nude mice and their heterozygous littermates during ageing. *Immunology*, **40**, 539.

MINK J.G., RADL J., VAN DEN BERG P., VAN MUISWINKEL W.B. & VAN OOSTEROM R. (1979) Homogeneous immunoglobulins in the serum of irradiated and bone marrow reconstituted mice: the role of thymus and spleen. *Immunology*, **37**, 889.

MUYLLE L., DE SMET D., COLE J. & PEETERMANS M.E. (1982) HLA-DR and monoclonal gammapathy. *Tiss. Antigens*, **20**, 397.

PATTENGALE P.K. & TAYLOR C.R. (1983) Experimental models of lymphoproliferative disease. *Am. J. Pathol.* **113**, 237.

RADL J. (1979) Idiopathic paraproteinemia—a consequence of age-related deficiency in the T immune system. Three-stage development—a hypothesis. *Clin. Immunol. Immunopathol.* **14**, 251.

RADL J. (1981a) Benign monoclonal gammopathy (mice). Animal models of Human Disease. 11th fascicle, model n. 234. *Am. J. Pathol.* **105**, 91.

RADL J. (1981b) Immunoglobulin levels and abnormalities in humans and mice. In: *Immunological Techniques Applied to Aging Research* (eds W. H. Adler and A. A. Nordin), p. 121. CRC Press, Boca Raton, Florida.

RADL J. (1985a) Monoclonal gammapathies. An attempt at a new classification. *Neth. J. Med.* **28**, 134.

RADL J. (1985b) Four major categories of monoclonal gammapathies. Introductory remarks. In: *MG-Clinical Significance and Basic Mechanisms* (eds J. Radl, W. Hijmans and B. van Camp), Vol. 5, p. 3. Eurage, Rijswijk, The Netherlands.

RADL J., DE GLOPPER B., SCHUIT H.R.E. & ZURCHER C. (1979) Idiopathic paraproteinemia. II. Transplantation of the paraprotein producing clone from old to young C57BL/KaLwRij mice. *J. Immunol.* **122**, 609.

RADL J., DE GLOPPER B., VAN DEN BERG P. & VAN ZWIETEN M.J. (1980a) Idiopathic paraproteinemia. III. Increased frequency of paraproteinemia in thymectomized aging C57BL/KaLwRij and CBA/BrARij mice. *J. Immunol.* **125**, 31.

RADL J., HEIDT P.J., KNAAN-SHANZER S. & VAN ZWIETEN M.J. (1984) Idiopathic paraproteinaemia. IV. The role of genetic factors in the development of monoclonal B cell proliferative disorders—a study in the ageing C57BL/KaLwRij and CBA/BrARij mouse radiation chimeras. *Clin. exp. Immunol.* **57**, 213.

RADL J., HOLLANDER C.F., VAN DEN BERG P. & DE GLOPPER B. (1978) Idiopathic paraproteinaemia. I. Studies in an animal model—the ageing C57BL/KaLwRij mouse. *Clin. exp. Immunol.* **33**, 395.

RADL J., MINK J.G., VAN DEN BERG P., VAN ZWIETEN M.J. & BENNER R. (1980b) Increased frequency of homogeneous immunoglobulins in the sera of nude athymic mice with aging. *Clin. Immunol. Immunopathol.* **17**, 469.

RADL J., VAN DEN BERG P. & JOL-VAN DER ZIJDE C.M. (1980c) IgD idiopathic paraproteinemia in the aging C57BL/KaLwRij mouse. *J. Immunol.* **124**, 2513.

RADL J., VIEVEEN M.H.M., VAN DEN AKKER T.W., BENNER R., HAAIJMAN J.J. & ZURCHER C. (1985) Idiopathic paraproteinaemia. V. Expression of the Igh1 and Igh5 allotypes within the homogeneous immunoglobulins in the ageing (C57BL/LiARij × CBA/BrARij) F_1 mouse. *Clin. exp. Immunol.* **62**, 405.

VAN ZWIETEN M.J., ZURCHER C., SOLLEVELD H.A. & HOLLANDER C.F. (1981) Pathology. In: *Immunological Techniques Applied to Aging Research* (eds W. H. Adler and A. A. Nordin), p. 1. CRC Press, Boca Raton, Florida.

ZURCHER C., VAN ZWIETEN M.J., SOLLEVELD H.A. & HOLLANDER C.F. (1982) Aging Research. In: *The Mouse in Biomedical Research* (eds H. L. Foster, J. D. Small and J. G. Fox), Vol. 4, p. 11. Academic Press, New York.

CHAPTER VII

IDIOPATHIC PARAPROTEINEMIA. V. EXPRESSION OF Ighl AND Igh5 ALLOTYPES WITHIN THE HOMOGENEOUS IMMUNOGLOBULINS OF AGING (C57BL/LiARij x CBA/BrARij)F1 MOUSE

This chapter has been published in Clinical experimental Immunology 62, 405-411 (1985).

Clin exp. Immunol. (1985) **62**, 405–411.

Idiopathic paraproteinaemia V. Expression of Igh1 and Igh5 allotypes within the homogeneous immunoglobulins of ageing (C57BL/LiARij × CBA/BrARij)F1 mouse

J. RADL,* MARGIT H. M. VIEVEEN,* T. W. VAN DEN AKKER,† R. BENNER,† J. J. HAAIJMAN* & C. ZURCHER* **TNO Institute for Experimental Gerontology, Rijswijk, and †Department of Cell Biology and Genetics, Erasmus University, Rotterdam, the Netherlands*

(Accepted for publication 8 May 1985)

SUMMARY

The role of genetic factors linked to the immunoglobulin loci and the development of idiopathic paraproteinaemia (IP)—a benign B-cell proliferative disorder—was investigated in F1 hybrid mice of low (CBA/BrARij) and high (C57BL/LiARij) IP frequency strains. Igh1 and Igh5 allotypes were used as markers for the (parental type) origin of homogeneous immunoglobulins (H-Ig) which appeared in the sera of the F1 mice with ageing. The frequencies of H-Ig in the F1 mice were intermediate with those of the parental strains. The isotype distribution of the H-Ig was 27%, 24%, 12%, 12%, 11%, 10%, 3% and 1% for IgG2a, IgM, IgG1, IgG3, IgG2b, IgD, IgA and IgE, respectively. H-Ig of the IgG2 subclass carried the Igh1b (C57BL) allotype in 98% and the Igh1a (CBA) allotype in 2% cases. Of the IgD H-Ig, 70% carried the Igh5b and 30% the Igh5a determinant. The Igh1 allotype distribution in the bone marrow and spleen plasma cells showed a large variation in the Igh1a/Igh1b ratio among old individual mice and often also between bone marrow and spleen within a single animal with or without a H-Ig component. The categorization of the paraproteinaemias on the basis of their origin showed that 10% of the H-Ig were the result of a transient monoclonal B-cell proliferation; multiple myeloma or lymphoma was found to be responsible for about 1% of the paraproteinaemias; H-Ig fulfilling the criteria for IP were detected in about 42% of cases. The origin of the remaining old age paraproteinaemias could not be determined. These data indicate that the F1 mice develop monoclonal proliferative disorders in a manner more similar to the C57BL than to the CBA parental strain. The allotype associated genetic material from the parental C57BL strain was shown to be mainly responsible for the development of IP in ageing F1 mice.

Keywords idiopathic paraproteinaemia benign monoclonal gammapathy genetic factors ageing

INTRODUCTION

Studies performed in a mouse model system indicate that idiopathic paraproteinaemia (IP) or benign monoclonal gammapathy is the result of an irreversible intrinsic defect within one B cell clone (Radl, 1981a,b, 1982). Factors extrinsic to the affected clone, however, may contribute to the development of IP in its early stages (Radl, 1979). A deficiency in the T immune system which develops with ageing plays a crucial role in this respect (Radl *et al.*, 1980a). Moreover, chronic and

Correspondence: Dr J. Radl, TNO Institute for Experimental Gerontology, P.O. Box 5815, 2280 HV Rijswijk, the Netherlands.

excessive antigenic stimulation increased the frequency of IP in ageing athymic nude mice (Radl *et al.*, 1980b).

A strong indication of genetic influences on the development of IP was the finding of various frequencies of homogeneous immunoglobulins (H-Ig) in ageing mice of different strains (Radl *et al.*, 1978); this view was supported by the results of transplantation experiments in which B cells from mice of a strain with a high IP frequency led to the development of IP in radiation chimeras of a strain with a low IP frequency but not vice versa (Radl *et al.*, 1984).

The present study extends this observation and shows that within the F1 generation of the low IP frequency strain CBA and the high IP frequency strain C57BL, the paraproteins of the IgG2a and IgD classes which develop with ageing (and against which antiallotype sera were available) carry predominantly the b allotype of the C57BL strain.

MATERIALS AND METHODS

Mice. Over a period of 5 years, a total of 254 (177 male and 77 female) F1 (C57BL/LiARij × CBA/BrARij) mice from the colonies of the Institute for Experimental Gerontology TNO were subjects of this study; they were maintained under conventional conditions and were allowed to live out their life-spans. Every 3 months, blood samples of about 0·2 ml were taken from tail vein and the serum was stored frozen at −20°C. Complete necropsies were performed on animals which were killed for transplantation studies and on those dying spontaneously, if not severely autolysed. Histological examinations were done on mice which showed abnormal serum immunoglobulins. Necropsies and histological examinations were performed according to a standard protocol (Zurcher *et al.*, 1982; van Zwieten *et al.*, 1981).

Detection of homogeneous immunoglobulins. All sera were investigated for the presence of H-Ig by agar electrophoresis according to Wieme (1959) and/or by immunofixation performed on Wieme's agar plates (Radl, 1981b) with a sheep antiserum against Fab fragments of mouse IgG. The limit of the sensitivity of this technique (for H-Ig in the presence of a normal heterogeneous Ig background) is 0·05 mg/ml. Sera with a H-Ig component were further investigated by immunofixation with sheep antisera specific for Igh1a and Igh1b allotypes. In addition, all sera were tested for the presence of IgD in a high concentration by a double radial immunodiffusion technique (Radl, van den Berg & Jol-v.d. Zijde, 1980c). Positive sera were additionally investigated by immunofixation with a specific rabbit antiserum against IgD and a monoclonal antibody against the Igh5b allotype of IgD. Finally, sera from all mice which reached the age of 30 months were investigated for isotype distribution within H-Ig by immunoelectrophoresis with specific antisera to the individual mouse immunoglobulin isotypes.

Immunofluorescence studies. Cytocentrifuge slides of cell suspensions from spleen and femoral bone marrow from 10 mice with H-Ig and 18 control normal mice were prepared as described previously (Haaijman *et al.*, 1979). The ratio Igh1a/Igh1b positive plasma cells was determined by double immunofluorescence with fluorescein-labelled sheep antibodies against Igh1b and rhodamine-labelled sheep antibodies against Igh1a allotypes.

Antisera. All polyclonal sheep, goat and rabbit antisera and fluorescence conjugates were prepared in our laboratory as described earlier (Radl, 1981b). The monoclonal antibody to the Igh5b allotype (MAM 021c) was purchased from SERA-LAB Ltd, Crawley Down, Sussex, England.

Transplantation experiments. Spleen and bone marrow cells from 13 mice with H-Ig components of different isotypes were transplanted as described previously (Radl *et al.*, 1979). Usually, 10 F1 mice of 3 months of age were injected intravenously with $2–5 \times 10^6$ bone marrow cells or $8–10 \times 10^6$ spleen cells. Blood samples from these recipient mice were taken every 2 months and the sera were tested for the presence of H-Ig of the donor type by the above described techniques.

RESULTS

The survival times of the F1 mice (three different male and two different female cohorts) did not

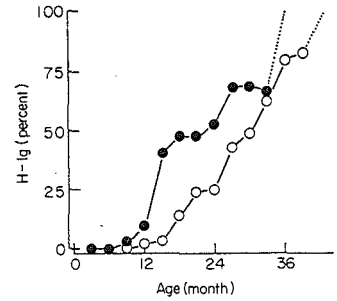

Fig. 1. Frequency of homogeneous immunoglobulin components in the sera of male (○; $n=177$) and female (●; $n=77$) (C57BL/LiARij × CBA/BrARij)F1 mice on ageing. Dotted line indicates data from less than five mice.

differ substantially over the observation period of 5 years. The values for 90%, 50% and 10% survival time were 21·5, 29·5 and 35·7 months for males and 21·7, 28·5 and 34·5 months for females, respectively.

The increasing frequency of H-Ig with ageing in the sera of male and female F1 mice is shown in Fig. 1. Female mice developed H-Ig with higher frequency than males. Of these H-Ig components, 10% were transient and 41% were persistent (i.e. they were detectable in the sera for longer than 6 months); the remaining 49% could not be evaluated in this way because the H-Ig components appeared in old animals in which a longer follow-up was not possible. In the sera of some mice, more

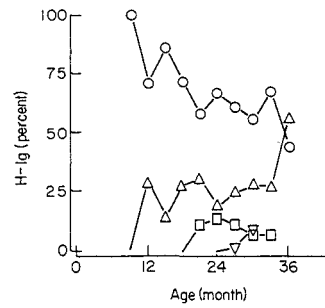

Fig. 2. Relative representation of single and multiple H-Ig as a percentage of all H-Ig components found at given ages in the sera of (C57BL/LiARij × CBA/BrARij)F1 mice. (○) 1 H-Ig; (△) 2 H-Ig; (□) 3 H-Ig; (▽) 4 H-Ig. ♂+♀, $n=254$.

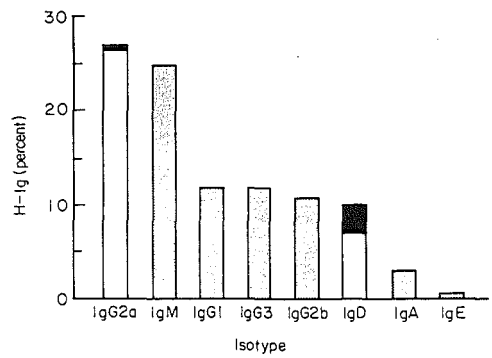

Fig. 3. Isotype and allotype distribution in 208 H-Ig components in the sera of ageing (C57BL/LiARij × CBA/BrARij)F1 mice. (■) Igh a allotype: (□) Igh b allotype; (⊡) Isotype.

than one H-Ig component was detected (Fig. 2); the frequency of these multiple H-Ig components also increased with ageing.

The isotype distribution of the H-Ig components and the representation of the a or b allotypes within the IgG2a (Igh1) and the IgD (Igh5) classes is shown in Fig. 3. The most frequent isotype of the H-Ig was shown to be the IgG2a subclass. Of the 57 H-Ig of this isotype, 56 (98%) carried the Igh1b allotype determinant (of the parent C57BL strain) and only one (2%) was shown to be of the Igh1a allotype (of the parent CBA strain). Of the 20 H-Ig of the IgD class, 14 (70%) carried the Igh5b determinant and six (30%) the Igh5a determinant. One mouse developed two H-IgD components, one of Igh5a and the other of Igh5b allotype (Fig. 4). In the sera of mice with multiple H-Ig components, the combination of IgM and the IgG3 isotypes seemed to be more frequent than other combinations.

In order to determine the representation of the Igh1a versus the Igh1b allotypes at the cellular level in F1 mice with and without H-Ig of the IgG2a subclass, plasma cells from bone marrow and spleen were investigated in 28 mice of different ages (Fig. 5). At the age of 3 months, there were not enough IgG2a$^+$ plasma cells in the bone marrow or the spleen to be evaluated. From the age of 8 months, a slight preponderance of plasma cells of the Igh1b allotype was found. A clear-cut increase in variability with age in allotype distribution was demonstrated for both bone marrow and spleen and for mice both with and without H-Ig of the IgG2a isotype. In mice with a H-Ig component of the Igh1b allotype, a predominance of plasma cells of this allotype was found in about 50% of cases. In six mice, the spleen cell suspensions did not contain enough Igh1$^+$ plasma cells to be counted.

Categorization of the paraproteinaemias on the basis of their origin in the F1 mice was possible in the majority of cases. As mentioned above, 10% of the H-Ig were the result of a transient monoclonal B-cell proliferation. In 41% of the cases, the H-Ig persisted for longer than 6 months, in fact, in all cases until the death of the animal. Of these mice, 19 were submitted to necropsy and histological examination. No plasma cell malignancy was found, to indicate that the observed H-Ig was the result of malignant cell proliferation. These long-lasting H-Ig were, therefore, considered to fulfill the criteria for IP (Radl *et al.*, 1978; Radl, 1981b). The origin in the remaining 49% of mice of

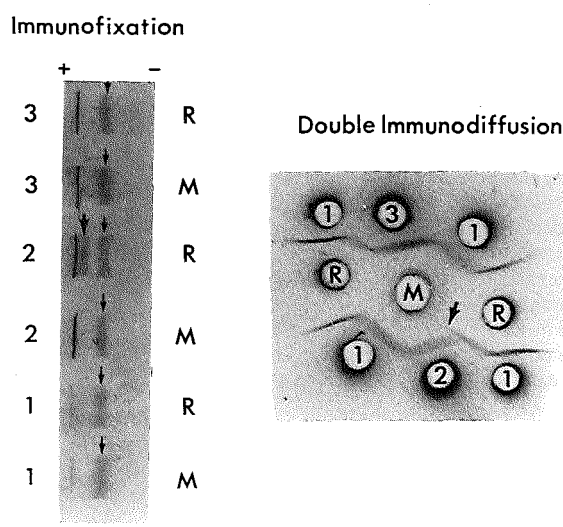

Fig. 4. Demonstration of H-Ig of the IgD class in sera of three F1 mice (1, 2, 3) by immunofixation. While mice 1 and 3 each showed only one H-IgD component reacting with both rabbit anti-IgD isotype specific serum (R) and mouse monoclonal anti-Igh5b antibodies (M), mouse 2 developed two H-IgD, one of which did not react with the anti-Igh5b reagent. This second IgD component caused spur formation (arrow) in the precipitating system developed with the same reagents (R, M) in the double immunodiffusion Ouchterlony plate.

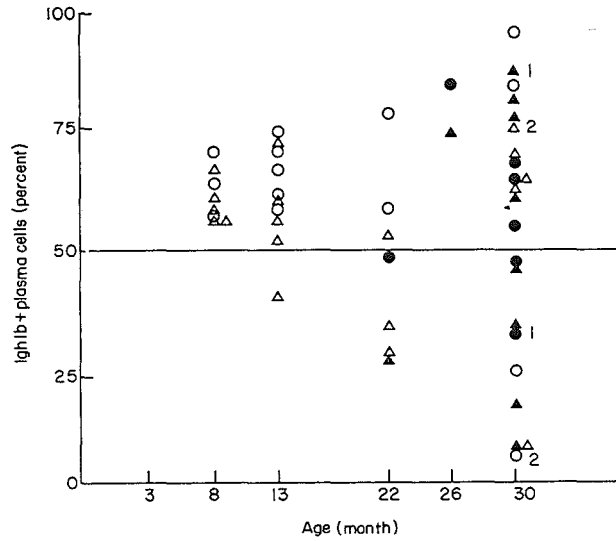

Fig. 5. Percentage distribution of Igh1b$^+$ plasma cells in bone marrow and spleen in ageing (C57BL/LiARij × CBA/BrARij)F1 mice. (○) spleen normal mouse; (●) spleen, mouse with H-Igh1b; (△) bone marrow, normal mouse; (▲) bone marrow, mouse with H-Igh1b. Numbers 1 and 2 designate two mice showing a marked difference in Igh1b representation between bone marrow and spleen. In six cases, data from bone marrow only are given because there were not enough Igh1 cells to be counted in the spleen.

the H-Ig components which appeared for the first time in aged mice (after the 50% survival time) remained obscure (except for nine mice which were used as donors of bone marrow and spleen cells for transplantation into young F1 recipients; see below).

Transplantation experiments were performed with 13 donor mice having H-Ig; four had a long lasting paraproteinaemia (group A), nine developed H-Ig in old age and shortly before transplantation (group B). Five (two in group A, three in group B) of the transplantation experiments failed because of a malignancy in the lymphoreticular system (histiocytic sarcoma or malignant lymphoma) of the donor; all recipients died without developing a paraproteinaemia and death followed the transplanted non-B-cell malignancy. In two cases (group B), no take was achieved; at histological examination of the donors, no lymphoproliferative disorder within the immune system was found. These H-Ig may have been an expression of a transient B cell proliferation. In three cases (two in group A, one in group B) (two IgD's, of which one was of the Igh5a and one of the Igh5b allotype and one an IgG2a (Igh1b) H-Ig), takes were achieved, with decreasing frequence in subsequent transplantations; this is typical for IP. In the remaining three cases (all group B), a take was achieved, but histopathological examination of the donor revealed the presence of malignant lymphoma (H-Ig of IgM and IgM + IgG3 isotypes) or myeloma (H-Ig of the IgG3 isotype).

DISCUSSION

In studies of genetic influences on the development of IP (an autonomous, but benign B cell monoclonal proliferation) the question arose whether the F1 hybrid mice of the high IP frequency strain C57BL and the low frequency strain CBA would develop this proliferative disorder in a pattern resembling that in one of the parental strains rather than the other. If genetic factors linked to the Ig loci are involved in the development of IP, the allotype of H-Ig developing in the F1 mice would be expected to be mainly that of the high IP frequency parental strain.

The F1 hybrids are long lived mice with a life span comparable with that of the CBA parental strain (Zurcher *et al.*, 1982). While the frequency with which H-Ig components can be detected in the sera of these mice during ageing is markedly higher and their onset is much earlier than that of the CBA mice, their incidence remains below that in the C57BL mice (Radl *et al.*, 1978 and unpublished results).

The analysis of the isotype distribution among H-Ig components in the F1 mice showed the IgG2a subclass to be of the highest frequency, followed closely by the IgM isotype. While IgG2a H-Ig components are commonest in the C57BL mouse (Radl *et al.*, 1978), IgM H-Ig are most frequent in thymectomized ageing CBA mice (Radl *et al.*, 1980a). Rather unexpected was the relatively frequent finding (10%) of H-Ig of IgD class. While H-IgD components of a low concentration are by no means rare findings in old C57BL mice, they have not been detected yet in the sera of the CBA mice (Radl *et al.*, 1980c). The determination of the allotypes within the H-Ig of the IgG2a and the IgD isotypes demonstrated a clear-cut predominance of the b (C57BL) allotype (98% for IgG2a and 70% for IgD). As far as the IgG2a H-Ig are concerned, the same dominance of the Igh1b allotype within IgG2a H-Ig was also found in ageing radiation chimeras of the C57BL and CBA mouse strains (Radl *et al.*, 1984). These results indicate that the F1 mice inherit the potential to develop monoclonal B-cell proliferative disorders from the C57BL parental strain. Assessment of the distribution of the Igh1a and Igh1b allotypes in the sera of F1 mice, as tested by immunofixation, indicated that the level of the Igh1b allotype was often somewhat higher than that of the Igh1a allotype; this was also seen in mice without a H-Ig component of this allotype. The distribution of these two allotypes among IgG2a producing plasma cells was therefore also investigated in bone marrow and spleen cell suspensions from F1 mice of different ages with and without H-Ig of the IgG2a isotype. In mice without H-IgG2a, a progressive increase in the variability of the Igh1a/Igh1b ratio among the individual mice in both spleen and bone marrow with ageing was found (Fig. 5). In some of the aged mice, there was a pronounced difference in expression of Igh1a and Igh1b allotypes between plasma cells from bone marrow and spleen in the same animal. A slight predominance of plasma cells producing IgG2a of the Igh1b allotype was seen at all ages. Similar results were obtained in mice with H-Ig2a. Against expectation, a clear-cut predominance of Igh1b allotype was not always expressed in both bone marrow and spleen in these mice. This implies that the monoclonal proliferation did not involve the entire bone marrow compartment and spleen in all cases, but only parts of them. A paradoxical increase in the numbers of cells producing the Igh1a allotype found in some mice in one of the organs investigated could perhaps be explained as a compensatory proliferation of the residual polyclonal IgG2a producing cells.

This study demonstrated that the F1 mice developed monoclonal B cell proliferative disorders of all three categories (i.e. B-cell malignancies, B-cell benign neoplasias and a monoclonal B-cell proliferation due to an immunodeficiency) which are known to be age related (Radl, 1984, 1985) and which are observed also in man and in high frequencies in the C57BL strain but only rarely in CBA mice (Radl *et al.*, 1978; Radl *et al.*, 1980a). A transient paraproteinaemia at low concentration is typical of an age-related immunodeficiency (Radl, 1982); it was found in at least 10% of the ageing F1 mice with H-Ig. A malignant paraproteinaemia due to a B-cell lymphoma or multiple myeloma was found or suspected in about 1% of the cases. Here, a progressively increasing paraprotein level, with the possibility of a continuous propagation of the malignant clone by transplantation and typical morphological findings at histopathology examination led to the correct diagnosis (Radl *et al.*, 1978; Radl, 1985). Of the three monoclonal gammapathies, IP presents most difficulties as far as correct diagnosis is concerned. The criteria for the diagnosis of mouse IP are in principle the same as those in man (Radl *et al.*, 1978). An additional important criterion for IP in the C57BL mouse model is the finding that IP is transplantable into young healthy recipients, but with decreasing 'take' frequency and limited life-span in subsequent transplantation generations (Radl *et al.*, 1979). From the practical point of view, this last criterion can be tested only in a limited number of cases. In the transplantation experiments described above, IP was proved in three out of 13 cases. This relatively low frequency can be explained by the fact that all the donors with H-Ig were of advanced age, at which non-B cell lymphoreticular malignancies (which can distort the take pattern of IP) are also very frequent, and that selection for long-lasting paraproteinaemia in the donors was applicable in only four cases. In comparison with our previous experiments in the C57BL mouse

(Radl *et al.*, 1979), this may have resulted in a lower proportion of successful transplantation of the IP pattern. However, these experiments still show a higher rate of success than in the CBA mice, where in seven transplantation experiments of bone marrow and spleen cells from thymectomized old CBA donors with H-Ig, only one take was achieved and that only from a mouse with an IgM producing lymphoma (unpublished results).

On analysis of all these results together with the histopathology findings, it is clear that the F1 mice developed IP in a similar manner but in a somewhat lower frequency than the C57BL parental strain. The distribution of the allotypes of the two isotypes tested demonstrates that it was allotype associated genetic material from the parental C57BL strain which was mainly responsible for the development of IP in the ageing F1 mice. It is interesting that, in addition to the benign B-cell neoplasia, the F1 mice also developed its malignant couterparts (multiple myeloma and Waldenström's macroglobulinaemia-like lymphoma) which were also noted in the C57BL but not in the CBA mice (Radl *et al.*, 1979; Radl *et al.*, 1985). Because of these differences, the three mouse strains used in our studies provide excellent models for studies not only of clinico-pathological but also cellular and subcellular aspects (e.g. the role of the oncogene expression) of benign as compared with malignant proliferative disorders.

This work was supported in part by the Netherlands Cancer Foundation, grant EUR 80-5. The authors thank R. J. Brondijk and P. van den Berg for their technical assistance and Lutien Vermeer, Marion Roggenkamp and Dr A. C. Ford for their assistance in preparing the manuscript.

REFERENCES

HAAIJMAN, J.J., SLINGERLAND-TEUNISSEN, J., BENNER, R. & VAN OUDENAREN, A. (1979) The distribution of cytoplasmic immunoglobulin containing cells over various lymphoid organs of congenitally athymic (nude) mice as a function of age. *Immunology*, **36**, 271.

RADL, J. (1979) Idiopathic paraproteinemia—A consequence of age-related deficiency in the T immune system. Three-stage development—A hypothesis. *Clin. Immunol. Immunopathol.* **14**, 251.

RADL, J. (1981a) Benign monoclonal gammopathy (idiopathic paraproteinemia). *Am. J. Pathol.* **105**, 91.

RADL, J. (1981b) Immunoglobulin levels and abnormalities in humans and mice. In *Immunological Techniques Applied to Aging Research* (ed. by W.H. Adler & A.A. Nordin) pp. 121–139. CRC Press, Boca Raton, USA.

RADL, J. (1982) Effects of aging on immunoglobulins. In *Pathology of Immunoglobulins: Diagnostic and Clinical Aspects. Protein Abnormalities.* (ed. by S.E. Ritzmann) Vol. 2 pp. 55–69. Alan R. Liss, New York, USA.

RADL, J. (1984) Differences among the three major categories of paraproteinaemias in aging man and the mouse. *Mech. Age. Dev.* **28**, 167.

RADL, J. (1985) Monoclonal gammapathies—an attempt at a new classification. *Neth. J. Med.* **28**, 134.

RADL, J., CROESE, J.W., ZURCHER, C., VAN DEN ENDEN-VIEVEEN, M.H.M., BRONDIJK, R.J., KAZIL, M., HAAIJMAN, J.J., REITSMA, P.H. & BIJVOET, O.L.M. (1985) Influence of treatment with APD-bisphosphonate on the bone lesions in the mouse 5T2 multiple myeloma. *Cancer*, **55**, 1030.

RADL, J., VAN DEN BERG, P. & JOL-VAN DE ZIJDE C.M. (1980c) IgD idiopathic paraproteinemia in the aging C57BL/KaLwRij mouse. *J. Immunol.* **124**, 2513.

RADL, J., DE GLOPPER, B., VAN DEN BERG, P. & VAN ZWIETEN M.J. (1980a) Idiopathic paraproteinemia. III. Increased frequency of paraproteinemia in thymectomized aging C57BL/KaLwRij and CBA/BrARij mice. *J. Immunol.* **125**, 31.

RADL. J., DE GLOPPER, B., SCHUIT, H.R.E. & ZURCHER, C. (1979) Idiopathic paraproteinaemia. II. Transplantation of the paraprotein producing clone from old to young C57BL/KaLwRij mice. *J. Immunol.* **122**, 609.

RADL. J., HEIDT, P.J., KNAAN-SHANZER, S. & VAN ZWIETEN, M.J. (1984) Idiopathic paraproteinaemia. IV. The role of genetic factors in the development of monoclonal B cell proliferative disorders—study in the ageing C57BL/KaLwRij mouse radiation chimeras. *Clin. exp. Immunol.* **33**, 213.

RADL, J., HOLLANDER, C.F., VAN DEN BERG, P. & DE GLOPPER, B. (1978) Idiopathic paraproteinaemia. I. Studies in an animal model—the ageing C57BL/KaLwRij mouse. *Clin. exp. Immunol.* **33**, 395.

RADL, J., MINK, J.G., VAN DEN BERG, P., VAN ZWIETEN, M.J. & BENNER, R. (1980b) Increased frequency of homogeneous immunoglobulins in the sera of nude athymic mice with aging. *Clin. Immunol. Immunopathol.* **17**, 469.

WIEME, R.J. (1959) Studies on agar gel electrophoresis. *Techniques-Applications*. Arscia. Brussels.

ZURCHER, C., VAN ZWIETEN, M.J., SOLLEVELD, H.A. & HOLLANDER, C.F. (1982) Aging research. In: *The Mouse in Biomedical Research.* (ed. by H.L. Foster, J.D. Small & J.G. Fox) Vol. 4 p. 11. Academic Press, New York, USA.

VAN ZWIETEN, M.J., ZURCHER, C., SOLLEVELD, H.A. & HOLLANDER C.F. (1981) Pathology. In: *Immunological Techniques Applied to Aging Research* (ed. by W.H. Adler & A.A. Nordin) p. 1, CRC Press, Boca Raton, USA.

CHAPTER VIII

THE INFLUENCE OF GENETIC FACTORS ASSOCIATED WITH THE IMMUNOGLOBULIN HEAVY CHAIN LOCUS ON THE DEVELOPMENT OF BENIGN MONOCLONAL GAMMAPATHY IN AGING Igh CONGENIC MICE

Th.W. van den Akker, E. de Glopper-van der Veer, J. Radl* and R. Benner

Department of Cell Biology, Immunology and Genetics, Erasmus University Rotterdam and *TNO Institute for Experimental Gerontology, Rijswijk, The Netherlands

Submitted for publication.

SUMMARY

The role of genetic factors associated with the immunoglobulin heavy chain locus (Igh) in the development of benign monoclonal gammapathy (BMG) - a benign B-cell proliferative disorder - was investigated in six Igh congenic mouse strains during aging. The strains used had a C57BL or BALB background. C57BL/6, BALB.Ig^b and CB-20 carrying the C57BL Igh (Igh^b allotype), BALB/c and C57BL/6.Ig^a carrying the BALB/c Igh (Igh^a allotype) and BAB-14, that is of BALB/c origin with the exception of the constant part of the Igh, which is of C57BL origin. The frequency of homogeneous immunoglobulins (H-Ig), both single and multiple, was the highest in C57BL/6 mice followed by C57BL/6.Ig^a. The frequencies of H-Ig in BALB.Ig^b and CB-20 mice were higher than those of BALB/c and BAB-14, although somewhat lower than in C57BL/6.Ig^a mice. Multiple H-Ig were found especially in the sera of C57BL/6 mice. Categorization of the monoclonal gammapathies (MG) on the basis of their origin showed a single transient monoclonal B-cell proliferation in 0-8% of the mice of all strains. Persistent, non-progressive MG, presumably BMG, were detected in 64% of C57BL/6, 30% of C57BL/6.Ig^a, 22% of BALB.Ig^b, 17% of CB-20, 13% of BAB-14 and 6% of BALB/c mice. Multiple myeloma or Waldenström-like B-cell lymphoma were found to be responsible for 2-4% of the paraproteinemias in all strains. The remaining H-Ig varying from 11% of the C57BL/6 to 70% of the BAB-14 mice, could not be evaluated in time. The most frequent isotypes of the BMG within C57BL/6 and C57BL/6.Ig^a were IgG2a and IgG2b, respectively; IgM was the most frequent isotype within the four BALB congenic strains. The immunoglobulin heavy chain allotypes under investigation appeared to be only partly related to the onset, occurrence, multiplicity and persistence of the BMG developing in these Igh congenic C57BL and BALB strains during aging. The immunoglobulin heavy chain allotypes, however, were not related to the major isotype of the BMG. The results obtained in CB-20 and BALB.Ig^b on the one hand and in BAB-14 on the other hand may suggest a role for the variable part of the Igh in the development of BMG. Since no absolute influence could be ascribed to the Igh, we assume that primarily other genetic sequences regulating proliferative B-cell functions account for the pathogenesis of BMG.

INTRODUCTION

Benign monoclonal gammapathy (BMG) is the result of an irreversible intrinsic defect within a single B-cell clone (Radl, 1982). Factors extrinsic to the affected B-cell clone, such as a T-immune system deficiency and chronic and excessive antigenic stimulation, contribute to the development of BMG in its early stages (Radl, 1979; Van den Akker et al., 1987a submitted, 1987c submitted). A strong indication for genetic influences in the development of BMG has been obtained by the finding of various frequencies of homogeneous immunoglobulins (H-Ig) in aging mice of different mouse strains (Radl et al., 1978). In that study, C57BL mice showed a high frequency and early onset of BMG in contrast to CBA and BALB/c mice in which a low frequency and late onset of H-Ig was observed. Further support for the influence of genetic factors on the pathogenesis of BMG was obtained in experiments using radiation chimeras of the C57BL and CBA strains (Radl et al., 1984). Considering the genes which could be candidates for possible pathogenetic factors in the occurrence of monoclonal gammapathies (MG), the major histocompatibility complex (MHC) and the immunoglobulin heavy chain locus (Igh) appeared among the most obvious. However, no correlation between the H-2 haplotype and BMG could be determined (Van den Akker et al., 1987b). With regard to the Igh, we recently found that within the F1 generation of the low BMG frequency strain CBA and the high BMG frequency strain C57BL, the H-Ig of the IgG2a and IgD classes which developed with aging carried predominantly the b allotype of the C57BL strain (Radl et al., 1985). These data suggested a role of Igh associated genetic material from the parental C57BL strain in the development of BMG in the aging F1 mice.

In the present study, the influence of the Igh in the development of MG was investigated in six aging Igh congenic C57BL and BALB strains. A partial correlation between a high frequency of MG and the presence of the b (C57BL) allotype of the Igh was found. Especially genes coding for the variable part of the Igh might play a role in the development of MG. However, the influence of the Igh on the pathogenesis of BMG was not found to be a dominant one.

MATERIALS AND METHODS

Mice

BALB/c and C57BL/6 mice were purchased from OLAC Ltd., Bicester, Oxon, England. The BALB.Ig^b breeding stocks were obtained from Prof. E. Kölsch, Department of Immunology, Münster, F.R.G. The CB-20, BAB-14 and C57BL/6.Ig^a breeding stocks were obtained from the Basel Institute for Immunology, Basel, Switzerland. The BALB.Ig^b, CB-20, BAB-14 and C57BL/6.Ig^a mice were bred in our own department. The C57BL/6.Ig^a strain is Igh congenic to the original C57BL/6 (Igh^b) strain. CB-20 and BALB.Ig^b are Igh congenic to the original BALB/c (Igh^a) strain. The BAB-14 strain is fully syngeneic to BALB/c except for the constant part of the Igh which is of C57BL origin. A survey of the mouse strains used and their Igh characteristics is presented in Table 1. Samples of the sera of all mouse strains were taken and tested initially for the presence of the correct Igh product. All strains were found to have the right Igh-allotype. The C57BL/6, C57BL/6.Ig^a, CB-20, BALB.Ig^b, BAB-14 and BALB/c groups consisted of 53, 83, 101, 100, 86 and 50 mice, respectively. All mice were maintained under clean-conventional con-

TABLE 1. Origin and Igh characteristics of the congenic mouse strains used

Strain	Inbred background	Strain source of introduced Igh	IgC*	IgV**
C57BL/6J	C57BL/6J	-	b	b
C57BL/6.Ig^a	C57BL/6	BALB/c	a	a
CB-20	BALB/c	C57BL/Ka	b	b
BALB.Ig^b	BALB/c	C57BL/6	b	b
BAB-14	BALB/c	C57BL/Ka	b	a
BALB/c	BALB/c	-	a	a

(Modified according to R. Lieberman, 1978)

* IgC: constant part of Igh
** IgV: variable part of Igh

ditions, where they were allowed to live out their life-spans. The mice received non-autoclaved diet AM II (Hope Farms, Woerden, The Netherlands) and acidified water (pH 3.5) ad libitum. Blood samples were taken at 3 monthly intervals and the serum was stored frozen at -20°C for later examination. In order to obtain sufficient material of the different mouse strains for histopathological examination, the experiments were finished at 30 months of age, when about 20% of all mice was still alive.

Detection of homogeneous Ig components

Sera were investigated for the presence of H-Ig by agar electrophoresis according to Wieme and by immunofixation performed on Wieme's agar plates (Radl, 1981) using a goat antiserum against all mouse Ig. Sera with a H-Ig component were further investigated by immunoelectrophoresis using goat or sheep antisera specific for mouse IgM, IgG1, IgG2a, IgG2b, IgG3, IgA and lambda light chain isotypes (Radl, 1981). All polyclonal sheep, goat and rabbit antisera were prepared at the TNO Institute for Experimental Gerontology, Rijswijk, The Netherlands as described earlier (Radl, 1981).

Mice were considered as positive for H-Ig, and therefore included in the calculations, when serum analysis by combination of agar electrophoresis, immunoelectrophoresis and immunofixation revealed a H-Ig component in the immunoglobulin spectrum.

RESULTS

Survival data

The survival data of the six different Igh congenic strains are shown in Fig. 1. All mice had a similar life-span. The 50% survival-time values were 105, 110, 111, 113, 113 and 115 weeks for C57BL/6, BALB.Ig^b, CB-20, C57BL/6.Ig^a, BAB-14 and BALB/c, respectively. No clear association between the survival pattern and the Igh of the congenic mouse strains was found (Fig. 1). During the observation period no serious infections occurred which could have influenced the survival of the mice.

Frequency of H-Ig

The frequencies of H-Ig in the sera of the Igh congenic C57BL and BALB

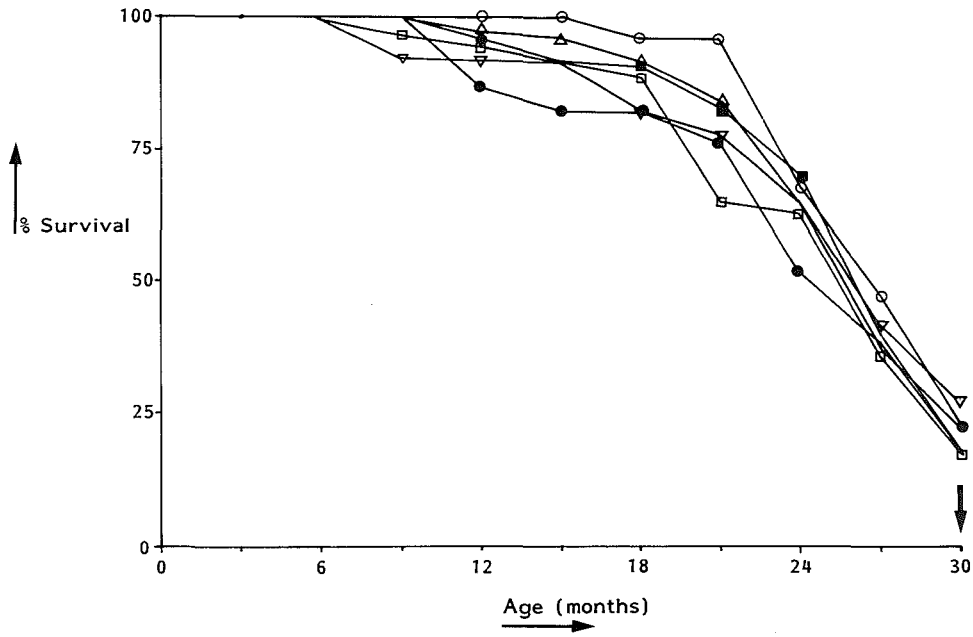

Figure 1. Survival of the Igh congenic C57BL and BALB mice used. ● , C57BL/6; ■ , C57BL/6.Ig^a; △ , CB-20; □ , BALB.Ig^b; ▽ , BAB-14; ○ , BALB/c. Arrow indicates termination of the study at 30 months of age.

strains increased with aging as shown in Fig. 2. C57BL/6 control mice developed H-Ig in the highest frequencies and with an earlier onset than did the other 5 strains. BALB/c control mice and BAB-14 mice showed the lowest frequencies of H-Ig. The C57BL/6.Ig^a mice showed a lower age-related H-Ig frequency than C57BL/6 control mice, but a somewhat higher one than CB-20 and BALB.Ig^b mice. The latter two strains developed H-Ig in a higher frequency than BALB/c and BAB-14 mice did. In the sera of C57BL/6 control mice and, especially, of some of the other mice, more than one H-Ig component was detected (Fig. 3). The frequency of mice with multiple H-Ig also increased with aging. Of the Igh congenic mice, 53% of the C57BL/6, 24% of the C57BL/6.Ig^a, 16% of the CB-20, 10% of the BALB.Ig^b, 8% of the BAB-14 and 6% of the BALB/c mice showed multiple H-Ig in their sera.

Origin of H-Ig

MG form a large, heterogeneous group of B-cell proliferative disorders with a H-Ig component. Most MG can be classified in one of four major categories (Radl, 1985) : 1) B-cell malignancies; 2) BMG; 3) immunodeficiencies with a T < B immune system dysbalance; and 4) antigen-driven MG. Only 3 conditions show a clear-cut relationship with age: Category 1, 2 and the immunodeficiency-due-to-aging group of category 3. The data presented in Fig. 2 do not allow to conclude whether the H-Ig observed during aging belonged to Category 1, 2 or 3. Therefore the MG of all mice with H-Ig were classified on the basis of individual follow-up investigations (Table 2). In this way, the H-Ig present in 89%, 60%, 56%, 48%, 30% and 35% of, respectively, the C57BL/6, C57BL/6.Ig^a, CB-20, BALB.Ig^b, BAB-14 and BALB/c mice were classified according to their origin. Malignant monoclonal B-cell proliferations

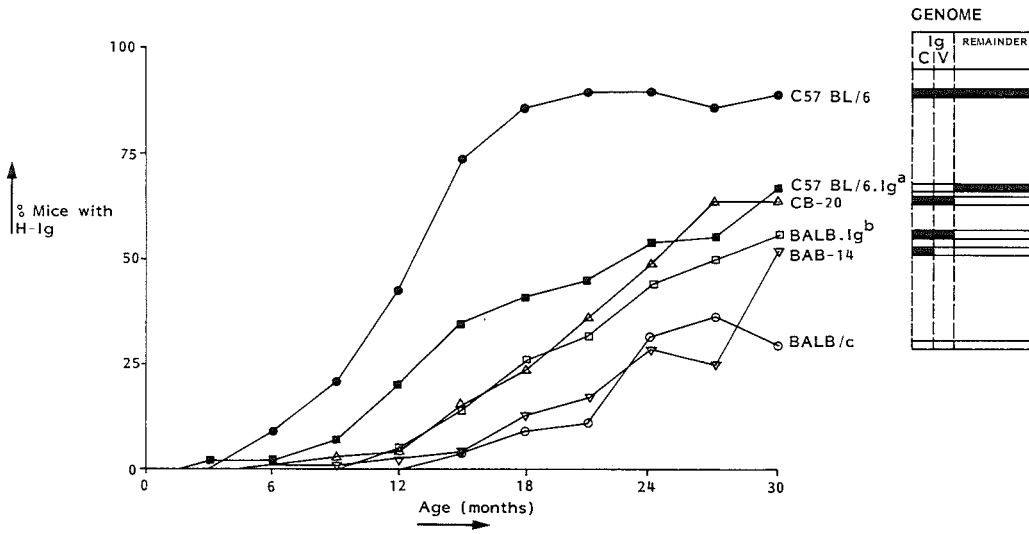

Figure 2. Frequency of H-Ig in the sera of Igh congenic C57BL and BALB mice during aging. ● , C57BL/6; ■ , C57BL/6.Ig^a; △ , CB-20; □ , BALB.Ig^b; ▽ , BAB-14; ○ , BALB/c. IgC: constant part of Igh; IgV: variable part of Igh.

(category 1) were found or suspected in 2 to 4% of the mice in all strains. In 6% to 64% of the mice the H-Ig persisted for longer than 6 months, usually until the death of the animals and without any sign of plasmacellular malignancy. Therefore, these long lasting H-Ig were considered to fulfill the criteria for BMG (category 2) as known in man (Radl et al., 1978). A single transient monoclonal B-cell proliferation belonging to the immunodeficient category 3 was found in 0% to 8% of the mice. The remaining H-Ig of 14% to 31% of the congenic mice with H-Ig could not be evaluated in

TABLE 2. Distribution (%) of malignant (MM), non-malignant persistent (presumably BMG) and transient H-Ig components detected in the sera of aging Igh congenic C57BL and BALB mice calculated on the basis of individual follow-up case histories

Mouse strain	n	H-Ig					Onset BMG
		MM	presumably BMG	transient only	unclassifiable	absent	< 12 months*
C57BL/6	53	2	64	6	17	11	53
C57BL/6.Ig^a	83	4	30	7	19	40	44
CB-20	101	2	22	1	31	44	14
BALB.Ig^b	100	2	17	3	26	52	18
BAB-14	86	3	13	0	14	70	18
BALB/c	50	2	6	8	19	65	0

* Frequency (%) of mice with persistent H-Ig starting before 12 months of age.

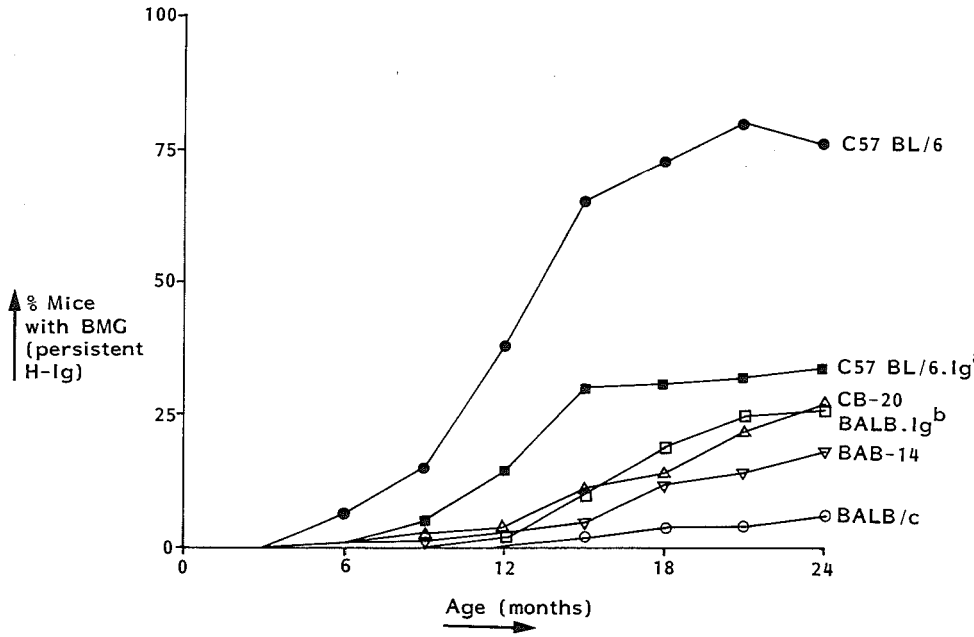

Figure 4. Frequency of non-malignant persistent H-Ig (presumably BMG) in the sera of Igh congenic C57BL and BALB mice during aging. ● , C57BL/6; ■ , C57BL/6.Ig^a; △ ,CB-20; □ , BALB.Ig^b; ▽ , BAB-14; ○ , BALB/c.

time because the H-Ig appeared in old animals in which death prevented a sufficiently long follow-up.

Since Fig. 2 presents the data of the H-Ig of all categories observed during aging, it is not possible to draw conclusions from Fig. 2 with regard to the role of the Igh in the development of BMG (category 2). Using the combined data of Fig. 2 and Table 2 a BMG-frequency figure was constructed (Fig. 4). This figure shows the age-related increase of nonmalignant persistent H-Ig, presumably BMG, in the six congenic strains. Taking into account the survival of the mice and the required observation period of 6 months for BMG, the construction of Fig. 4 required the omission of the 27- and 30-months time-points. During aging, the highest frequencies of BMG were found in C57BL/6 mice, the lowest in BALB/c mice. C57BL/6.Ig^a mice showed an intermediate frequency of persistent H-Ig. Persistent H-Ig developed in BAB-14 in frequencies higher than those found in BALB/c but lower than found in CB-20 and BALB.Ig^b mice.

H-Ig isotype distribution

The heavy and light chain isotype distribution of the first appearing persistent H-Ig, presumably BMG, is shown in Table 3. The most frequent heavy chain isotypes of the presumed BMG in the C57BL/6 and C57BL/6.Ig^a mice were shown to be the IgG2a and IgG2b isotype, respectively. In the BALB congenic mice the IgM class was revealed to be the most frequent isotype among the first appearing presumed BMG. In contrast, in C57BL/6 and C57BL/6.Ig^a mice the frequencies of IgM were low. In parallel, the BALB Igh congenic

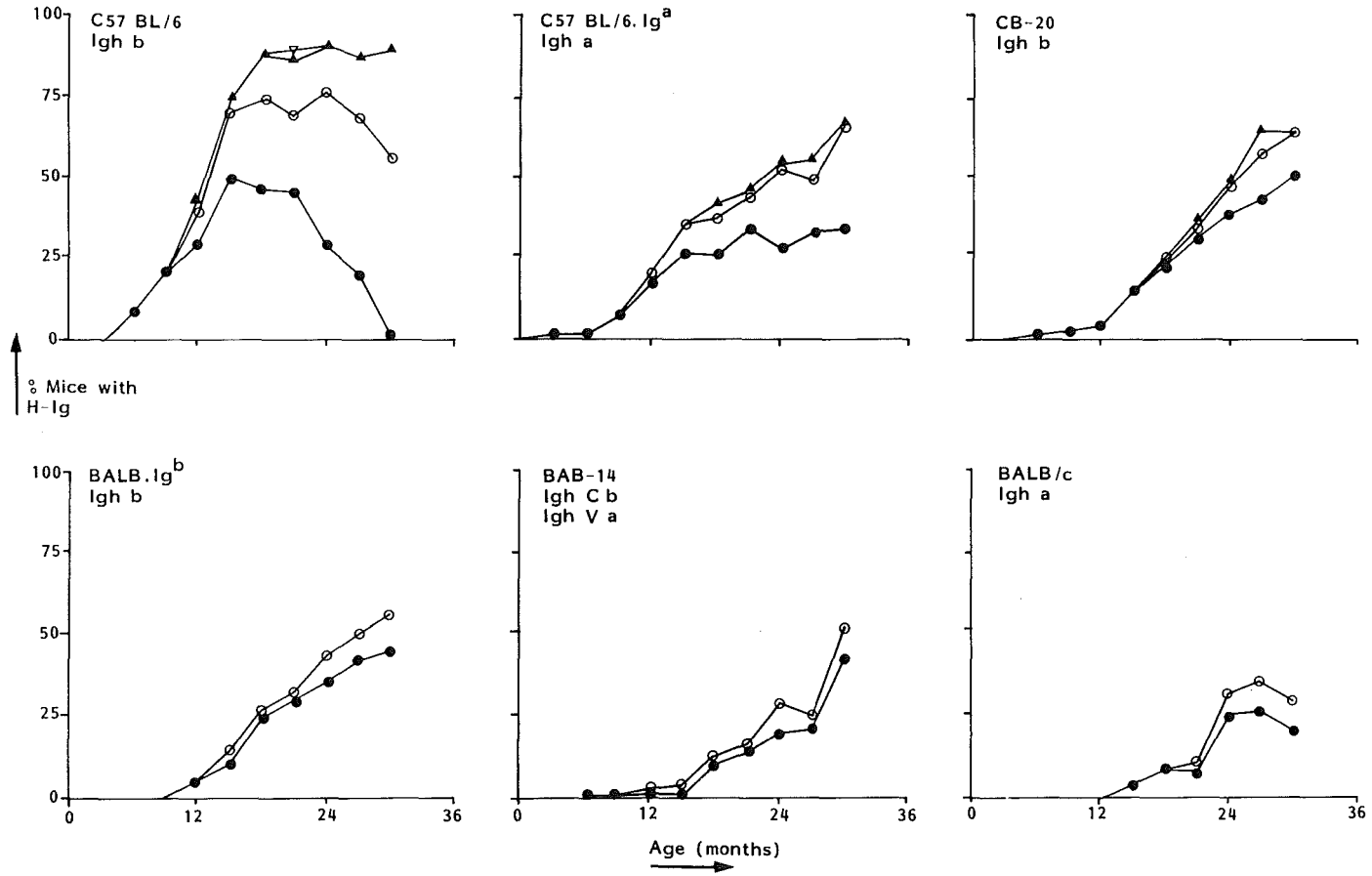

Figure 3. Cumulative representation of single and multiple H-Ig components in the sera of Igh congenic C57BL and BALB mice during aging. ● , 1 H-Ig; ○ , 1-2 H-Ig; ▲ , 1-3 H-Ig; ▽ , 1-4 H-Ig. Igh C: constant part of Igh. Igh V: variable part of Igh.

TABLE 3. Heavy and light chain isotype distribution (%) among first-appearing non-malignant persistent H-Ig (presumably BMG) detected in the sera of aging Igh congenic C57BL and BALB mice.

Mouse strain	(n)	H-Ig observed (n)	Presum. BMG (n)	%						
				IgM	IgG1	IgG2a	IgG2b	IgG3	IgA	λ
C57BL/6	53	83	34	9	26	33	21	9	2	12
C57BL/6.Ig^a	83	74	25	11	12	11	41	22	3	8
CB-20	101	79	22	40	15	12	22	10	1	25
BALB.Ig^b	100	59	17	31	31	6	19	13	-	12
BAB-14	86	34	9	56	17	10	17	-	-	11
BALB/c	50	24	3	67	-	33	-	-	-	-

mice except the BALB/c controls had low IgG2a serum concentrations (as assessed by immunoelectrophoresis) and a low frequency of persistent H-Ig of the IgG2a isotype. An unexpectedly high frequency of lambda bearing presumed BMG (25%) was found in CB-20 mice. This observation was related to the high incidence (16%) of small IgM lambda carrying presumed BMG in that strain.

A similar pattern of the heavy and light chain isotype distribution was obtained, when the H-Ig of all categories were included in the calculation.

DISCUSSION

In this study, some influence of loci within the Igh complex on the development of MG in aging Igh congenic C57BL and BALB mice was found. None of the five congenic strains approached the H-Ig frequencies of the C57BL/6 control strain. With respect to the onset, occurrence, multiple appearance and persistence of the H-Ig, a decrease from C57BL/6 via C57BL/6.Ig^a, CB-20, BALB.Ig^b and BAB-14 to BALB/c was observed. Regarding the isotype distribution of the presumed BMG, it is remarkable that the major isotype was the same as in the original strain. Thus, it seems that the genetic influence on the development of BMG is very complex and cannot be ascribed simply to a certain H-2 (Van den Akker et al., 1987b) or Igh locus. From our data it appeared that the Igh contributes to the development of BMG to some extent; however, probably other subcellular genetic factors, e.g. those which regulate certain clonal cell functions, also contribute to the pathogenesis of BMG. These postulated factors should be related to the C57BL genome. Viral factors intrinsic to the C57BL genome, derepression of genes or gene amplification (Waldenström 1982, 1983) or the activation and/or expression of particular oncogenes (Goustin et al., 1986; Klein and Klein, 1986), may be among these factors. They all may affect sequences which exert a negative control on cell proliferation resulting in the monoclonal proliferation of B cells carrying the Igh of C57BL.

The data of the C57BL/6 and BALB/c mice are in agreement with those of C57BL/10.ScSn and BALB/c mice from our previous studies on the influence of H-2 haplotypes on the development of MG (Van den Akker et al., 1987b). With respect to the isotype distribution of the presumed BMG, the most frequent in the BALB congenic strains (including BALB/c) was the IgM isotype; this

finding is at variance with earlier data (Van den Akker et al., 1987b). In the latter study, IgG1 was found to be the most frequent isotype in BALB/c mice. The high IgM lambda frequency among the presumed BMG of CB-20 was another peculiar finding, we cannot explain at this moment. Frequencies and isotype distribution of H-Ig in the C57BL/6 mice are fully comparable with those reported earlier for C57BL/KaLwRij mice (Radl et al., 1978).

In the construction of the BMG frequency figure (Fig. 4), BMG was defined as a H-Ig component persisting for longer than 6 months, usually until death and without any sign of plasmacellular malignancy. Evidence that the persistent H-Ig are true BMG should be confirmed by transplantation studies. Persistent H-Ig from C57BL mice could be transplanted up to three or four times into young, healthy syngeneic mice by bone marrow grafting (Radl et al., 1979) indicating an intrinsic defect but a limited life-span of the B-cell clone affected in BMG. So far no data on transplantation of bone marrow cells from BALB/c mice with spontaneously appearing persisting H-Ig are available.

An interesting observation is the divergence in BMG frequencies between the BAB-14 on the one hand and the BALB.Ig^b and CB-20 mice on the other hand. The latter strains showed higher frequencies of H-Ig than BAB-14, while the only genetic difference between these strains concerns the variable part of the Igh (IgV). Since IgV codes for sequences including the antigen combining site of the immunoglobulin molecule and the idiotype, the way in which mice deal with antigenic stimulation may influence the development of MG. This suggestion is supported by our recent observation, that chronic excessive stimulation with dinitrophenylated human albumin, ovalbumin and pneumococcal polysaccharide resulted in the development of higher frequencies of MG in aging C57BL mice than in a non-immunized control group (Van den Akker, Brondijk and Radl, 1987a submitted). Although many reports deal with the regulation of the assembly and expression of variable region genes (reviewed a.o. by Yancopoulos and Alt, 1986), very little if anything is known about the regulation of the B-cell clone size or immunoglobulin production rate by the IgV.

In conclusion, our data show that factors associated with the allotype of the Igh do not play a dominant role in the development of BMG. Although they may play a contributing role, there must still be other genetic factors inherent to the C57BL background, which have a larger and complex influence on the development of BMG.

ACKNOWLEDGMENTS

We gratefully acknowledge Ms. G. de Korte and Ms. J. de Goey-van Dooren for typing the manuscript. This investigation was supported by the Netherlands Cancer Foundation (Koningin Wilhelmina Fonds).

REFERENCES

Goustin, A.S., Leof, E.B., Shipley, G.D. and Moses, H.L. (1986) Growth factors and cancer. Cancer Res. 46, 1015.

Klein, G. and Klein, E. (1986) Conditioned tumorigenicity of activated oncogenes. Cancer Res. 46, 3211.

Lieberman, R. (1978) Genetics of IgCH (allotype) locus in the mouse. Springer Seminars Immunopathol. 1, 7.

Radl, J. (1979) Idiopathic paraproteinemia - A consequence of an age-related deficiency in the T immune system. Three-stage development - A hypothesis. Clin. Immunol. Immunopathol. 14, 251.

Radl, J. (1981) Immunoglobulin levels and abnormalities in aging humans and mice. In Immunological Techniques Applied to Aging Research (ed. by W.H. Adler and A.A. Nordin) pp. 121-139. CRC Press. Boca Raton, Florida, USA.

Radl, J. (1982) Effects of aging on immunoglobulins. In Pathology of Immunoglobulins: Diagnostic and Clinical aspects. Protein Abnormalities. Vol. 2 (ed. by S.E. Ritzmann) pp. 55-69. Alan R. Liss, Inc., New York.

Radl, J. (1985) Monoclonal gammapathies. An attempt at a new classification. Neth. J. Med. 28, 134.

Radl, J., De Glopper, E., Schuit, H.R.E. and Zurcher, C. (1979) Idiopathic paraproteinemia. II. Transplantation of the paraprotein producing clone from old to young C57BL/KaLwRij mice. J. Immunol. 122, 609.

Radl, J., Heidt, P.J., Knaan-Shanzer, S. and Van Zwieten, M.J. (1984) Idiopathic paraproteinaemia. IV. The role of genetic factors in the development of monoclonal B cell proliferative disorders - a study in the ageing C57BL/KaLwRij and CBA/BrARij mouse radiation chimeras. Clin. exp. Immunol. 57, 213.

Radl, J., Hollander, C.F., Van den Berg, P. and De Glopper, E. (1978) Idiopathic paraproteinaemia. I. Studies in an animal model - the ageing C57BL/KaLwRij mouse. Clin. exp. Immunol. 33, 395.

Radl, J., Vieveen, M.H.M., Van den Akker, Th.W., Benner, R., Haaijman, J.J. and Zurcher, C. (1985) Idiopathic paraproteinaemia. V. Expression of Ighl and Igh5 allotypes within the homogeneous immunoglobulins of ageing (C57BL/LiARij x CBA/BrARij)F1 mouse. Clin. exp. Immunol. 62, 405.

Van den Akker, Th.W., Brondijk, R. and Radl, J. (1987a) Influence of long term antigenic stimulation started in young C57BL mice on the development of age-related monoclonal gammapathies. Submitted for publication.

Van den Akker, Th.W., Tio-Gillen, A.P., Benner, R., Zurcher, C. and Radl, J. (1987b) The influence of H-2 genetic factors on the development of benign monoclonal gammopathy in ageing H-2 congenic C57BL and BALB mice. Immunology, 61, 403.

Van den Akker, Th.W., Tio-Gillen, A.P., Solleveld, H.A., Benner, R. and Radl, J. (1987c) The influence of T cells on homogeneous immunoglobulins in sera of athymic nude mice during aging. Submitted for publication.

Waldenström, J.G. (1982) Benign monoclonal gammapathy. Ergeb. Inn. Med. Kinderheilkd. 50, 31.

Waldenström, J.G. (1983) Stable gene amplification and its importance in clinical medicine. Lancet 1, 1306.

Yancopoulos, G.D. and Alt, F.W. (1986) Regulation of the assembly and expression of variable-region genes. Ann. Rev. Immunol. 4, 339.

CHAPTER IX

GENERAL DISCUSSION
- of pathogenetic factors in benign monoclonal gammapathy -

In the chapters III-VIII, the influence of several factors on the development of transient monoclonal gammapathy (MG) and benign MG (BMG) has been investigated on the basis of the 'three-stage' hypothesis on the pathogenesis of BMG (Radl, 1979a).

In general, predictions proposed on the basis of this hypothesis were confirmed with regard to the pathogenetic factors investigated: aging, T-immune system impairment, suppressor T-cell function, chronic antigenic stimulation and genetic influences, among which the immunoglobulin heavy chain locus (Igh). In the following, some remarks will be made concerning the role of pathogenetic factors in the development of BMG as described in the Chapters III-VIII.

IX.1. AGING

An age-related increase of homogeneous immunoglobulins (H-Ig) could be detected in the sera of mice of all strains followed up during their life-span. Not only previous animal studies (Radl et al., 1978; 1980a; 1980b) but also those described in this thesis (Chapters III and V-VIII) clearly demonstrate the role of aging in the development of H-Ig. In a narrower sense, BMG (category 2 of Radl's classification (1985)) defined in C57BL mice as an autonomous but not immortal B-cell proliferative disorder, which covers the majority of MG developing in that mouse strain, also showed an age-related increase (Radl et al., 1978; 1979). A similar age-related increase in the occurrence of BMG, but also in that of multiple myeloma (MM) has been found frequently in man (e.g., Radl et al., 1975; Blattner, 1980).

All the data collected concerning the role of aging in BMG are compatible with the notions embodied in stage 1 of the 'three-stage' hypothesis. Involution of the thymus is the first and possibly most important immunological phenomenon occurring during aging. In mice, it starts as soon as 15 days of age, when the maximal relative thymus weight is reached (Pepper, 1961). T-cell functions also decrease with aging, probably reflecting the involution of the thymus and a selective decrease in certain T-cell subpopulations. This decline precedes a decrease of B-cell functions and the onset of most aging phenomena (Adler, Jones and Nariuchi, 1977; Weksler, Innes and Goldstein, 1978). The age-dependent loss of regulatory T-cell function can be regarded as a starting-point for the further evolution of BMG.

IX.2. T-IMMUNE SYSTEM INFLUENCES

1. The T-immune system

The results of the study of the T-immune system with regard to the occurrence of BMG were presented in Chapter III. Impairment of the T-immune system is especially involved in stages 1 and 2 of the development of BMG according to the 'three-stage' hypothesis. Predictions made on the basis of the hypothesis could be confirmed. Firstly, athymic nude mice with a C57BL background showed higher incidences of H-Ig than their high H-Ig frequency background strain. In fact, the athymic C57BL mice developed the highest frequencies of all mouse strains investigated so far. These data are in accord with those of athymic BALB/c mice and controls (Radl et al., 1980b). Athymic BALB/c nudes also showed a higher age-related frequency of H-Ig than the control mice. Secondly, the apparent pathogenetic factor, the impairment of the T immune system was investigated by the infusion of immunocompetent corticosteroid resistant thymocytes (CRT) into 9-months-old BALB/c nude mice. The experimental mice showed a 54% decrease in the frequency of H-Ig, whereas the control group showed a 70% increase during aging.

With regard to the origin of the H-Ig spontaneously appearing in BALB/c and BALB/c nude athymic mice, no data on the transplantability of persistent H-Ig are available. No properly performed transplantation experiments with a sufficiently long follow-up have been performed. Therefore, in contrast to the persistent H-Ig of C57BL/Ka mice, those of athymic BALB/c mice and controls were not shown to be true BMG (Radl et al., 1979).

However, in the T-cell supplementary experiment, the CRT infusion contributed to the revelation of the nature and origin of the H-Ig detected in the sera of the 9-months-old BALB/c nude mice. On the basis of the 'three-stage' hypothesis on the development of H-Ig (Radl, 1979a), transient H-Ig of the immunodeficiency category (stage 2; Radl, 1985) are the result of a principally reversible dysbalance in T-B lymphocyte cooperation and thus prone to correction by T-cell regulatory signals. BMG however, showing an intrinsic B-cell defect (stage 3), would be insensitive to T-cell reconstitution. After infusion of CRT at 9 months of age, 22% of the 35 BALB/c nude mice with H-Ig showed a MG that was insensitive to CRT regulatory signals during an 11-month follow-up. So these MG probably represent BMG. In 43% of the 35 CRT infused BALB/c nude mice with H-Ig at 9 months of age the H-Ig disappeared during follow-up and thus were held for transient MG. The MG of the remaining 35% of the CRT infused mice were

unclassifiable because of untimely death of the mice.

The prospective study using CRT infusion into BALB/c nude mice can be regarded as the conclusion of a series of studies on the influence of the T-immune system as a whole on the development of transient H-Ig of the immunodeficiency category 3 and of BMG (category 2). Starting with the clinical and laboratory observations of about twenty years ago, transient H-Ig appeared especially in conditions with impaired T-cell functions, while the B-cell functions were relatively unaffected. They were found in old, healthy people, in children suffering from various immunodeficiency diseases such as the Nezelof syndrome, Di George syndrome, Wiskott-Aldrich syndrome and in some cases of severe combined immunodeficiency (SCID) (Radl, 1979b). Multiple transient H-Ig were described in sera of three children with SCID after successful bone marrow transplantation treatment (Radl et al., 1972; Radl and Van den Berg, 1973). A restriction in the number of newly developing B-cell clones capable of responding to an antigenic stimulus was tentatively suggested as an explanation (Radl et al., 1972). In order to understand the mechanisms of the development of the MG in these immunodeficiencies, animal models mimicking the clinical situations were investigated. The following models of immunodeficiency were studied: 1) lethal irradiation and bone marrow reconstitution in monkeys and mice; 2) primary T-cell deficiency in athymic nude mice; 3) secondary T-cell deficiency in neonatally and adult thymectomized mice; and 4) secondary age-related immunodeficiency in aging mice. In summary, with respect to the occurrence of transient H-Ig of the immunodeficiency category 3 (Radl, 1985), which can also be considered as the expression of the first two out of the three stages of the developing BMG (Radl, 1979a), the conclusions were:

1) Animal models of human immunodeficiency disorders proved to be useful in elucidating the mechanisms of the development of restricted heterogeneity (RH) of immunoglobulins (Ig) and monoclonal B-cell proliferation in different immunodeficiencies as listed in the third category of the monoclonal gammapathies (Radl, 1985).
2) In all these studies, one common denominator was found: T < B immune system imbalance.
3) The investigation of the pattern of RH of Ig and of the appearance of transient monoclonal Ig components in the serum offers a sensitive test for T-cell mediated control of the heterogeneity of the antibody response.

With regard to the non-malignant persistent H-Ig, presumably BMG, it could be concluded that all T-cell deficient groups of mice showed higher incidences of presumable BMG, somewhat lower incidences of transient MG and fewer normal conditions than the groups with a properly functioning T-immune system. Thus, a shift from normal via dysregulated to intrinsically defective B-cell clones can consistently be seen in T-immune system deficiencies. In terms of the hypothesis, the greater the T-cell impairment, the greater the number of dysregulated B-cell clones which are at risk for developing the intrinsic defect described in stage 3 of the hypothesis.

2. T-cell subpopulations and role of purine metabolism

The influence of T-cell subpopulations, i.e. suppressor T cells (Ts cells) on the development of transient MG and thus within the first two stages of the development of BMG (Radl, 1979a) was assessed by daily administration of 2'-deoxyguanosine (dGuo) to lethally irradiated and reconsti-

tuted C57BL/Ka mice (Chapter IV). In the dGuo treated group, an increased incidence of transient H-Ig was found preceded by a decreased Ts-cell generation and activation. The data indicated an important role of Ts-cell activity in the development of transient H-Ig and probably in that of BMG. The latter may be concluded when it is assumed that impaired suppressor or control T-cell activity results in a greater T-B immune system dysbalance with more B-cell clones at risk for developing the intrinsic defect of the third stage of the hypothesis (Radl, 1979a).

The way, in which dGuo exerted its action was further examined. It was found (Bril et al., 1984) that delayed-type hypersensitivity (DTH)-reactive T cells, the majority of which are probably identical to helper T cells (Bianchi et al., 1981; Mosmann and Coffman, 1987), have a 1000-fold higher resistance to dGuo than Ts cells. The same data were reported by Gelfand et al. for helper T cells and Ts cells (Gelfand, Lee and Dosch, 1979). It was concluded in that study that all T-cell proliferative events were susceptible to dGuo toxicity. These authors explained the differential effect of dGuo on helper T cells and Ts cells by assuming that helper T cells in contrast to Ts cells, do not need to proliferate in order to become functionally active.

In the graft-versus-host (GvH) model of Bril et al. (1985), the induction and further differentiation of DTH-reactive T cells was dependent on proliferation (Wolters and Benner, 1979) and insensitive to daily doses of guanosine (Guo) and dGuo as high as 1 mg per mouse (Bril et al., 1984). The induction and further differentiation of Ts cells induced according to our protocol (Bril et al., 1984) is also proliferation dependent (Bril et al., 1985). However, this process is sensitive to 1 mg dGuo but not Guo. Furthermore, the expression phase of both Ts-cell activity and DTH-reactive T-cell activity is proliferation independent and not susceptible to dGuo and to Guo. Thus, it was concluded that all Ts-cell proliferative events but not the helper T-cell proliferative events are susceptible to dGuo but not Guo toxicity. These data are in accord with the hypothesis that DTH-reactive T cells and Ts cells have a different enzymatic make up with regard to the purine metabolism, resulting in inhibition of DNA synthesis by the accumulation of deoxyguanosine triphosphate (dGTP) in Ts cells but not in DTH-reactive T cells and helper T cells (Carson et al., 1981). Cloned murine T-cell subpopulations seem to be the appropriate material for studying purine enzyme activities and accumulation of purine metabolites after administration of dGuo.

Recently, Scharenberg (1987) reported different pathways for dGuo toxicity in human T lymphocytes of various developmental stages. He showed, that the dGuo mediated toxicity in immature $CD3^-$ thymocytes is caused by dGTP, and in more mature $CD3^+$ thymocytes and in peripheral blood T cells by guanine ribonucleotides. Furthermore, it was found that the presence of dGuo in the culture of peripheral blood T lymphocytes only interfered with the later stages of T-cell activation (Scharenberg, 1987). It was proposed that, possibly, guanosine triphosphate (GTP) interferes with signal transduction following interaction of interleukin-2 with its receptor. Regulatory G-proteins, which govern signal transduction, can probably serve as a target for GTP (Evans et al., 1987). Whether this mechanism of GTP toxicity may play a role in the dGuo-mediated inhibition of Ts-cell activity remains to be studied.

IX.3. ANTIGENIC STIMULATION

The influence of long term antigenic stimulation by multiple antigens (dinitrophenylated human serum albumin (DNP-HSA), ovalbumin and pneumococcal polysaccharide) in young C57BL mice resulted in the development of H-Ig during aging in frequencies higher than those found in the control group (Chapter V). This finding is in agreement with experiments performed in athymic nude mice (Radl et al., 1980b). Conventionalized athymic nude mice exposed to a relatively high antigenic load showed an earlier onset and higher frequencies of H-Ig than barrier maintained athymic nude mice exposed to a relatively low antigenic load. Apparently, long lasting antigenic stimulation exerts an influence on the frequency of H-Ig during aging. The finding of an increased frequency of H-Ig in the experimental groups can possibly be explained in the following way: long lasting immunizations started in young mice expanded a number of B-cell clones involved in the immune response to the immunizing agents. During aging, this increased number of antigen-specific B cells was at risk for developing a monoclonal proliferative disorder and thus more H-Ig appeared in the immunized than in the control group. Consequently, one would expect that the antibody activity of this extra number of H-Ig of the experimental group was related to the immunizing agents. Antibody activity was indeed shown to one of the original immunizing agents (DNP-HSA), but, using the technique of in situ adsorption on Wieme's agar plates, not to the idiotypes of specific antibodies of B cells which had responded to the original stimulation. The antigen specific MG most likely belonged to the category of BMG. The findings indicate that long lasting antigenic stimulation contributes to the development of age-related B-cell proliferative disorders, namely of BMG. Further elucidation of the mechanisms by which chronic diseases with a long lasting antigenic stimulation may contribute to the development of benign and possibly also malignant proliferative disorders of the immune system is necessary by the performance of larger and more detailed studies.

Our data are in agreement with those of Seligmann, Sassy and Chevalier (1973). They described a patient who had received two injections of horse anti-tetanus serum 30 years before he developed an IgG MM with antibody activity to horse α_2-macroglobulin (α_2M) and cross-reactivity with α_2M from other species but not with human α_2M. Another patient with a monoclonal IgG with high antistreptolysin activity had three recurrent attacks of rheumatic fever in childhood (Seligmann et al., 1968). These findings are difficult to reconcile with the occurrence of a first transforming event at the level of a stem cell or of a B-cell precursor not yet able to bind antigen. In fact, these data fit well to the above suggested mechanism of a transforming event at the level of the antigen-activated B cell.

It has been reported that the vast majority of the ligands reacting with human H-Ig are autologous components present on cells (erythrocytes, neurons, other tissue cells), cell constituents (cytoskeleton proteins, nuclear components), plasma proteins (IgG, apolipoproteins, coagulation factors) and ubiquitous phospholipids (Merlini, Farhangi and Osserman, 1986). These authors also conclude that activities against synthetic ligands such as DNP and drugs may represent cross reactions to some autologous components, IgG or nucleic acids. In order to explain the very high frequency of human H-Ig with auto-antibody activity, Seligmann (1983) suggested that the proliferating clones in those MG originate from the human CD5+ B-cell subset, which is associated with autoimmunity. This B-cell subset may mostly use a restricted set of highly conserved germ line V genes (Seligmann, 1985).

IX.4. GENETIC INFLUENCES

For the study of genetic influences on the etiology and pathogenesis of BMG, the mouse model was successfully used. As stated before an age-dependent increase in the appearance of H-Ig has been found in the serum of all mouse strains investigated (Radl et al., 1978). Various frequencies and times of onset of H-Ig in aging mice of different strains strongly suggested the influence of genetic factors on the development of BMG. The highest frequency of H-Ig was found in female C57BL/KaLwRij mice and the lowest in male BALB/c and male and female CBA mice. Further investigations afforded the conclusion that C57BL and human BMG are, with the exception of minor differences, comparable (cf. Chapter I, table III). The fact that the transplantation of bone marrow and spleen cells from old mice with BMG resulted in the persistent appearance of the same H-Ig in the sera of young healthy recipients (Radl et al., 1979), indicated an intrinsic cellular defect within the affected B-cell clone rather than an effect due to specific age-related extrinsic factors.

1. Major histocompatibility complex

Since the high BMG frequency strain C57BL differs from the low BMG frequency strains CBA and BALB/c in H-2 haplotype, the influence of genetic factors associated with the H-2 complex on the development of BMG during aging was investigated in six H-2 congenic female C57BL and BALB strains (Chapter VI). C57BL/10.ScSn and BALB.B mice (both $H\text{-}2^b$), B10.D2 and BALB/c mice (both $H\text{-}2^d$), B10.BR and BALB.K mice (both $H\text{-}2^k$) were included in the study. It was found that the frequencies of H-Ig in all three C57BL congenic strains were higher than those in all BALB congenic strains. Multiple H-Ig were found in higher frequencies among the C57BL strains than among the BALB strains. The major isotype of the H-Ig among the C57BL strains was IgG2a, among the BALB strains IgG3 (BALB.B and BALB.K) and IgG1 (BALB/c). Categorization of the paraproteinemias on the basis of their origin showed 2-9% of the H-Ig to be transient in all strains investigated; 59-70% of the H-Ig in the C57BL congenic strains and 27-39% in the BALB congenic strains were non-malignant and persistent, i.e. were detectable in the sera for longer than 6 months, presumably BMG. The remaining 21-35% of the H-Ig in the C57BL congenic mice and 56-71% of these in the BALB congenic mice could not be evaluated in this respect because of insufficient observation periods. It was concluded that the H-2 haplotypes investigated were not related to the occurrence, onset, multiplicity, persistence and isotype of the H-Ig components in these H-2 congenic C57BL and BALB strains during aging.

2. Immunoglobulin heavy chain locus

Further support for the role of genetic influences in BMG development was obtained from the results of additional transplantation experiments. The infusion of bone marrow cells from C57BL mice, which carry the Igh^b allotype and have a high BMG frequency, into irradiated CBA mice with the Igh^a allotype and a low BMG frequency led to the development of BMG but not vice versa (Radl et al., 1984). Using sheep antisera against $Ighl^a$ and $Ighl^b$ allotypes of the IgG2a subclass, the development of IgG2a BMG with increasing age was investigated. After having received C57BL bone marrow cells, 4 out of 18 CBA recipients developed the $Ighl^b$ (donor) allotype BMG,

whereas none of the 23 C57BL recipients of CBA bone marrow cells showed BMG with the Igh1^a allotype. These data are consistent with the proposition that intrinsic cellular genetic factors of the affected B-cell clone are mainly responsible for the development of BMG.

In a subsequent study (Chapter VII), the sera of aging F1 hybrid mice of the low CBA and high C57BL BMG frequency strains were studied for the occurrence and parental type origin of the H-Ig using the Igh1 and Igh5 allotypes as markers. The frequencies of H-Ig in the aging F1 mice were intermediate to those of the parental strains. Of the IgG2a H-Ig 98% carried the Igh1^b (C57BL) allotype and 2% the Igh1^a (CBA) allotype. IgD H-Ig were 70% of the Igh5^b allotype and 30% of the Igh5^a allotype. The H-Ig could be divided into several categories, 42% being determined as BMG, 1% as MM or lymphoma and about 10% as H-Ig of the immunodeficiency category. The remaining old age paraproteinemias were of uncertain origin. Summarizing these data, the BMG of the F1 mice resemble BMG of the C57BL more closely than those of the CBA parental strain. Thus, the factor contributing to the high frequency development of BMG in the aging F1 mice seemed to be associated with allotype linked genetic material from the parental C57BL strain.

Therefore, the influence of genetic factors associated with the Igh on the development of BMG during aging was investigated in six Igh congenic female C57BL and BALB strains (Chapter VIII): C57BL/6, BALB.Ig^b and CB-20 carrying the C57BL Igh (Igh^b allotype), BALB/c and C57BL/6.Ig^a carrying the BALB/c Igh (Igh^a allotype) and BAB-14 that is of BALB/c origin with the exception of the the constant part of the Igh, that is of C57BL origin. The frequency of H-Ig, both single and multiple, was the highest in C57BL/6 mice followed by C57BL/6.Ig^a. The frequencies of H-Ig in BALB.Ig^b and CB-20 were higher than those of BALB/c and BAB-14, although somewhat lower than that of C57BL/6.Ig^a. Multiple H-Ig were found especially in the sera of C57BL/6 mice. Categorization of the MG on the basis of their origin showed a single transient monoclonal B-cell proliferation in 0-8% of the mice of all strains investigated. Persistent, non-progressive MG, possibly BMG was detected in 64%, 30%, 22%, 17%, 13% and 6% of the C57BL/6, C57BL/6.Ig^a, BALB.Ig^b, CB-20, BAB-14 and BALB/c mice, respectively. MM or Waldenström-like B-cell lymphoma (MW) were found to be responsible for 2-4% of the paraproteinemias in all strains. The remaining H-Ig varying from 11% to 70% of the, respectively, C57BL/6 and BAB-14 mice, could not be evaluated in time. The most frequent isotypes of the BMG within C57BL/6 and C57BL/6.Ig^a were IgG2a and IgG2b, respectively; IgM was the most frequent isotype within the four BALB congenic strains. The immunoglobulin heavy chain allotypes under investigation appeared to be only partly related to the onset, occurrence, multiplicity and persistence but not to the major isotype of the BMG developing in these Igh congenic C57BL and BALB strains during aging. The results obtained in CB-20 and BALB.Ig^b on the one hand and in BAB-14 on the other hand may suggest a role for the variable part of the Igh in the development of BMG. Since no absolute influence could be ascribed to the Igh, we assume that other genetic sequences regulating proliferative cell functions are mainly involved in the pathogenesis of BMG.

3. Oncogenes

From the preceding paragraphs, it seems that the genetic influences on the development of BMG are complex and cannot be ascribed simply to a

certain H-2 or Igh locus. It appeared that the H-2 complex did not (Chapter VI) and the Igh did only partly (Chapter VII and VIII) contribute to the development of BMG. However, other subcellular C57BL related genetic factors which regulate certain clonal B-cell functions, are more likely candidates for pathogenetic influences on BMG. Viral influences intrinsic to the C57BL genome, derepression of genes or gene amplification (Waldenström 1982; 1983) or the activation and/or expression of oncogenes may be among these factors. If these factors involve sequences which exert a negative control on cell proliferation, the clone will continue to proliferate even after the original antigenic stimulation has disappeared. In fact, these considerations represent stage 3 of the 'three-stage' hypothesis on the development of BMG (Radl, 1979). This hypothesis suggests that the intrinsic defect in cell regulation in BMG is different from that in B-cell malignancies.

On the basis of observations of the natural history of both experimental and clinical myelomatosis as well as detailed studies on the proliferative kinetics of myeloma cells in man, Salmon and Seligmann (1973) postulated a two-hit hypothesis of the evolution of MM. In that scheme, the first hit is 'triggering' by a specific antigen, leading to the required monoclonal expansion, while the second hit is the oncogenic event. In the light of recent discoveries of the role of the different oncogenes and their dysfunction in the development of malignant neoplasias (in particular in human Burkitt lymphoma and mouse plasmacytoma (rev. by Klein and Klein, 1985)) it is attractive to postulate that dysfunction of a given oncogene can lead to the initiation of a benign monoclonal B-cell proliferation, a BMG. Further, activation and/or expression of at least two oncogenes will result in the development of a malignancy, MM or MW. Probably, the oncogene involved in BMG is not the same as the oncogene(s) involved in the development of MM. If so, BMG would be a pre-myeloma condition, which is not supported by the observations in many clinical studies (p.e. Axelsson, 1986) and those of the experimental studies in the mouse model of BMG (Radl et al., 1979). In rare reports on the development of clearcut MM after years of a nonprogressive MG (Kyle and Greipp, 1980; Rödjer, Swolin and Westin, 1983), the proliferative disorder could have been malignant from the beginning. In the plasmacytoma model, it was demonstrated that malignant cells still respond to regulatory impulses from the immune system (Lynch and Milburne, 1984). These control mechanisms may become impaired during aging, which may result in a fulminant growth of the neoplasm. Alternatively, a new mutation may lead to changes in the nature of the original neoplasm, which would permit an escape from the controlling immune system.

These notions ascribe the regulatory defect in BMG to the activation and/or expression of oncogenes, which are different from those in malignant MG. Further studies using molecular biological and immunological techniques in clinical laboratory experiments and the mouse models of benign and malignant MG are in progress in order to contribute to the elucidation of this problem.

REFERENCES

Adler W.H., Jones K.H. and Nariuchi H. (1977) Ageing and immune function. In Recent advances in clinical immunology (ed. by R. Thompson) pp. 77-100. Churchill Livingstone, Edinburgh, London and New York.

Axelsson U. (1986) A twenty-year follow-up study of 64 subjects with M-components. Acta Med. Scand. 219, 519.

Bianchi A.T.J., Hooijkaas H., Benner R., Tees R., Nordin A.A. and Schreier M.H. (1981) Clones of helper T cells mediate antigen-specific, H-2 restricted delayed type hypersensitivity. Nature 290, 62.

Blattner W.A. (1980) Epidemiology of multiple myeloma and related plasma cell disorders: An analytical review. In Progress in myeloma (ed. by M. Potter) pp. 1-65. Elsevier North Holland Inc., The Netherlands.

Bril H., Van den Akker Th.W., Hussaarts-Odijk L.M. and Benner R. (1985) Differential influence of 2'-deoxyguanosine on the induction and expression of suppressor T lymphocytes in vivo. Cell. Immunol. 90, 531.

Bril H., Van den Akker Th.W., Molendijk-Lok B.D., Bianchi A.T.J. and Benner R. (1984). Influence of 2'-deoxyguanosine upon the development of DTH effector T cells and suppressor T cells in vivo. J. Immunol. 132, 599.

Carson D.A., Lakow E., Wasson D.B. and Kamatani N. (1981) Lymphocyte dysfunction caused by deficiencies in purine metabolism. Immunol. Today 2, 234.

Evans S.W., Beckner S.K. and Farrar W.L. (1987). Stimulation of specific GTP binding and hydrolysis activities in lymphocyte membrane by interleukin-2. Nature 325, 166.

Gelfand E.W., Lee J.J. and Dosch H.M. (1979) Selective toxicity of purine deoxynucleosides for human lymphocyte growth and function. Proc. Natl. Acad. Sci. USA 76, 1998.

Klein G. and Klein E. (1985) Evolution of tumours and the impact of molecular oncology. Nature 315, 190.

Kyle R.A. and Greipp P.R. (1980) Smoldering multiple myeloma. N. Engl. J. Med. 302, 1347.

Lynch R.G. and Milburn G.L. (1984) In The biology of idiotypes (ed. by M.I. Greene and A. Nisonoff) pp. 299-313. Plenum publishing corporation, New York.

Merlini G., Farhangi, M. and Osserman E.F. (1986) Monoclonal immunoglobulins with antibody activity in myeloma, macroglobulinemia and related plasma cell dyscrasias. Semin. Oncol. 13, 350.

Mosmann T.R. and Coffman R.L. (1987) Two types of helper T-cell clone. Implications for immune regulation. Immunol. Today 8, 223.

Pepper F.J. (1961) The effect of age, pregnancy and lactation on the thymus gland and lymph nodes of the mouse. J. Endocrinol. 22, 335.

Radl J. (1979a) Idiopathic paraproteinemia. A consequence of an age-related deficiency in the T immune system. Three-stage development. A hypothesis. Clin. Immunol. Immunopathol. 14, 251.

Radl J. (1979b) The influence of the T immune system on the appearance of homogeneous immunoglobulins in man and experimental animals. In Humoral immunity in neurological diseases (ed. by D. Karcher, A. Lowenthal and A.D. Strosberg) pp. 517-522. Plenum press, New York.

Radl J. (1985) Monoclonal gammapathies. An attempt at a new classification. Neth. J. Med. 28, 134.

Radl J., De Glopper E., Schuit H.R.E. and Zurcher C. (1979) Idiopathic paraproteinemia. II. Transplantation of the paraprotein-producing clone from old to young C57BL/KaLwRij mice. J. Immunol. 122, 609.

Radl J., De Glopper E., Van den Berg P. and Van Zwieten M.J. (1980a) Idiopathic paraproteinemia. III. Increased frequency of paraproteinemia in thymectomized aging C57BL/KaLwRij and CBA/BrARij mice. J. Immunol. 125, 31.

Radl J., Dooren L.J., Eijsvoogel V.P., Van Went J.J. and Hijmans W. (1972) An immunological study during post-transplantation follow-up of a case of severe combined immunodeficiency. Clin. exp. Immunol. 10, 367.

Radl J., Heidt P.J., Knaan-Shanzer S. and Van Zwieten M.J. (1984) Idiopathic paraproteinaemia. IV. The role of genetic factors in the development of monoclonal B cell proliferative disorders - study in the ageing C57BL/KaLwRij mouse radiation chimeras. Clin. exp. Immunol. 33, 213.

Radl J., Hollander C.F., Van den Berg P. and De Glopper E. (1978) Idiopathic paraproteinaemia. I. Studies in an animal model - the ageing C57BL/KaLwRij mouse. Clin. exp. Immunol. 33, 395.

Radl J., Mink J.G., Van den Berg P., Van Zwieten M.J. and Benner R. (1980b) Increased frequency of homogeneous immunoglobulins in the sera of nude athymic mice with aging. Clin. Immunol. Immunopathol. 17, 469.

Radl J., Sepers J.M., Skvaril F., Morell A. and Hijmans W. (1975) Immunoglobulin patterns in humans over 95 years of age. Clin. exp. Immunol. 22, 84.

Radl J. and Van den Berg P. (1973) Transitory appearance of homogeneous immunoglobulins - "paraproteins" - in children with severe combined immunodeficiency before and after transplantation treatment. In Protides of the biological fluids (ed. by H. Peeters) Vol. 20 pp. 263-266. Pergamon press, Oxford.

Rödjer S., Swolin B. and Westin J. (1983) Monoclonal gammapathy - a diagnostic challenge. Acta Med. Scand. 214, 325.

Salmon S.E. and Seligmann M. (1974) B-cell neoplasia in man. Lancet 2, 1230.

Scharenberg J.G.M. (1987) Purine metabolite mediated toxicity in human lymphoid cells. Relevance for congenital immunodeficiency in man. Dissertation, University of Utrecht.

Seligmann M. (1983). Human lymphocyte subsets in health and disease. Progress in Immunol. 5, 1047.

Seligmann M., Brouet J.-C. and Dellagi K. (1985) Antibody activities of human monoclonal gammopathies with special emphasis on monoclonal IgM in patients with peripheral neuropathy. In MG-clinical significance and basic mechanisms (ed. by J. Radl, W. Hijmans and B. van Camp) Vol. 5 pp. 49-55. Eurage, Rijswijk, The Netherlands.

Seligmann M., Danon F., Basch A. and Bernard J. (1968) IgG myeloma cryoglobulin with antistreptolysin activity. Nature 220, 711.

Seligmann M., Sassy C. and Chevalier A. (1973) A human IgG myeloma protein with anti-α_2-macroglobulin antibody activity. J. Immunol. 110, 85.

Waldenström J.G. (1982) Benign monoclonal gammapathy. Ergeb. Inn. Med. Kinderheilkd. 50, 31.

Waldenström J.G. (1983) Stable gene amplification and its importance in clinical medicine. Lancet 1, 1306.

Weksler M.E., Innes J.G. and Goldstein G. (1978) Immunological studies of aging. IV. The contribution of thymic involution to the immune deficiencies of aging mice and reversal with thymopoietin. J. Exp. Med. 148, 996.

Wolters E.A.J. and Benner R. (1979) Immunobiology of the Graft-versus-Host reaction. II. The role of proliferation in the development of specific anti-host immune responsiveness. Transplantation 27, 39

SUMMARY

Monoclonal gammapathies (MG) can be defined as monoclonal B-cell proliferations characterized by the production of a component of homogeneous immunoglobulins (H-Ig) that usually can be detected in the serum (Chapter I). The increase in sensitivity of the techniques used for the detection of H-Ig during the past 40 years have resulted in the discovery of a broad array of conditions accompanied by H-Ig. The H-Ig, especially occurring in elderly persons, turned out to be far more often benign than malignant (frequency ratio about 200 : 1). On the basis of recent clinical and experimental investigations, most MG could be classified into one of four major categories: 1) B-cell malignancies; 2) B-cell benign neoplasias (BMG); 3) immunodeficiencies characterized by a T < B immune system imbalance; and 4) homogeneous antibody response due to a particular antigenic stimulation.

Our attention was directed at category 2, BMG. In fact, very few data on the etiology and pathogenesis of BMG were available from studies in humans. Therefore, studies were performed in a suitable animal model, the aging C57BL/KaLwRij mouse. Results of BMG transplantation experiments indicated that BMG could be regarded as an autonomous proliferative disorder, characterized by a limited lifespan of the clonal B cells and thus different from malignant MG. These data and those from studies performed in thymectomized and athymic nude mice as well as data from clinical studies resulted in the proposition by Radl (1979) of the 'three-stage' hypothesis on the development of BMG. According to this hypothesis, in the first stage an involution of the thymus takes place, followed by a genetically determined selective decrease in certain T-cell subpopulations and an impairment especially of the regulatory T-cell function during aging. The selectivity and extent of this process is influenced by chronic antigenic stimulation. The resulting imbalance in the T < B immune system network results in restriction of the heterogeneity of the antibody response and in excessive B-cell clonal proliferations, i.e. MG of a reversible transient nature (stage 2). Repeated clonal expansions lead, on a susceptible genetic background, to a higher probability for a mutation; if it involves sequences which exert a negative control on cell proliferation, the clone will continue to proliferate even after the original antigenic stimulus has disappeared (stage 3). This intrinsic defect in cell regulation in BMG is assumed to be different from that in B-cell malignancies.

On the basis of the hypothesis on the development of BMG, experiments have been devised and performed to test the hypothesis. Also the investigations described in this thesis were aimed at testing the hypothesis, and especially at the elucidation of the influence of aging, T-immune system impairment, chronic antigenic stimulation and genetic factors as possible pathogenetic factors in BMG (outlined in Chapter II).

In Chapter III, studies are described aimed at the influence of the T-immune system on the development of BMG. Using a T-cell depletion model, C57BL/Ka nude mice were shown to develop single and multiple H-Ig during aging in the highest frequencies known so far. Ninety percent of the C57BL/Ka nude mice displayed one or more H-Ig at 12 months of age. Using a T-cell supplementation model, infusion of corticosteroid resistant T cells into 9-months-old BALB/c nude mice resulted in a decrease of the frequency

of H-Ig from 43% at 9 months down to 20% at 15 months of age. In contrast, the frequency of H-Ig in the control group increased from 40% at 9 months up to 68% at 12 months of age. The results show that normally functioning T cells are essential for the generation of a normal heterogeneous Ig spectrum; they further support the validity of the 'three-stage' hypothesis with regard to the role of an impairment of the T-immune system in the pathogenesis of BMG.

In Chapter IV, it is described that prolonged administration of micromolar amounts of 2'-deoxyguanosine (dGuo) to lethally irradiated and reconstituted mice led to an increased incidence of transient H-Ig in their sera. The increased incidence of H-Ig was preceded by a decreased suppressor T-cell generation and activation in the dGuo treated group. These data indicate that deficient suppressor T-cell activity plays an important role in the first two stages of the development of BMG, namely in the development of transient H-Ig.

Long term antigenic stimulation by multiple antigens (DNP conjugated to human serum albumin (DNP-HSA), ovalbumin and pneumococcal polysaccharide) in young C57BL mice resulted in the development of H-Ig during aging in frequencies higher than those in the control group (Chapter V). Antibody activity to one of the immunizing agents (DNP-HSA) could be demonstrated for 10% of the H-Ig appearing in the immunized mice at old age, but in none of the H-Ig of the aging control mice. The MG of the mice with those antigen-specific B-cell clones belonged most likely to the category of BMG. These findings indicate that long lasting antigenic stimulation contributes to the development of age-related B-cell proliferative disorders, namely of the BMG.

The role of genetic influences in the development of BMG is discussed in Chapters VI, VII and VIII. Genetic factors in the pathogenesis of BMG associated with the H-2 complex were investigated in six H-2 congenic C57BL and BALB strains (Chapter VI). Our data showed that factors governed by the H-2 loci under investigation ($H\text{-}2^b$, $H\text{-}2^d$ and $H\text{-}2^k$) did not influence the onset, occurrence, multiplicity, persistence or isotype of the MG developing with aging in these congenic strains.

In Chapter VII, it is described that within the F1 generation of the low BMG frequency strain CBA and the high BMG frequency strain C57BL, the H-Ig of the IgG2a and IgD classes, which developed with aging, carried predominantly the b allotype of the C57BL strain (98% and 70%, respectively). These data suggest a role of immunoglobulin heavy chain locus (Igh) associated genetic material from the C57BL strain in the development of BMG in the aging F1 mice.

This suggested role of Igh associated genetic material in the pathogenesis of BMG was further investigated in six aging Igh congenic C57BL and BALB strains (Chapter VIII). The Igh allotypes under investigation (Igh^a and Igh^b) appeared to be only partly related to the onset, occurrence, multiplicity and persistence but not to the major isotype of the BMG developing with aging in these Igh congenic strains. Differences in BMG frequency between CB-20 and BALB.Ig^b on the one hand and BAB-14 on the other hand suggested a modest role for the variable part of the Igh in the development of BMG.

The preceding data were discussed in Chapter IX. In accord with the 'three-stage' hypothesis on the development of BMG, it was concluded that an age-related impairment of the T-immune system and especially a deficient suppressor T-cell activity play an important role in the pathogenesis of BMG in its first two stages. The possible contributing role of chronic

antigenic stimulation to the development of BMG could be confirmed. Genetic influences in the pathogenesis of BMG appeared to be complex and could not be ascribed simply to a certain H-2 or Igh locus. The H-2 complex did not and the Igh did only to a limited extent contribute to the development of BMG. Probably, a defect in other subcellular C57BL related genetic factors, which regulate proliferation of B cells such as activated oncogenes, plays a decisive role in the pathogenesis of BMG.

SAMENVATTING

Monoclonale gammapathieën (MG) kunnen worden gedefinieerd als monoclonale B-cel proliferaties die worden gekenmerkt door de productie van homogene immuunglobulinen (H-Ig). Deze H-Ig componenten kunnen gewoonlijk worden gedetecteerd in het serum (Hoofdstuk I). De toename in gevoeligheid van de technieken, die de afgelopen 40 jaar gebruikt zijn voor de detectie van H-Ig, heeft geresulteerd in de ontdekking van een reeks ziekten die gepaard gaan met H-Ig. De H-Ig, die vooral bij oudere mensen voorkomen, bleken veel vaker bij een benigne dan bij een maligne MG te behoren (frequentieratio ongeveer 200 : 1). Op grond van recente klinische en experimentele onderzoeken konden de meeste MG worden ingedeeld in één van de volgende vier categorieën: 1) B-cel maligniteiten; 2) benigne monoclonale gammapathieën (BMG); 3) immunodeficiënties waarbij het T-immuunsysteem ernstiger tekort schiet dan het B-immuunsysteem; en 4) homogene antistofrespons ten gevolge van een bepaalde antigene stimulatie.

Onze aandacht richtte zich op categorie 2, de BMG. In feite waren er weinig gegevens over de etiologie en pathogenese van BMG naar voren gekomen uit onderzoeken die bij de mens waren verricht. Daarom werd onderzoek verricht in een geschikt diermodel, de verouderende C57BL/KaLwRij muis. De resultaten van BMG transplantatie experimenten gaven aan dat BMG beschouwd kan worden als een autonome proliferatieve aandoening die gekenmerkt wordt door een beperkte levensduur van de klonale B cellen en daarom verschilt van maligne MG. Deze gegevens en die van onderzoek met gethymectomeerde en thymusloze 'nude' muizen, alsmede gegevens van klinische studies, leidden in 1979 tot het opstellen van de 'drie-stadia' hypothese over de ontwikkeling van BMG door Radl. Volgens deze hypothese vindt in het eerste stadium een involutie van de thymus plaats, gevolgd door een genetisch bepaalde, selectieve afname van bepaalde T-cel subpopulaties en een aantasting van vooral regulatoire T-cel functies tijdens veroudering. De selectiviteit en omvang van dit proces worden beïnvloed door chronische antigene stimulatie. Als gevolg van deze veranderingen treedt een verstoring op van het evenwicht binnen het regulatie netwerk dat de interactie tussen het T en B immuunsysteem verzorgt. Deze evenwichtsverstoring leidt tot een beperking van de heterogeniteit van de antistof respons en tot overmatige proliferaties van B-cel klonen, d.w.z. tot reversibele MG van voorbijgaande aard (stadium 2). Als er een geschikte genetische achtergrond is, bestaat er bij herhaaldelijk voorkomende klonale expansies een grotere kans op het optreden van een mutatie. Als deze mutatie betrekking heeft op sequenties die een negatieve controle op de cellulaire proliferatie uitoefenen, zal de B-cel kloon doorgroeien, zelfs nadat de oorspronkelijke antigene stimulatie is verdwenen (stadium 3). Aangenomen wordt dat dit intrinsieke defect in de cellulaire regulatie bij BMG verschilt van dat bij B-cel maligniteiten.

Op grond van de hypothese betreffende de ontwikkeling van BMG, zijn experimenten ontworpen en uitgevoerd om deze te toetsen. Ook de in dit proefschrift beschreven studies betroffen toetsing van de genoemde hypothese. Zoals aangegeven in hoofdstuk II richtte het onderzoek zich op de betekenis van veroudering, aantasting van het T-immuunsysteem, chronische antigene stimulatie en genetische factoren in de pathogenese van BMG.

In hoofdstuk III, wordt onderzoek naar de invloed van het T-immuunsysteem op de ontwikkeling van BMG beschreven. In een T-cel depletie model

werd aangetoond dat tijdens veroudering bij C57BL/Ka nude muizen de tot nu toe hoogste frequenties van enkele en meervoudige H-Ig voorkomen. Op de leeftijd van 12 maanden had 90% van de C57BL/Ka nude muizen één of meer H-Ig in het serum. In een T-cel suppletie model veroorzaakte infusie van immunocompetente, cortocosteroid resistente T cellen in 9 maanden oude BALB/c nude muizen een daling van de H-Ig frequentie van 43% op de leeftijd van 9 maanden tot 20% op de leeftijd van 15 maanden. De frequentie van H-Ig in de controle groep daarentegen steeg van 40% op de leeftijd van 9 maanden tot 68% op de leeftijd van 12 maanden. De resultaten laten zien dat normaal functionerende T cellen essentieel zijn voor het ontstaan van een normaal heterogeen immunoglobuline spectrum. Verder ondersteunen de resultaten de 'drie-stadia' hypothese met betrekking tot de rol van een tekortschietend T-immuunsysteem in de pathogenese van BMG.

In hoofdstuk IV wordt beschreven dat langdurige toediening van micromolaire hoeveelheden 2'-deoxyguanosine (dGuo) aan letaal bestraalde en gereconstitueerde muizen leidde tot een toename van H-Ig van voorbijgaande aard in het serum. De toegenomen incidentie van H-Ig werd voorafgegaan door een verminderde vorming en activatie van suppressor T cellen in de met dGuo behandelde groep. Deze gegevens duiden erop dat deficiënte suppressor T-cel activiteit een belangrijke rol speelt in de eerste twee stadia van de ontwikkeling van BMG, met name in de ontwikkeling van H-Ig van voorbijgaande aard.

C57BL muizen, die op jonge leeftijd veelvuldig worden geïmmuniseerd met meerdere antigenen (DNP geconjugeerd aan humaan serum albumine (DNP-HSA), ovalbumine en pneumococcen polysaccharide) ontwikkelden tijdens veroudering hogere frequenties H-Ig dan de controle groep (hoofdstuk V). Bij 10% van de op oudere leeftijd in geïmmuniseerde muizen verschijnende H-Ig, maar in geen enkele H-Ig van de controle groep, kon antistof reactiviteit tegen één van de immunogenen (DNP-HSA) worden aangetoond. De MG van de muizen met die antigeen-specifieke B-cel klonen behoorden waarschijnlijk tot de BMG categorie. Deze gegevens tonen aan, dat langdurige antigene stimulatie een bijdrage levert aan de ontwikkeling van proliferatieve aandoeningen van B cellen die optreden tijdens veroudering, met name aan die van BMG.

De rol van genetische invloeden op de ontwikkeling van BMG wordt besproken in de hoofdstukken VI, VII en VIII. Genetische factoren die geassocieerd zijn met het H-2 complex werden op hun betrokkenheid bij de pathogenese van BMG onderzocht door bestudering van zes H-2 congene C57BL en BALB stammen (hoofdstuk VI). Onze gegevens laten zien dat factoren die onder invloed staan van de bestudeerde H-2 loci ($H\text{-}2^b$, $H\text{-}2^d$ en $H\text{-}2^k$) geen rol spelen bij het moment van verschijnen, het vóórkomen, het aantal, de persistentie en het isotype van de MG, die tijdens veroudering bij deze congene stammen ontstaan.

In hoofdstuk VII wordt beschreven dat de F1 generatie van de CBA (lage BMG frequentie) en C57BL stam (hoge BMG frequentie) tijdens veroudering voornamelijk IgG2a en IgD H-Ig van het b allotype ontwikkelde (98% respectievelijk 70%). Deze gegevens suggereren dat genetisch materiaal van de C57BL stam, dat geassocieerd is met het immunoglobuline zware keten gen (Igh), een rol speelt bij de ontwikkeling van BMG bij verouderende F1 muizen.

Deze gesuggereerde rol voor met Igh geassocieerd genetisch materiaal in de pathogenese van BMG werd verder onderzocht bij zes Igh congene C57BL en BALB stammen tijdens veroudering (hoofdstuk VIII). De bestudeerde Igh

allotypes (Igh^a en Igh^b) bleken slechts gedeeltelijk gerelateerd te zijn aan het moment van verschijnen, het vóórkomen, het aantal en de persistentie, maar niet aan het belangrijkste isotype van de BMG, die tijdens veroudering bij deze Igh congene stammen ontstaan. Verschillen in BMG frequentie tussen aan de ene kant CB-20 en BALB.Ig^b (beide identiek aan de BALB/c stam behalve voor Igh, dat van C57BL afkomstig is) en aan de andere kant BAB-14 (die identiek is aan de BALB/c stam, behalve voor het constante deel van Igh, dat van C57BL afkomstig is) duiden op enige invloed van het variabele deel van het Igh in de ontwikkeling van BMG.

De hierboven beschreven gegevens worden besproken in hoofdstuk IX. In overeenstemming met de 'drie-stadia' hypothese betreffende de ontwikkeling van BMG wordt geconcludeerd dat een tekortschieten van het T-immuunsysteem, zoals dat ontstaat tijdens veroudering, en vooral een deficiënte suppressor T-cel activiteit, een belangrijke rol spelen in de pathogenese van BMG in de eerste twee stadia. De veronderstelde bijdrage van chronische antigene stimulatie aan de ontwikkeling van BMG kon worden bevestigd. De genetische invloeden op de pathogenese van BMG bleken complex te zijn en konden niet eenvoudig worden toegeschreven aan een bepaald H-2 of Igh locus. Het H-2 complex en het Igh droegen niet, respectievelijk in beperkte mate, bij aan de ontwikkeling van BMG. Waarschijnlijk speelt een defect in andere aan het C57BL genoom gerelateerde genetische factoren, die de proliferatie van B cellen reguleren, zoals bijvoorbeeld geactiveerde oncogenen, een beslissende rol in de pathogenese van BMG.

ABBREVIATIONS

Ag	Antigen
α_2M	α_2-Macroglobulin
ATx	Thymectomy performed at young adult age
B cell	Bone marrow-derived lymphocyte
BMG	Benign monoclonal gammapathy
BSS	Balanced salt solution
CD	Cluster of differentiation
CRT	Corticosteroid resistant thymocytes
dGTP	Deoxyguanosine triphosphate
dGuo	2'-Deoxyguanosine
DNA	Deoxyribonucleic acid
DNP	Dinitrophenyl
DNP-HSA	Dinitrophenylated human serum albumin
DTH	Delayed type hypersensitivity
Fab	Antibody binding fragment of the immunoglobulin molecule
GTP	Guanosine triphosphate
Guo	Guanosine
GvH	Graft-versus-host
H-Ig	Homogeneous immunoglobulins
HLA	Human leucocyte antigens; major histocompatibility complex of man
HPLC	High performance liquid chromatography
HSA	Human serum albumin
H-2	Major histocompatibility complex of the mouse
Ig	Immunoglobulin(s)
IgC, Igh C	Constant part of the immunoglobulin heavy chain locus
Igh	Immunoglobulin heavy chain locus
Igh1	Locus coding for immunoglobulin heavy chain G2a
Igh5	Locus coding for immunoglobulin heavy chain D
IgV, Igh V	Variable part of the immunoglobulin heavy chain locus
i.p.	Intraperitoneal(ly)
IP	Idiopathic paraproteinemia
i.v.	Intravenous(ly)
LPS	Lipopolysaccharide
M-component	Monoclonal component
MG	Monoclonal gammapathy, gammapathies
MGUS	Monoclonal gammapathy of undetermined significance
MHC	Major histocompatibility complex
MM	Multiple myeloma
MW	Macroglobulinemia of Waldenström
NTx	Thymectomy performed neonatally
OVA	Ovalbumin
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PPS	Pneumococcal polysaccharide
RH	Restricted heterogeneity
s.c.	Subcutaneous(ly)
STx	Sham-thymectomy
T cell	Thymus-derived lymphocyte
Ts cell	Suppressor T cell

DANKWOORD

Bij het gereedkomen van dit proefschrift gaat mijn dank uit naar mijn promotoren, Prof. Dr. R. Benner en Prof. Dr. O. Vos voor de mogelijkheden die zij mij hebben geboden het in dit proefschrift beschreven onderzoek uit te voeren. Beste Rob, hartelijk bedankt voor je stimulerende invloed, steun, vertrouwen en enthousiasme in de afgelopen onderzoeksperiode. Jouw inspanningen waren van essentiële betekenis voor het welslagen van dit proefschrift. Ook professor Vos bedank ik hartelijk voor zijn voortdurende belangstelling voor mijn onderzoeksonderwerp en de discussies tijdens de werkbesprekingen. De overige leden van de 'kleine' promotie-commissie, Prof. Dr. J. Abels en Prof. Dr. R.O. van der Heul wil ik graag bedanken voor het nauwkeurig doornemen van het manuscript.

Een zeer belangrijke plaats in mijn onderzoeksproject werd ingenomen door Dr. J. Radl van het TNO Instituut voor Experimentele Gerontologie (IVEG) in Rijswijk. Beste Jiri, bedankt voor de constructieve samenwerking gedurende de afgelopen jaren, je kritische evaluatie van de experimenten en de vele waardevolle en boeiende discussies die we hebben gevoerd over monoclonale gammapathieën en andere onderwerpen.

De resultaten van dit proefschrift zijn mede tot stand gekomen door de grote inzet van aanvankelijk Anne Tio-Gillen en later Betsy de Glopper-van der Veer. Anne en Betsy, ik heb jullie geduld, betrouwbaarheid en toewijding ten opzichte van het werk bijzonder gewaardeerd. Talloze elektroforese-plaatjes voor de analyse van talloze serummonsters hebben jullie zorgvuldig bewerkt. Een grootse prestatie. Anne wil ik tevens bedanken voor haar correcties en adviezen met betrekking tot de Engelse taal.

In het IVEG te Rijswijk werd de pathologie van de in de experimenten bestudeerde muizen geanalyseerd door Dr. Henk Solleveld en vooral Dr. Chris Zurcher. Hartelijk dank voor de zorgvuldige verwerking van de vele histologische coupes. Ook Dr. Joost Haaijman, die destijds aan het IVEG was verbonden, wil ik bedanken voor de kritische noten en nuttige gesprekken over monoclonale gammapathieën. Roelie Brondijk en Margit van den Enden-Vieveen hebben prima werk gedaan aan de experimenten die in dit proefschrift beschreven zijn in hoofdstuk V en VII. Ik ben jullie zeer erkentelijk. Pleunie van den Berg wil ik bedanken voor het zuiveren van de in ons onderzoek gebruikte antisera.

Van vele medewerkers van de afdelingen Celbiologie, Immunologie en Genetica heb ik de afgelopen jaren steun gekregen. Alle collega's en deelnemers aan de Immunologie werkbesprekingen hebben door vragen, opmerkingen, kanttekeningen en suggesties op hun eigen wijze bijgedragen aan het slagen van het in dit proefschrift beschreven onderzoek. Marleen Bakkus en Yvonne Punt (IVEG), die aan de moleculair-biologische aspecten van monoclonale gammapathieën werken, wil ik in het bijzonder bedanken voor de prettige samenwerking. Elsa Franken-Postma, die de chromosomale analyse van monoclonale gammapathieën voor haar rekening neemt, bedank ik hartelijk voor haar nauwgezette werk.

Voordat de resultaten van het onderzoek hun vorm vonden in dit proefschrift moest veel typewerk worden verricht. Jacqueline de Goey-van Dooren en Geertje de Korte verdienen daarom speciale vermelding. Rein Smid en Tom Vermetten zorgden steeds voor snelle, efficiënte bestellingen van de benodigde materialen. De muizen werden uitstekend verzorgd door aanvankelijk Joop Bos en later Joop Brandenburg, bijgestaan door Ferry Siefert, Marry en

Yvonne Steinvoort en Saskia Kalkman. Technische problemen werden regelmatig opgelost door Piet Hartwijk. De figuren van dit proefschrift werden getekend door Pim Visser, Piet Hartwijk en medewerkers van het Audiovisueel Centrum (AVC) onder leiding van Cor van Dijk. De deskundig uitgevoerde fotografie was in handen van Tar van Os, Joop Fengler en het AVC. Mevrouw Godijn, Jopie Bolman en Lia de Wever zorgden niet alleen voor schoon glaswerk, maar ook voor versterking van de inwendige mens.

Tenslotte wil ik van deze gelegenheid gebruik maken mijn ouders hartelijk te bedanken voor de goede sfeer thuis en de prima mogelijkheden die ze mij altijd geboden hebben om te studeren.

CURRICULUM VITAE

Na het behalen van het diploma Gymnasium β in 1973 aan 'Het Christelijk Lyceum' te Dordrecht begon de schrijver van dit proefschrift in hetzelfde jaar de studie Geneeskunde aan de Medische Faculteit van de Erasmus Universiteit Rotterdam (E.U.R.). In het kader van het keuzepracticum werd in 1976 onderzoek verricht naar het verschil in resultaat van behandeling van migraine-patiënten door 'alternatieve' en 'officiële' genezers. Van september 1976 tot en met augustus 1978 was de schrijver als student-assistent verbonden aan de afdeling Pathologische Anatomie I van de E.U.R.

In februari 1980 werd het artsexamen afgelegd, waarna binnen de vakgroep Celbiologie en Genetica van de E.U.R. gestart werd met onderzoek naar monoclonale gammapathieën. Binnen de sectie Immunologie van deze vakgroep werd het in dit proefschrift beschreven onderzoek verricht onder leiding van Prof. Dr. R. Benner en Dr. J. Radl van het TNO Instituut voor Experimentele Gerontologie te Rijswijk.

In 1981 werd het C-diploma Stralingsbescherming van het J.A. Cohen Instituut (I.R.S.) behaald.

Gedurende de onderzoeksperiode werd in het nieuwe medische curriculum van de E.U.R. Immunologie-onderwijs ingevoerd. De auteur van dit proefschrift heeft aan de vormgeving van het eerste- en derdejaars onderwijs Immunologie veel aandacht besteed.

Naast de appendix publicaties die in dit proefschrift zijn opgenomen (hoofdstuk III-VIII) was de auteur betrokken bij onderzoek dat uitmondde in de volgende publicaties:

Benner R., Van den Akker Th.W. and Radl J. (1985) Monoclonal gammapathies in immunodeficient animals - a review. In MG - Clinical Significance and Basic Mechanisms (ed. by J. Radl, W. Hijmans and B. van Camp) Vol. 5 pp. 97-102. Eurage, Rijswijk, The Netherlands.

Bril H., Van den Akker Th.W., Hussaarts-Odijk L.M. and Benner R. (1985) Differential influence of 2'-deoxyguanosine on the induction and expression of suppressor T lymphocytes in vivo. Cell. Immunol. 90, 531.

Bril H., Van den Akker Th.W., Molendijk-Lok B.D., Bianchi A.T.J. and Benner R. (1984) Influence of 2'-deoxyguanosine upon the development of DTH effector T cells and suppressor T cells in vivo. J. Immunol. 132, 599.

De Bruyn C.H.H.M., Baudoin P, Van den Akker Th.W., Spierenburg G. Th. and Van Laarhoven J.P.R.M. (1983) Enzyme des Purinstoffwechsels in alternden lymphoiden Zellen. Berichte der Österreichischen Gesellschaft für Klinische Chemie 6, 58.

Van den Akker Th.W., Benner R. and Radl J. (1987) Transient monoclonal gammapathies with lambda light chains. Transplantation 44, 725.

Van den Akker Th.W., Bianchi A.T.J., Bril H. and Benner R. (1984) Inhibition of murine suppressor T cell development by 2'-deoxyguanosine in vivo. Adv. Exp. Med. Biol. 165B, 183.

Van den Akker Th.W., Brondijk R.J., Tio-Gillen A.P., Benner R. and Radl J. (1985) Homogeneous Ig components in serum and secretions of aging C57BL/KaLwRij mice. In Protides of the Biological Fluids (ed. by H. Peeters) Vol. 32 pp. 99 -102. Pergamon Press, Oxford and New York.

Van den Akker Th.W., Stuy M.C., Bianchi A.T.J. and Benner R. (1986) Decreased in vivo functional T-cell capacity in the murine autoimmune strains MRL/Mp-lpr/lpr and male BXSB/Mp. Immunobiol. 171, 45.

Van den Akker Th.W., Tio-Gillen A.P., Benner R. and Radl J. (1984) Increased sensitivity of detection of homogeneous immunoglobulins in murine B cell proliferative disorders with special emphasis on the IgA isotype. In Protides of the Biological Fluids (ed. by H. Peeters) Vol. 31 pp. 671-674. Pergamon Press, Oxford and New York.

Van den Akker Th.W., Tio-Gillen A.P. and Radl J. (1985) The influence of T cell infusion on homogeneous immunoglobulins in the sera of athymic nude mice. In MG - Clinical Significance and Basic Mechanisms (ed. by J. Radl, W. Hijmans and B. van Camp) Vol. 5 pp. 213-215. Eurage, Rijswijk, The Netherlands.

Van den Akker Th.W., Van den Enden-Vieveen M., Tio-Gillen A.P. and Radl J. (1985) Genetic influences in the development of murine idiopathic paraproteinemia - a minireview. In MG - Clinical Significance and Basic Mechanisms (ed. by J. Radl, W. Hijmans and B. van Camp) Vol. 5 pp. 209-211. Eurage, Rijswijk, The Netherlands.

Van den Akker Th.W., Ziere G., Gillen A.P., Radl J. and Benner R. (1984) Increased incidence of homogeneous immunoglobulins in irradiated, reconstituted mice after prolonged treatment with 2'-deoxyguanosine. Adv. Exp. Med. Biol. 165B, 193.