Pathogenetic aspects of monoclonal gammopathies
A study in aging mice

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Pathogenetic aspects of monoclonal gammopathies
A study in aging mice

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Een studie in verouderende muizen

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Sapere Aude

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Introduction

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CHAPTER 1

INTRODUCTION

Monoclonal B cell proliferative disorders, when at the differentiation stage of a plasma cell, are often able to produce large quantities of an immunoglobulin (Ig) product. These monoclonal or homogenous Ig components (H-Ig), also called paraproteins or M-components, become detectable in the serum (section 1.1). The monoclonal bands in the gamma-region of the serum protein spectrum has given the name to these B-cell proliferative disorders: monoclonal gammopathies (MG) [1]. This heterogenous group of serologically diagnosed disorders include a wide variety of benign and malignant B-cell proliferations (section 1.2).

1.1 Detection of H-Ig components

Routine assessment of serum protein spectrum abnormalities in hospital and research laboratories is based on electrophoretic techniques. As early as in 1939, Tiselius and Kabat used free boundary electrophoresis for the detection of H-Ig [2]. A major improvement of this technique was the agar-gel electrophoresis on microscope slides, developed by Wieme in 1959 [3,4]. The increased resolving power of his technique enabled him, for the first time, to separate normal human serum into at least 9 fractions: one pre-albumin, one albumin, one α_1-globulin, two α_2-globulins, three ß-globulins and a continuous spectrum of γ-globulins. In a slightly modified form, this technique has been used in this thesis to screen for the presence of H-Ig in the sera. It has a detection limit for H-Ig of about 100 μg/ml. Even more sensitive techniques are needed in experimental settings. Usually a protein blotting technique is used, called immunoblotting. The procedure starts with the agar-gel electrophoresis followed by a protein transfer step to a membrane carrier. The Ig spectrum is further analyzed on this membrane, using specific antibodies or antigens. The detection limit of this technique can be as low as 0.5 to 1 μg/ml [5].

1.2 Classification of monoclonal gammopathies

Several attempts have been made to classify the MG. Based on their possible origin and/or pathogenesis, Radl [6,7] described four major categories of MG (see chapter 2.1, table 1).

Category 1: B-cell malignancies [8]. These include multiple myeloma (MM), Waldenström's macroglobulinaemia (WM), (extra-)medullary plasmacytoma, light chain disease, heavy chain disease, B-cell chronic lymphocytic leukemia (B-CLL) and lymphoma. These disorders are frequently accompanied by the occurrence of Bence-Jones proteins (Ig light-chain complexes) in the urine. The incidences of these B-cell malignancies increase with age.

Category 2: B-cell benign tumor. This common B-cell proliferative disorder is also known as benign monoclonal gammopathy (BMG) or idiopathic paraproteinemia (IP). Like the disorders of the first category, the incidence of BMG increases with age. Many authors regard this entity as an early stage in the development of myeloma [8,9]. This is understandable from the viewpoint that in the early phase of the malignant disease diagnostic parameters may be lacking. The single use of the H-Ig level as a tumor marker is than insufficient to discriminate between benign and malignant disorders. In this phase it is good practice to use the term monoclonal gammopathy of undetermined significance (MGUS). Experimental and clinical data, however, show that BMG may remain stable during many years without progression to malignancy [10-14].

Category 3: MG due to immunodeficiency with T-cell / B-cell imbalance. This group includes rare primary immunodeficiency syndromes (Wiskott-Aldrich, Nezelof, DiGeorge, and SCID) [15], as well as secondary immune disturbances due to aging, immunosuppressive treatment [16], viral infections, malignancies of the immune system other than from B-cell origin [17], or carcinomas [18], and the reconstitution phase after bone marrow transplantation [19-22].

Category 4: MG due to particular antigenic stimulation, as is seen in response to certain haptens and polysaccharides, and in some autoimmune disorders.
Categories 3 and 4 represent generally disorders with low levels of H-Ig that are transient in time. This is in contrast to categories 1 and 2, with high levels of persistent H-Ig.

1.3 Animal model of age-related monoclonal gammopathies

Although a large amount of clinical data is available on MG of neoplastic origin (categories 1 and 2), there is still not much known about the exact etiology of these B-cell proliferative disorders. The possible relationship among the different disorders, the location and the stage in the differentiation pathway of the B-cell at the moment of the neoplastic transformation, and the causes of this transformation are among the questions that asked for a suitable animal model.

It turned out that not only aging humans developed malignant MG and, about a 100 times more frequent, BMG [23-25], but also aging laboratory animals: monkeys [26], dogs, rabbits, rats, and mice. A particularly useful mouse strain is the C57BL/KaLwRij mouse [27-34]. Aging mice of this strain develop spontaneously various monoclonal B-cell proliferative disorders, which are indistinguishable from those that occur in humans. The vast majority of MG are BMG (about 80%), while about 1% of the mice older than two years develop MM or WM.

A BMG in these mice usually becomes manifest by an H-Ig in the serum that is detectable for at least 6 months and mostly until the death of the mouse. The concentration of the H-Ig reflects the size of the plasma cell clone in the bone marrow (BM) and the spleen, and is usually less than 4 mg/ml. As in the human situation, the concentration of the other Ig in the spectrum is not affected and the level of the H-Ig is non-progressive.

Occasionally, a rapid progressive H-Ig develops in an old C57BL mouse, often with concentrations of more than 10 mg/ml. Histopathological examination of the bone and BM shows often the typical picture of MM. X-ray analysis of the skeleton of these mice can even show the typical osteolytic bone lesions of MM.

The first intriguing information from this mouse model came from transplantation experiments [35]. After transplantation of BMG clones into young syngeneic mice these recipient mice developed the same H-Ig as the donor mice. This result indicated that BMG represents an intrinsic B-cell abnormality that leads to continued proliferation of the plasma cell clone. Within the young recipient mice, the BMG clones maintained their non-progressive character. However, the engraftment or "take" frequency in subsequent transplantations of the same BMG clone decreases, and propagation of a BMG clone for more than three or four generations was never achieved. This is in sharp contrast with the results of mouse MM and WM. Both are continuously transplantable, with a take frequency of approximately 100% and a progressive development of a paraproteinemia. The latter always caused the marked shortened survival-times of the affected mice.

Thus, although BMG, MM, and WM cell clones proliferate after transplantation, BMG cells are not immortal, while those of the B-cell malignancies are.

The immortal character of the C57BL/KaLwRij mouse MM and WM resulted in a rich myeloma- and macroglobulinemia cell bank with BM cells, spleen cells and cells from lymph nodes of the mice that were engrafted with earlier generations of the malignant mouse MG. This so-called 5T series of mouse B-cell tumors is therefore easily accessible for research purposes.

1.4 Introduction to the experimental work

B-cell lineages

The first part of this thesis addresses the question whether B-cell lineages are differentially involved in the development of age-related monoclonal B-cell proliferative disorders. The T-cell differentiation antigen CD5 has been found on B-cells in the peritoneal and thoracal cavities. Demonstrated by adoptive transfer experiments, these B-cells comprise a separate compartment of self-replenishing B-cells: the B-1 lineage [36-38]. An introduction to the B-cell subsets in the peritoneal cavity will be given in Chapter 2.1. This short overview of the B-1 cell phenotype and other cell characteristics also addresses the question whether the B-1 cells have a distinctive role in the development of MG. Although CD5 expression has been found on the malignant cells of B-CLL in humans and in certain mouse B-cell lymphomas and cell lines [39], no information was available on the lineage origin of the more mature B-cell proliferative disorders (BMG, MM, WM). Two experimental approaches have been used to investigate the latter question.

First, a life-long follow-up study of μ,κ-transgenic Sp6 mice [40] was started. In these mice, peripheral B-cells expressed exclusively the transgenic IgM of μ^a allotype. Only a small proportion of B-cells showed endogenous μ^b-allotype expression; these B-cells possessed a B-1 phenotype, were especially enriched in the peritoneal cavity, and could not fully reconstitute endogenous IgM-positive B-cells in irradiated recipient mice. Therefore it was concluded that the B-1 cells in these transgenic mice were the producers of endogenous Ig isotypes [41]. Determination of the isotype (and allotype in case of IgM) of the H-Ig that developed during aging should give insight into the involvement of the B-1 cell lineage in the development of MG. This study is described in Chapter 2.2.

Second, we used IgH allotype-congenic C57BL mouse chimeras in

order to be able to evaluate the H-Ig that originated from the B-1 cell lineage separately. Lethally irradiated mice were reconstituted with BM cells and IgH allotype congenic peritoneal wash cells. The peritoneal cells provided for the B-1 cells [42-45]. The H-Ig of aging C57BL mice originating from the B-1 cell lineage in the peritoneal cavity could be discriminated from the BM derived B-2 cell Ig product by allotype difference, and is described in detail in Chapter 2.3.

Clonal dominance of specific antibody responses

Immunization of mice with carbohydrates or certain other antigens with a limited number of different antigenic determinants frequently elicits an antibody response that shows markedly restricted heterogeneity [46]. Using isoelectric focussing (IEF), the specific antibodies can be divided into discrete bands, the so-called spectrotypes or clonotypes. This is a sensitive technique to show clonality at the protein level [47]. The spectrotype of the highest concentration is thus regarded as the dominant clone. Furthermore, recurrent spectrotype patterns between individuals might indicate a favourable and therefore dominant antibody response.

Since clonal dominance seems to be a physiological phenomenon in antibody responses, the question arises whether a control mechanism is present, and if so, does disruption or impairment of this control mechanism facilitate the development of even bigger dominant B-cell clones? The Ig secreted by these bigger clones might than become detectable in the serum as a transient H-Ig or even a persistent one.

We investigated the effects of age, genetic background (CBA and C57BL mouse strains), and impaired T-cell system (neonatal thymectomy) on the levels and the heterogeneity of the specific antibody response to dinitrophenylated human serum albumin (DNP-HSA). The individual parameters have been shown to be involved in the development of MG: persistent H-Ig is age-related, and aging C57BL strains are especially sensitive to development of MG, whereas CBA mice have a low incidence of these B-cell proliferative disorders (section 1.3). Furthermore, a normally functioning T-cell system is essential for the generation of a normal heterogenous Ig spectrum [16,48-51]. Whether the idiosyncrasy of an individual to respond to an antigen with a restricted set of clonotypes correlates with the tendency to develop MG is studied in Chapter 3.

Immunosuppressive treatment and the incidence of MG

The number of patients that are treated with immunosuppressive drugs for a prolonged time increases steadily. This treatment is especially indicated after solid organ transplantation and in some autoimmune disorders. Short term evaluation studies have shown a markedly increased incidence of lymphoproliferative disorders, most of them B-cell neoplasms associated with Epstein-Barr virus (EBV) [52-54]. Terminal differentiation to plasma cells in these B-cell malignancies is rare, and is only published as case reports [reviewed in 55]. Information on the late effects in life-long treated individuals is scarce. Preliminary data suggested that the increased frequencies of MM and BMG in kidney recipients were more related to the higher age of the individual than to the duration of the immunosuppressive treatment [16].

In Chapter 4 we report a study of two widely used treatment protocols (azathioprine/prednisolone and Cyclosporin A/prednisolone) applied to young (4 to 5 months old) and adult (14 months old) C57BL/KaLwRij mice. The aim of this study was to investigate the influence of long-term maintenance immunosuppressive treatment on the incidence of age-associated monoclonal gammopathies: MM, WM, and BMG. Extensive postmortem histopathological examination of the mice was required for the definitive diagnosis and the evaluation of other pathological effects of the treatment protocols.

Immunoglobulin V_H gene sequence analysis of spontaneous mouse MG

The specificity of an Ig-molecule for an antigen is made up by three complementarity-determining regions (CDR) in the variable (V) regions of the heavy (H) and light (L) chains. Each B-cell expresses a single and unique combination of these CDR that in total forms the Ig repertoire of an individual. The V-regions are responsible for binding of the antigen, while the constant (C) regions determine the isotype or (sub)class, *i.e.* κ and λ light chains, and (in the mouse) μ, δ, $\gamma 1$, $\gamma 2a$, $\gamma 2b$, $\gamma 3$, ϵ, and α heavy chains. At the molecular level, V-regions of the L-chains are encoded by two gene segments: the V_L (variable) genes and the J_L (joining) genes. V-regions of H-chains are encoded by V_H, D_H (diversity), and J_H genes. The germ line V_H and V_L genes each encode two of the CDR, while the third (CDR3) is generated when the forementioned gene segments are rearranged.

Several mechanisms contribute to the diversity of antibodies, such as many different genes of the V-, D-, and J-families, junctional diversity by imprecise joining of the individual gene segments and random insertion of nucleotides, combination of the H- and L-chains in the complete Ig molecule,

and somatic hypermutation. Antigen-driven somatic mutation in the germinal centers and subsequent clonal selection results in the production of Ig with higher affinity for the antigen [56-61].

These mechanisms that generate antibody diversity act in subsequent steps of the B-cell differentiation pathway. Thus the presence of somatic mutations tells about a former encounter of the antigen. Sequence analysis of V-regions of MM Ig in humans showed that there were replacement mutations clustered in CDR regions in either V_H or V_L [62-64]. This can be regarded as evidence for antigen selection of the corresponding myeloma cell clone. Intraclonal variation was not detected in human MM, indicating that the malignant plasma cells are no longer able to accumulate further mutations. However, a proportion of cases of MGUS (quite often they turn out to be a BMG) did show intraclonal variation and therefore these tumor cells may still be under the influence of the somatic mutation mechanism [63].

In Chapter 5 we report the sequences of Ig V_H genes of spontaneously developed mouse MM (5T2, 5T7, 5T13, 5T14, 5T33) and WM (5T10, 5T16) lines, and of one biclonal BMG. Their sequences were analyzed in comparison with human disease counterparts in order to reveal the role of antigenic selection of the precursor cells of MG in the C57BL/KaLwRij mouse model. Furthermore, the intraclonal variation of the mouse tumors could be studied. Information on these subjects should give insight into whether the mouse MG model is equivalent to human age-related MG.

1.5 Aim of the experimental work

In **Chapter 2** the question is addressed whether the B-1 and B-2 cell lineages are differentially involved in the development of age-related monoclonal B-cell proliferative disorders. Two experimental approaches have been used. First, a life-long follow-up study of μ,κ-transgenic mice was started. B-1 cells in these mice were the producers of endogenous Ig isotypes, and therefore MG originating from the B-1 cells produce endogenous Ig. Second, we used IgH allotype-congenic C57BL mouse chimeras. The H-Ig of aging C57BL mice originating from the B-1 cell lineage in the peritoneal cavity could be discriminated from the BM derived B-2 cell Ig product by allotype difference.

In **Chapter 3** we investigated the effects of age, genetic background, and impaired T-cell system on the levels and the heterogeneity of the specific antibody response to dinitrophenylated human serum albumin (DNP-HSA).

In **Chapter 4** we investigated the influence of long-term maintenance immunosuppressive treatment on the incidence of age-associated monoclonal

gammopathies: MM, WM, and BMG.

In **Chapter 5** we report the sequences of Ig V_H genes of spontaneously developed mouse MM, WM, and of one biclonal BMG. Their sequences were analyzed in order to reveal the role of antigenic selection of the precursor cells of MG in the C57BL/KaLwRij mouse model, and to give insight into whether the mouse MG model in this respect is equivalent to human age-related MG.

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B-cell subsets in monoclonal gammopathies 2

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CHAPTER 2.1

B-CELL LINEAGE INVOLVEMENT IN AGE-RELATED MONOCLONAL B-CELL PROLIFERATIVE DISORDERS *

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SUMMARY

The B-1 or B-2 lineage origin of monoclonal B-cell proliferative disorders is of interest but cannot easily be studied directly in humans; however, this is possible in experimental animal models that became available only recently. A short review of the literature relevant to such a study involves some recent data on the four major categories of monoclonal gammopathies (MG), on the phenotypes, biological features and on the behavior of the two B-cell lineages. Two examples of suitable experimental approaches are given.

INTRODUCTION

The immune system in aging individuals changes in several aspects. Thymus involution, appearance of $CD8^+$ T-cell clones of restricted heterogeneity in peripheral blood (PB) [1,2] and, with respect to the B-cell system, restriction of immunoglobulin (Ig) heterogeneity and appearance of monoclonal Ig components (M-Ig) in the serum are the most remarkable phenomena appearing in the aging immune system [3,4]. To what extent these changes reflect a decline in the immune function is not exactly known, but it seems plausible that the increased levels of autoantibodies, higher incidences of infections and lymphoid neoplasias in aging individuals are consequences of the hampering immune system.

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Recent findings indicated the existence of different B-cell lineages, designated B-1 and B-2 cells (reviewed in 5 and 6). In order to understand better the mechanisms by which the age-related monoclonal B-cell proliferative disorders develop, the cell origin of individual B-cell neoplasias became of interest. In this short review, we will focus on age-related monoclonal B-cell proliferative disorders and the possible pathogenetic role of B-cell subsets.

B-CELL PROLIFERATIVE DISORDERS: MONOCLONAL GAMMOPATHIES

An antigenic stimulation in healthy individuals initiates the activation of several B-cell clones, all specifically reactive to different antigenic determinants or epitopes. In this process often a dominance of one or a few B-cell clones occurs. The homogenous antibody component in the serum, produced by such relatively small dominant clones (less than 10^7 cells) can be detected only by very sensitive techniques, such as antigen-specific immunoblotting [7]. The sum of these homogenous antibody components usually gives a heterogenous (polyclonal) pattern, and no abnormal homogenous Ig components are detected by routine laboratory techniques. However, some autoimmune processes or T-cell independent antigenic stimulation on a certain genetic background may lead to a larger clonal expansion with a concentration of the monoclonal Ig in the serum of more than 0.5 mg/ml (category 4, Table 1). In these conditions, no intrinsic B- and T-cell defects have been detected [3].

Some T-cell dysfunctions and immunodeficiencies frequently give rise to larger monoclonal B cell expansions. An impaired T-cell regulation seems to be typical for aging of the immune system. Iatrogenic immunosuppression may cause a comparable T/B cell imbalance and subsequently give rise to a high incidence of MG [8]. Furthermore, MG of this category (category 3, table 1) can be observed in children and adults with some primary and secondary immunodeficiencies [9,10] and following bone marrow transplantation (BMT) [11]. Similar changes may appear within the T-cell system. In this respect, two recent findings of the restriction of the heterogeneity of the peripheral CD8+ T cell pool in aging mice and humans are of special interest [1,2]. MG that develop by these two mechanisms represent in general transient proliferative disorders of normal B cells, in contrast to the next two categories of autonomous, neoplastic proliferative disorders.

Benign monoclonal gammopathy (BMG) (category 2, table 1) is a

monoclonal proliferation of mature plasma cells without malignant characteristics of multiple myeloma, with production of a M-Ig of usually less than 2.5 mg/ml in man. Genetic susceptibility, T-cell dysfunction and long-lasting thymus dependent antigenic stimulation, all contribute to the genesis of this essentially benign and age-related proliferative disorder [reviewed in 3,4,12 and 13].

Malignant forms of MG are about 100 times less frequent than BMG. To this category (category 1, table 1) belong multiple myeloma (MM), (extramedullary) plasmacytomas, Waldenström's macroglobulinemia and some other non-Hodgkin lymphomas, heavy and light chain disease [3,4,15]. B-cell tumors, usually without a distinct monoclonal immunoglobulin serum com-

Table 1. Four major categories of monoclonal gammopathies.

Category	Condition
1. B-cell malignancies [a]	a. multiple myeloma (MM), Waldenström's macroglobulinemia b. plasmacytoma, lymphoma, B-CLL, heavy and light chain disease
2. B-cell benign proliferations [a]	benign monoclonal gammopathy (BMG)
3. Immunodeficiency with T/B immune system imbalance	a. primary: Wiskott-Aldrich, Nezelof-, DiGeorge-syndromes b. secondary: 1. due to aging [a] 2. immunosuppressive treatment 3. immune system malignancies other than of B-cell origin 4. acquired, viral, idiopathic c. reconstitution of the immune system after bone marrow transplantation (SCID, aplastic anemia) d. early ontogenesis with excessive antigenic stimulation
4. Homogenous antibody response	Due to particular antigenic stimulation a. excess stimulation with polysaccharides, haptens b. autoimmune disorders

[a] Categories 1, 2, and 3b1 are age-related (together 90% of all MG).

ponent, are leukemias and lymphomas. Within these groups we can distinguish a rapid growing and aggressive group of tumors, e.g. B-cell acute lymphocytic leukemia (B-ALL) and Burkitt's lymphoma, and more indolent, low grade tumor forms, e.g. B-cell chronic lymphocytic leukemia (B-CLL, which is the most common B-cell tumor in humans) and follicular lymphomas.

Animal model of MG

Animal models have been developed to facilitate the study of B-cell neoplasia development in vivo [16-18]. The aging C57BL/KaLwRij mouse appeared to offer an excellent model of B-cell proliferative disorders, strongly resembling those of human origin [13-15]. These mice spontaneously develop in a high frequency BMG and follicular center cell lymphomas and, less frequently like in the human situation, typical MM and WM. In this model, it was shown that BMG did not represent a premalignant condition of MM or WM. By transplantation of BM or spleen cells to unmanipulated recipient mice, the BMG clone could be propagated for maximally three to four generations and with decreasing take frequency. This is in contrast to malignant forms, which can be propagated indefinitely. Thus, the clonogenic cell of BMG differs substantially from that of B-cell malignancies by remaining mortal.

B-CELL LINEAGES

Initiated by the discovery of Ly-1$^+$ B-cells in mice and CD5$^+$ B-cells in humans the search for the pathophysiological role of this CD5$^+$ B-cell subset in the immune system became of interest [5,6,19-21]. The majority of data were obtained from *in vivo* and *in vitro* experiments, performed in mice.

According to phenotype, two B-cell subsets can be distinguished: B-1 (with B-1a and B-1b subpopulations) and B-2 cells (table 2) [22]. This nomenclature implies a different origin of these B-cell subsets. However, this is still a matter for discussion because some authors believe that antigenic stimulation and subsequent selection of cells are responsible for the dichotomy of B-cell subsets (for excellent discussion, see references 23 and 24).

The B-1 cells are preferentially located in the coelomic cavities - the peritoneal and thoracal cavities - between which there is a free migration of cells [25]. No other preferential localization is known. Thymic B cells have a B-1 cell phenotype [26], but are probably not of B-1 origin [27]. In low frequencies, B-1 cells are resident in the spleen. It is not known whether there

is a migrational route via peripheral blood (PB) to or from other organs, especially the bone marrow, although in some autoimmune diseases, such as primary Sjögren's Syndrome and rheumatoid arthritis [28], increased frequencies of these cells can be detected in PB, as is also obvious in autoimmune NZB mice [29]. Their role in pathogenesis is not clear, however.

Adoptive transfer studies, performed by various research groups, of embryonic splanchnopleura and fetal omentum in lethally irradiated or SCID recipient mice revealed that in these organs only B-1 cell precursors are present. Fetal liver reconstitutes both the B-1 and B-2 cell population, but adult bone marrow can only fully reconstitute the B-2 cell population [19-21,30,31]. These findings form the basis of the lineage theory, which implies that B-1 and B-2 cells are derived from different precursor cells, located in adult life in different organs and tissues [23].

B-cells of the B-2 lineage are readily available for investigation because they are abundant in bone marrow, spleen and peripheral blood. Most of the scientific data concerning B-cells in general are related to these cells. Less is known about the B-1 lineage, on which we focus in the next section.

Characteristics of the B-cell lineages

The biological function of the CD5 and CD11b membrane markers of the B-1 cell population is unknown. CD11b belongs to the family of adhesion molecules and it is very likely that this property is also preserved in B-1 cells. CD5 is associated with the immunoglobulin receptor [32], the membrane bound IgM-Igα/ß (CD79a/CD79b) complex, and may very well act, in conjunction with its ligand CD72 [33], as a modifier of intracellular signals after antigen binding by the IgM receptor [34,35].

Various *in vitro* experiments showed that CD5- B cells can be induced to express CD5 (by phorbolmyristateacetate (PMA) [36], anti-IgM [37] or *Staphylococcus aureus* Cowan strain I antigen [38] activation), but whether this is a functional and lasting expression is unknown. Zupo *et al.* found that only a distinct subset of human tonsillar CD5- B-cells could be induced to express CD5 by PMA and that these cells coexpress the activation marker CD38. This was prevented by T cell contact alone or by rIL-4 [39]. These inducible CD5+ B-cells may be the human analogues of the murine B-1b cells [40].

In B-2 cells, the mechanism of somatic mutation is responsible for so called affinity maturation. The B-1 cell population is characterized by a restricted usage of V_H immunoglobulin genes, with a relative resistance to

Table 2. B cell phenotypes in B-1 and B-2 nomenclature

	B cell types/lineages		
Markers	B-1a	B-1b	B-2
sIgM	+++	+++	+
sIgD	+	+	+++
CD5 (Ly-1)	+	-	-
CD11b (Mac-1α)[a]	+	+	-
IL-5R	+	+	±
CD72	+	+	++
CD45R (B220)	+	+	++

[a] Splenic B-1 cells are CD11b-negative.

somatic mutations as compared to B-2 cells [41-43]. Thus, it is not surprising that the antigenic specificity of B-1 cells is also restricted and biased to usually polyreactive activity, with low affinity against autoantigens and bacterial antigens. In addition to the production of multispecific Ig, a relatively large amount of IL-10 is secreted by peritoneal B-1 cells [44]. This suggests their regulatory role by inhibiting T_H1 reactions. Furthermore, IL-10 probably functions as an autocrine growth factor for B-1 cells, as anti-IL10 treatment selectively depleted the B-1 cell population in the peritoneal cavity [45].

Focussing on the physiological behavior of B-1 cells during life, it becomes apparent that their representation in the total B-cell pool shows two peaks. During fetal life and perinatally, the proportion of B-1 cells is relatively high, decreasing thereafter because of the expansion of the B-2 cell pool, which will remain the largest population of B cells throughout life [46]. However, in aging mice, the peritoneal B-1 cell population expands, whereas the V_H gene usage becomes more restricted. These emerging B-1 clones can even be detected in the spleen [29]. This is illustrated also by the observation of a selective increase in the phosphorylcholine specific B cells in aging mice, which is a typical B-1 cell specificity [47], although no B-1 cell data were collected in that study [48]. Recently, it appeared that sex-associated phenomena may play an important part in the homeostasis of this cell population. It was found that B-1 cells decreased in aged male mice and increased in aged female mice. B-1 cell responsiveness to mitogens and antigens is probably decreased in all aged mice [49,50].

INVOLVEMENT OF B-CELL LINEAGES IN DEVELOPMENT OF MG

The lineage origin of some B-cell neoplasias is known, e.g., the B-1 cell origin of $CD5^+$ B-CLL and the B-2 cell origin of follicular-center cell lymphoma. However, the lineage origin of the majority of B-cell proliferative disorders, including disorders that give rise to MG, is unknown. Different mechanisms may be responsible for abnormal clonal restrictions and expansions within B-cell populations. Most likely, multiple causes should be considered.

1. Because of a limited number of B-1 precursor cells and no supplementation by BM resident precursors, the B-1 cell pool becomes clonally restricted in time. This also implies that no significant number of somatic mutations occurs in the descendants of these long-living precursors, and that some clones are able to outgrow all others, or that most clones disappear and the remaining cells have to fill in the cellular gap by expanding (not for any other purpose) their cell number. The feedback regulatory mechanisms play an important role in the homeostasis of the B-1 cell population [51]. Independence from the BM of the B-1 cell population and longevity of their precursors in coelomic cavities, clonal restriction, and clonal expansion increasing with age all may subsequently lead to an increased chance of neoplastic transformation [29].

2. Repeated or prolonged antigenic stimulation throughout life may be a good reason for clonal expansions of both B-1 and B-2 cells. In that case, clonality may have a purpose. It is possible that autoantigens play a major role in this process in the case of B-1 cells, as is suggested by the production of large amounts of natural (auto)antibodies. However, autoantigens are present in abundance during the whole lifetime, whereas the B-1 cell clones become enlarged, but restricted in number, during aging only. Other unknown antigenic stimulations, possibly initiated at the respiratory and gastrointestinal mucosa, may be more important in inducing clonal expansions. A recent study by Murakami *et al.* showed in an autoantibody transgenic mouse model that orally administered LPS activated B-1 cells in the lamina propria of the gut, as well as in the peritoneal cavity. Systemic injection of LPS did not result in B-1 cell activation [52]. These findings imply that these B-1 cells readily migrate between both locations. The work of Kroese *et al.* leads to similar conclusions [53]. Together with the B-2 cells in Peyer's patches, the B-1 cells in the lamina propria take part in gut-associated lymphoid tissue (GALT) and possibly also in bronchus-associated lymphoid tissue (BALT). Their exact function in mucosal immune reactions have to be elucidated. Results of experiments in C57BL/Ka mice suggest that memory B-cells (the B-2 lineage) are involved in the development of BMG and MM.

3. In addition to these mechanisms, insufficient T-cell help may reduce the numbers of responding B-cell clones, and on the other hand, loss of T-cell suppression would allow excessive expansions of B-cell clones that did respond to certain (antigenic) stimuli. This mechanism may act especially in MG of B-2 cell origin because of the fact that in most B-2 responses T-cell help and control plays a major role. Responses of B-1 cells (e.g., to polysaccharides) may not need such a T-cell interference.

Independent of the mechanisms that are responsible for the development of B-cell proliferative disorders, the antigenic specificity and structural properties of M-Ig may be helpful in defining subgroups of B-cell neoplasias.

1. B-cell neoplasias with relatively few somatic mutations, as seen in $CD5^+$ B-CLL, Burkitt's lymphoma, WM, and some BMG. Most of the M-Ig with known antibody specificity were shown to be autoantibodies [16,54-56]. These tumors may very well be of B-1 cell origin.

2. B cell neoplasias with antigen-selected somatic mutations in V_H immunoglobulin genes (as in MM, CD5- B-CLL, hairy cell leukemia and possibly some BMG) [28,54,55, 57], or with ongoing somatic mutations as in follicular lymphomas. Most likely these tumors originate from B-2 cells.

An additional point should be considered. The CD5 expression alone on B-cell neoplasias must be interpreted carefully because it is not known whether neoplastic transformation can induce or inhibit the expression of CD5. However, in addition to other B-1 cell characteristics, such as antigenic specificity of secreted Ig and V_H gene family usage and mutational status of the complementarity determining regions (CDR), the expression of CD5 and CD11b can help to reveal the origin of B-cell tumors [28,41,54,55].

EXPERIMENTAL APPROACHES

To test whether B-1 or B-2 cells give rise to certain B-cell proliferative disorders with different frequency and characteristics, a murine model can be helpful. Allotype congenic chimeras, in which bone marrow-derived B-2 cells and peritoneal cavity resident B-1 cells are of different IgH allotype, enable the study of the two separate B-cell lineages in the same animal [58]. Alternatively, μ-κ transgenic Sp6 mice can be used as an experimental model. In these mice, B-2 cells express only the transgenic IgM on their cell membranes, while B-1 cells coexpress endogenous IgM molecules [59]. The M54 μ heavy-chain transgenic mouse model has been studied for the development of age-related MG by Gueret *et al.*, and showed a profound

effect of the transgene on the development of M-Ig, which were always of endogenous origin [60]. However, results from transgenic model experiments have to be interpreted carefully because the entire B-cell system may have been altered. In those animals, e.g., the peripheral B-cell number is remarkably reduced [62,63].

Elucidating the lineage origin of MG may help to better understand the mechanisms of their development, to improve the differential diagnosis of the underlying disorders, and possibly to provide us with new means of treatment of the health- or life-threatening forms of MG.

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2.2

CHAPTER 2.2

MONOCLONAL GAMMOPATHIES IN AGING μ,κ-TRANSGENIC MICE: INVOLVEMENT OF THE B-1 CELL LINEAGE *

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SUMMARY

Monoclonal gammopathies (MG) are monoclonal proliferative disorders of B cells at the differentiation stage of Ig production. They can be detected in the serum, either as transient or as persistent homogenous Ig components (H-Ig). The exact phenotype, localization, and cell lineage origin of the precursor cells of MG are unknown. In the μ,κ-immunoglobulin (Sp6) transgenic (Tg) mice a small proportion of B cells still can produce endogenous IgM. It has been shown, by virtue of their phenotype and localization, and in adoptive transfer experiments, that these cells are of B-1 cell origin. We studied aging μ,κ Tg mice for their ability to develop MG. It can be assumed that MG, producing an Ig of endogenous isotype, originated from the B-1 cells.

The Tg mice showed a stable expression of the transgene during aging. Male Tg mice survived 20 weeks shorter than littermate control mice. This could be attributed to more intercurrent acute inflammatory diseases in Tg mice. In the Tg mice Ig serum levels were decreased as compared to controls, except for IgG2b and IgM. The MG in Tg mice showed a later onset and a lower frequency than those in littermate control mice. The 10% of B cells that were able to produce endogenous Ig led to the development of MG in a frequency that was half the number of MG found in normal littermates. None of the MG in Tg mice produced an Ig of the Tg origin and therefore it can be concluded that they originated from B-1 cells. These results show that, in the presence of a genetic susceptibility to develop MG, B-1 cells in mice are frequently involved as precursor cells of transient monoclonal expansions of B cell clones as well as of neoplastic B cell proliferative disorders.

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INTRODUCTION

The competence of the immune system is greatly affected by aging of the individual. Changes in both B and T cell systems are responsible for the age-related increase in levels of autoantibodies, higher incidences of infections and the development of various neoplastic diseases. One of the most striking manifestations of aging of the immune system is the development of excessive monoclonal B cell proliferations [1]. They are often detectable in the sera by electrophoresis as homogenous or monoclonal immunoglobulin (H-Ig) components, therefore designated as monoclonal gammopathies (MG). Transient MG often accompany imbalances between the B and T cell systems. Persistent MG can either be benign or malignant and both categories are age-related neoplastic proliferative disorders of immunoglobulin (Ig) producing plasma cells, mainly localized in the bone marrow (BM). Benign monoclonal gammopathies (BMG) are much more frequent than the malignant MG, *i.e.* multiple myeloma, Waldenström's macroglobulinemia and (extra-)medullary plasmacytoma. Influences of T cell function, antigenic stimulation and genetic background on MG development have been reported earlier in mice as well as in man [2,3,4]. The question which cell becomes the target for the events leading to the development of benign and malignant B cell proliferation disorders may be crucial for both the correct diagnosis and for timely efficient treatment of the malignant forms.

Based on cell surface phenotype, anatomical localization, function and origin, B cells in mice can be divided into two lineages: the predominantly in thoracal and peritoneal cavities localized B-1 cells, expressing the CD11b molecule (B-1b cells), often in conjunction with CD5 expression (B-1a cells), and the conventional, bone marrow derived B-2 cells, which express neither CD11b, nor CD5. B-1 cells appear early in foetal life and migrate to the peritoneal and pleural cavity, where they maintain themselves as a self-renewing IgM-positive population. B-2 cells arise later and are continuously renewed by newly formed bone marrow precursor cells [5].

We used for the first time unmanipulated aging μ,κ-transgenic Sp6 mice [6] on C57BL background to investigate the incidence of MG in aging Tg mice and the B cell lineage origin in the development of MG. It was shown by Kroese *et al.* [7] that the majority of B cells in the periphery and in the BM of B6-Sp6 mice expressed exclusively transgenic IgM, bearing the μ^a allotype. However, a small proportion of B cells (about 10% of spleen cells) showed endogenous IgM (μ^b) expression, usually concomitant with transgenic IgM. These cells possessed a B-1 phenotype: expression of CD11b, low levels of (endogenous) IgD, and most cells also expressed CD5. Furthermore, they

were especially enriched in the peritoneal cavity. BM of the transgenic (Tg) mice could not fully reconstitute endogenous IgM positive B cells in irradiated recipients. From this experiment it was concluded that endogenous isotypes in Sp6 mice were produced by B-1 cells. We therefore assumed that B cell proliferative disorders arising from the B-1 cell population in these aging Tg mice would result in an H-Ig of endogenous isotype.

We found a stable expression of the transgene throughout the life-span of the Tg mice. Serum Ig levels were lower in Tg mice than in littermate (LM) controls. The frequency of H-Ig components in the serum of Tg mice was half of that in LM controls and they developed at a later age. These H-Ig were of endogenous isotypes and none of these produced the Tg μ^a allotype. Our findings suggest that the B-1 cell lineage can be responsible for at least a part of the MG in mice.

MATERIALS AND METHODS

Mice

Sp6 transgenic male mice on C57BL/6 background (designated B6-Sp6) were originally provided by Dr M.C.Lamers (Max Planck Institute for Immunobiology, Freiburg, Germany). They were backcrossed once with C57BL/KaLwRij female mice from the breeding stock of TNO-Prevention and Health, Leiden, The Netherlands and kept under conventional conditions in the animal housing of the Department of Histology and Cell Biology, University of Groningen. After one year they were transferred to the animal facilities of the Erasmus University Rotterdam and kept in isolators under positive pressure isolation.

The μ,κ-transgene originates from the BALB/c (Igh-a allotype) derived Sp6 hybridoma, which produces an antibody that specifically binds to TNP [6]. Backcrossing on C57BL Igh-b allotype background facilitated the detection of the transgenic IgM (Igh-6a) by allotype specific anti-IgM antibodies.

The C57BL/KaLwRij mouse strain has been established as a suitable animal model for research on MG, based on the relatively high frequency of spontaneously developing MG of all categories at old age [8]. The one time backcross of the Sp6-B6 mice with the C57BL/KaLwRij mice was useful to introduce at least some of the genetically determined characteristics for susceptibility to the development of MG.

The Tg phenotype was identified by ELISA, using coated DNP-BSA as the relevant antigen for transgenic IgM. Binding of Tg encoded antibody was revealed by goat anti-mouse (GaM) IgM conjugated to alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL). Control mice were LM of the Tg animals. Every 2-3 months a small blood sample was taken from the tail veins of 58 unmanipulated Tg mice (35 male, 23 female) and 68 LM (37 male, 31 female), starting at the age of 3.7-10.5 months and continued till the end of their lives. Seriously ill or moribund animals were terminated by carbon dioxide inhalation and submitted to necropsy. Microbiological evaluation of the animals showed no deviations from the established flora during the experiment.

Histopathological examination

Complete necropsy and histopathological examination was performed on 36 LM controls (24 males, 12 females) and 28 Tg mice (21 males, 7 females) according to a standard protocol [9]. All tissues were fixed with B5-fixative. The sections were stained with hematoxilin-phloxin-saffron. Examination of the sections was carried out without prior knowledge of the genetic constitution of the mice that corresponded to the tissue sections.

Ig-isotype ELISA

Microwell plates with high binding affinity (Greiner, Alphen aan de Rijn, The Netherlands) were coated overnight at 4 °C with 50 μl of diluted GaM-IgG1, GaM-IgG2a, GaM-IgG2b, GaM-IgG3, GaM-IgA, or GaM-IgM (7S-Ig fractions, Nordic Immunological Laboratories, Tilburg, The Netherlands) at a concentration of 10 μg/ml. The plates were washed six times with PBS, containing 0.05% Tween-20 (polyoxyethylene-sorbitan-monolaurate; Sigma, St.Louis, MO) (PBS-Tw), using the Scanwasher 300 version A (Skatron Instruments, Costar Europe Ltd, Badhoevedorp, The Netherlands). The wells were blocked with 300 μl PBS-Tw with 0.5% BSA (PBS-Tw-BSA; Fraction V, Sigma, St.Louis, MO) for 30 minutes at room temperature and washed again. Fifty μl of diluted samples and standards in PBS-Tw-BSA were added to each well and the plates were incubated for 2 hours at room temperature. On each plate three subsequent dilutions of a commercially available calibrated normal mouse serum (Nordic Immunological Laboratories) were treated as samples to serve as a positive isotype-specific control. After washing, 50 μl of a 1:250 dilution of alkaline phosphatase (AP) conjugated GaM-IgA-IgG-IgM (Kirkegaard & Perry Laboratories, Mutacon B.V., Leimuiden, The Netherlands) were added to the wells. The plates were incubated for 1 hour at room temperature. After washing the wells, 50 μl of a substrate solution (*p*-nitrophenylphosphate 1 mg/ml in a 1 M diethanolamine, 0.05 M $MgCl_2$ buffer (pH 9.8)) was added to the wells and incubated for approximately 40 minutes at 37 °C. The absorbance was read at 405 nm using a Bio-Rad Model 3550 Microplate Reader (Bio-Rad, Veenendaal, The Netherlands). The IgM-allotypes were measured using plates coated with RS3.1 and MB86 monoclonal mouse-anti-mouse Igh-6a and Igh-6b, respectively (kindly donated by dr A.Coutinho, Institut Pasteur, Paris), and GaM-IgM-AP (Kirkegaard & Perry Labs.) as a detecting antibody.

Two serum pools, one from CBA mice (Igh-a) and one from C57BL mice (Igh-b), were calibrated to be used as secondary allotype- and isotype-specific standards. Therefore affinity-chromatography purified mouse paraproteins of appropriate isotype and allotype were used as primary standards. The protein concentrations of the primary standards were measured using the Bio-Rad Protein-assay (Bio-Rad) and subsequently used to construct a standard curve for calibration of the standard sera. Concentrations of the individual sample dilutions were calculated using the calibrated standard serum. To minimize variability we analyzed the sera derived from a single mouse on the same plate. Per isotype all samples were tested on the same day.

Detection of homogenous Ig-components

Sera were investigated for the presence of H-Ig by a slightly modified high resolution agar electrophoresis according to Wieme [10,11]. Briefly, 8.2 x 8.2-cm glass plates were covered with 12 ml of 0.9% Agar Noble (Difco, Detroit, MI) in IEP buffer, pH 8.6, containing 10 mM 5.5'-diethylbarbituric acid and 50 mM sodium barbital (Merck, Darmstadt, Germany).

Each slit was filled with 4 μl of serum. Electrophoresis was performed at 190 V and 80 mA for 15 min with the agar plate completely submerged in petroleum ether (40-60 °C; BDH, Poole, U.K.) to prevent overheating of the gel. The proteins in the gels were fixated by 30 min soaking in a glacial acetic acid and methanol mixture (1:10). The gels were dried, covered with wet filter paper, in a stove at 60 °C, and colored with Amido Black /Thiazine Red in fixating solution for direct assessment of the serum protein spectrum.

Electrophoresis of the sera in dilutions 1:50 and 1:100 were used for the immunoblotting of H-Ig. Serum proteins were transferred from the agar by diffusion under pressure for 30 min onto polyvinylidene difluoride (PVDF) membranes (Immobilon; Millipore, Bedford, MA). Unoccupied protein binding sides on the blots were then blocked with PBS-0.05% Tw-1% BSA (Boseral DEM; Organon Tecnica, Oss, The Netherlands) by incubating 60 min at room temperature. After 90 min incubation with peroxidase conjugated isotype specific antibodies and subsequent washing, the blots were stained with a freshly prepared substrate solution of 0.02% (w/v) 3,3'-diaminobenzidine tertahydrochloride (DAB; Sigma, St.Louis, MO) and 0.03% (v/v) hydrogen peroxide (Perhydrol; 30% H_2O_2, Merck, Darmstadt, Germany) in PBS-Tw. The enzymatic reaction was stopped after 10 min by rinsing the blots in distilled water.

Since IgD is normally not detectable in the sera of mice [12], we first screened undiluted sera using the Ouchterlony immunoprecipitation technique in agar plates. As a positive control we used ascites from the TEPC1017 plasmacytoma line. Positive sera were subsequently tested in dilutions by immunoblotting.

An H-Ig component was defined as a narrow band in agar electrophoresis, confirmed by the detection of a band of the same mobility by immunoblotting with isotype specific anti-Ig antiserum conjugates.

Antibodies for immunoblotting

Peroxidase-labelled polyclonal GaM isotype-specific conjugates (Nordic Immunological Laboratories) were used for characterisation of H-Ig by immunoblotting in the following dilutions: GaM-IgG1-PO 1:10.000, GaM-IgG2a-PO 1:10.000, GaM-IgG2b-PO 1:5000, GaM-IgG3-PO 1:5000, GaM-IgA-PO 1:10.000, GaM-IgM-PO 1:5000, GaM-IgD-PO 1:7500. For determination of the H-Ig light chains, peroxidase conjugated polyclonal sheep anti-mouse (ShaM) kappa and ShaM lambda were used in 1:4000 and 1:10.000 dilutions, respectively.

To discriminate between transgenic IgM of a-allotype and the endogenous IgM of b-allotype, we used RS3.1-biotin anti-Igh-6a and MB86-biotin anti-Igh-6b conjugates (kind gifts from dr A.Coutinho, Institut Pasteur, Paris) in immunoblotting experiments, both in 1:5000 dilution. A second-step avidin-peroxidase (Calbiochem Co., La Jolla, CA) 1:1000 incubation was used to allow for detection with DAB substrate.

Classification of MG

Electrophoresis and immunoblotting data were assessed in combination with the clinical features of mice with H-Ig to allow for classification of MG into the categories as described by Radl [1,8]. Briefly, the following categories were distinguished: in category 1, the H-Ig showed a progressive development within two months, reaching paraprotein concentrations of more than 5 mg/ml (often more than 10 mg/ml). This pattern is typical for multiple myeloma. An H-Ig was classified as belonging to category 2 when the concentration of the paraprotein remained below 4 mg/ml and persisted for at least six months before the animal

died of other causes. This pattern is typical for BMG, i.e. benign neoplasias. Category 3 comprised transient H-Ig components, usually of low concentration. H-Ig that appeared shortly before death were considered unclassifiable. The first two categories represent neoplastic B cell disorders in which an intrinsic B cell defect is present. This is illustrated by the fact that MG of both categories are transplantable into congenic recipients. The third category of transient H-Ig components often accompanies an imbalance between B and T cell systems.

Statistical methods

Survival curves were estimated by the method of Kaplan-Meier with differences assessed by the logrank test. Proportions were compared by chi-square analysis, using Yates continuity correction, or Fisher's Exact test when a number was smaller than 5. ELISA-data were log transformed and analyzed by ANOVA and Mann-Whitney-U tests. P-values smaller than 0.05 were considered statistically significant.

RESULTS

Survival analysis

Survival probability curves of Tg and LM mice were constructed according to the method of Kaplan-Meier (Fig. 1A). The 50% survival times were 96.5 weeks for Tg mice and 108 weeks for LM controls. The life-span of Tg mice was significantly ($P < .02$) shorter than that of LM, both sexes taken together. Male LM mice lived significantly longer than females ($P < .001$) (Fig. 1B and C). Previously it has been shown for inbred C57BL strains that males lived longer than females [9], similar to our findings in our control group. In the Tg mice there was no apparent difference in survival time between the sexes and both sexes lived as long as female LM mice. In other words: only male Tg mice had a reduced (20 weeks) life-span as compared to their LM male controls ($P < .001$).

Histopathology

The histopathological diagnoses are given in table 1 with a summary in table 1a. No significant differences between Tg and LM mice were observed with the exception of the relatively high incidence of histiocytic sarcoma in LM mice and the absence of such lesions in Tg mice ($P = 0.0088$). Compared to historical data [9] the relatively high incidence of acute inflammatory lesions in LM as well as in Tg mice was remarkable. Such intercurrent disease would interrupt the development of aging pathology (e.g. neoplastic, chronic inflammatory and various degenerative lesions), decrease the number of lesions per mouse and increase the proportion of acute inflammatory lesions in Tg mice. As can be deduced from table 1a, a trend in

Figure 1. A. Survival probability curves of μ-κ Sp6 transgenic mice (Tg: ___) and littermate controls (LM: - - -), both sexes taken together. **B.** Survival probability curves of male μ-κ Sp6 Tg mice and LM controls. **C.** The same for female mice. The logrank test was used to assess the differences between the curves. Corresponding P-values are given. The horizontal (.....) line is the 50% survival value.

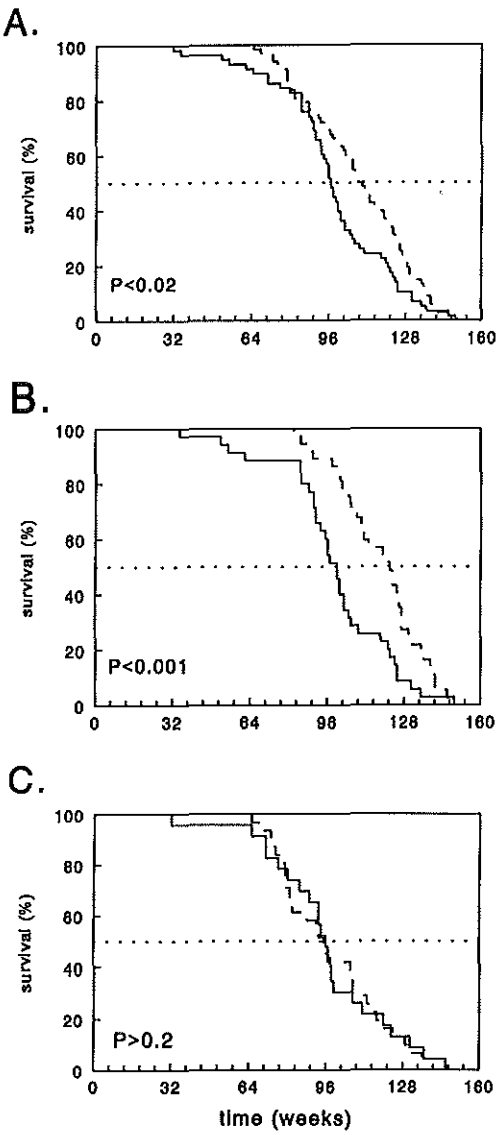

Table 1. Survey of neoplastic and non-neoplastic lesions in aged μ,κ Tg mice and littermate controls.

	LM mice		Tg mice	
	♂♂	♀♀	♂♂	♀♀
I. Neoplastic lesions				
Follicle center cell lymphoma (FCC)	7/24 [a]	5/11	8/20	2/7
Malignant lymphoma NOS [b]	5/24	0/11	4/20	1/7
Multiple myeloma [c]	1/24	0/12	0/21	1/7
Histiocytic sarcoma [d]	7/23	5/11	0/21	0/7
Hemangioma or hemangiosarcoma	5/24	2/11	5/21	2/7
Total other neoplastic lesions	5/24	2/11	6/21	0/7
II. Hyperplastic lesions				
Fibro-osseous hyperplasia	0/24	2/12	1/21	2/7
Lymphoid hyperplasia	1/24	2/11	0/20	0/7
Bone marrow plasma cell hyperplasia	9/24	2/12	5/21	0/7
idem, including pleomorphism	2/24	4/12	4/21	1/7
Extramedullary haemopoiesis	8/23	5/11	7/20	3/7
Total other hyperplasia	2/24	1/11	1/21	1/7
III. Non-neoplastic lesions:				
~ *Acute inflammatory*				
Parodontitis	2/17	0/3	0/17	0/3
Incisor dysplasia + inflammation	8/17	0/3	6/17	0/3
Acute lymphadenitis	2/24	0/11	2/20	2/7
Skin erosion / ulceration	2/16	1/7	3/16	3/5
Subcutaneous abscesses	1/16	0/6	3/16	0/5
Otitis media / rhinitis	3/8	0/1	4/9	3/3
Total other acute inflammatory	10/24	3/11	12/21	4/7
~ *Chronic inflammatory:*				
Polyarteritis	2/24	3/11	1/21	0/7
Sialoadenitis	2/22	1/11	2/21	0/7
Eosinophilic macrophage pneumonia	6/24	0/10	4/20	2/7
Total other chronic inflammatory	7/24	0/11	2/21	1/7
IV. Various other non-neoplastic lesions				
Incisor dysplasia	6/17	3/3	8/17	1/3
Amyloidosis (localised)	12/24	1/11	10/21	2/7
Hydronephrosis	5/21	1/9	2/21	0/5
Renal tubular atrophy + dilatation	5/21	0/9	5/21	2/5
Total other non-neoplastic lesions	19/24	9/11	10/21	3/7

a) Number of diagnoses per relevant necropsy.
b) NOS: not otherwise specified.
c) Diagnosis by histopathological and serological findings.
d) LM mice compared to Tg mice: two-sided P-value = 0.0088, both sexes together.

Table 1a. Total number of lesions in necropsy material of Tg and LM male mice.

	LM male mice (n=24)		Tg male mice (n=21)	
Diagnostic category	*n (lesions)*	*ratio*	*n (lesions)*	*ratio*
Neoplastic lesions	30	1.25	23	1.10
Hyperplastic lesios	22	0.92	18	0.86
Acute inflammation	28	1.17	30	1.43
Chronic inflammation	17	0.71	9	0.43
Various other non-neoplastic lesions	47	1.95	35	1.67
Total lesions per mouse	144	6.0	115	5.5

this direction is indeed present. This favours the explanation of the decreased survival of male Tg mice to be due to a decreased resistance to acute inflammatory disease. There were no differences detected between the two sexes except for a higher incidence of localized amyloidosis (mainly in the stomach) in the male mice (P = 0.016, Tg and LM together).

Immunoglobulin levels during aging

The fully rearranged μ,κ-transgene resulted in a relatively high concentration of serum IgM of Tg origin, and a suppression of the levels of the endogenous isotypes in young Tg mice [13]. We quantitated the serum concentrations of the predominant Ig isotypes in aging Tg and LM mice of both sexes; Tg-mice: n=16 males, n=17 females; LM-mice: n=10 males, and n=10 females. All selected groups had the same age distribution and none of the mice showed an H-Ig in agar electrophoresis. From each mouse a small blood sample was taken from the tail vein at 3 to 6 consecutive time points. The data were ordered according to age by using 7 age cohorts of 15 weeks duration (Fig.2). Neither in the Tg group, nor in the LM control group differences were observed between the two sexes (data not shown). Therefore we analyzed the data of both sexes together.

The mean isotype concentrations in the sera of the Tg mice were lower than those in the LM group, except for IgG2b (during their entire life) and for total IgM (in the first year of their life) (Fig. 2, table 2). The effects of aging on the Ig isotype levels in Tg mice were similar as in LM controls: a decreasing amount of IgG2b (P=0.0016 in the Tg mice, P=0.0051 in the LM mice; table 2), and a marginal increase in total IgM. The decrease in Tg mice

Figure 2. Immunoglobulin isotype levels (mean ± SD) during aging in Tg (black columns and dashed regression lines) and LM mice (open columns and pointed regression line). For analysis of data, see table 2.

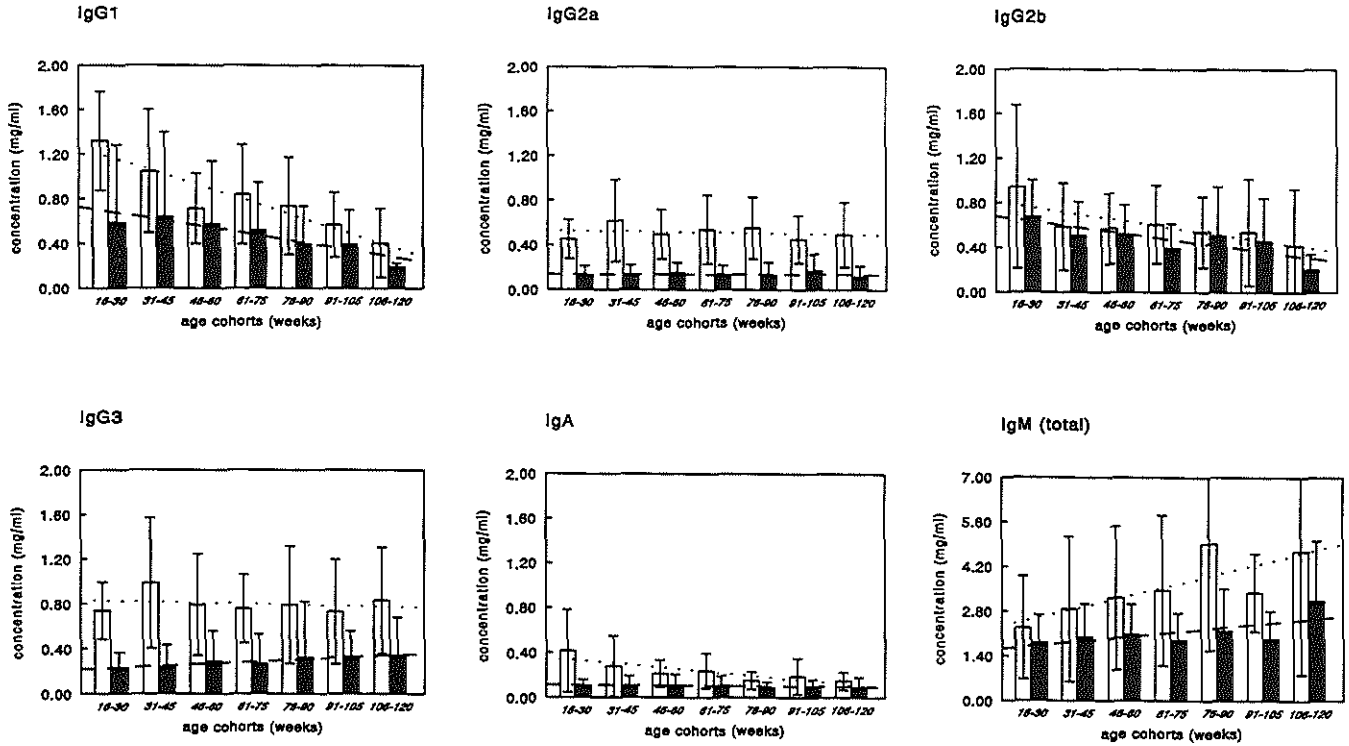

Table 2. Comparison of Ig-levels in Tg and LM mice during aging. Log transformed data were analyzed by ANOVA and Mann-Whitney-U tests.

		Age cohorts (weeks)						
Isotype		*16-30*	*31-45*	*46-60*	*61-75*	*76-90*	*91-105*	*106-120*
IgG1:		7/11 [a)]	21/26	20/23	26/17	10/3	18/13	10/3
2-sided P-value		0.007	0.002	0.03	0.01	0.004	0.05	NS
Linear trend - LM: P<.0001	Tg: NS							
IgG2a:		7/13	22/34	20/29	26/22	12/19	18/13	12/4
2-sided P-value		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
Linear trend - LM: NS	Tg: NS							
IgG2b:		9/12	22/33	24/32	25/24	15/19	16/17	7/5
2-sided P-value		NS	NS	NS	NS	NS	NS	NS
Linear trend - LM: P=.0051	Tg: P=.0016							
IgG3:		6/12	20/30	17/26	23/20	12/21	12/16	8/4
2-sided P-value		<.0001	<.0001	<.0001	<.0001	0.0007	0.0007	0.0315
Linear trend - LM: NS	Tg: NS							
IgA:		3/13	20/31	19/27	24/22	13/18	15/13	8/3
2-sided P-value		0.005	<.0001	<.0002	<.0001	0.007	0.034	NS
Linear trend - LM: P=.0073	Tg: NS							
IgM:		6/12	18/35	17/29	25/24	15/20	17/15	12/3
2-sided P-value		NS	NS	NS	0.01	0.004	0.0006	NS
Linear trend - LM: P=.0099	Tg: NS							

a) Number of mice analyzed (LM / Tg).

of the IgG1 levels with aging was not significant due to the large range of the results. As previously described [6,13], IgM was the predominant isotype in the serum of Tg mice. The low levels of IgG2a and IgG3 were mainly responsible for the low IgG levels in Tg mice at all ages tested.

Stable expression of the IgM-κ transgene during aging

The transgenic IgM (Igh-6a) and the endogenous IgM (Igh-6b) levels were also quantitated by ELISA (Fig. 3). At old age, the transgenic IgM was still produced in at least the same amount as in young Tg mice. We found that the cumulative amounts of both IgM allotype concentrations was lower than the amount measured by the total IgM-isotype ELISA. This observation was reported earlier by Grandien *et al.* [14] and can be ascribed to the production of "mixed" pentamers in Sp6-Tg mice [13,15], with approximately five times as much transgenic IgM (Igh-6a) as compared to endogenous IgM (Igh-6b). Although the results of the IgM allotype ELISA might not accurately represent the quantitative amounts that are actually present in Tg mice, they showed the same effect of aging as the results of the total IgM ELISA.

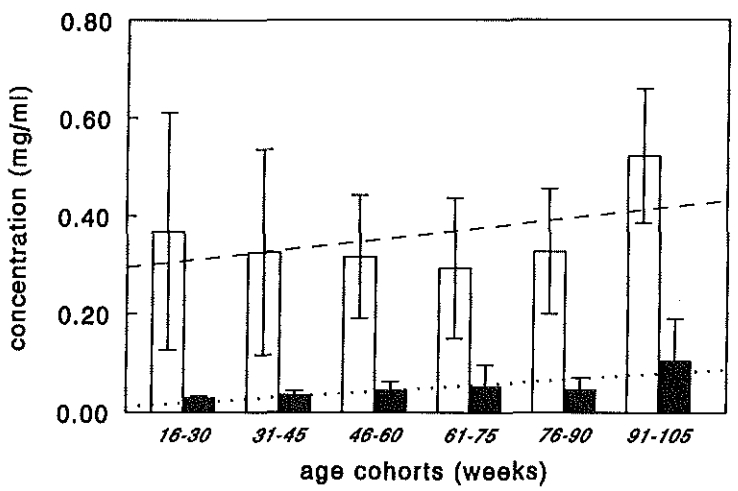

Figure 3. Concentration (mean ± SD) of transgenic IgM, Igh-6a (open columns and dashed regression line) and endogenous IgM, Igh-6b (black columns and pointed regression line) in serum of Tg mice during aging.

Frequency of MG-categories

The H-Ig were classified into the three categories as described (Table 3). No differences were observed between Tg and LM mice for the frequencies of multiple myeloma, transient MG and unclassifiable MG. The diagnosis of multiple myeloma was based on histopathology, confirming the serological data. The only one myeloma which developed in a Tg mouse produced IgG2b-kappa, the only one myeloma in a LM mouse produced IgG1-kappa. The aging Tg mice showed half as many MG as the LM ($P = 0.0016$), mainly because of a four times lower incidence of BMG ($P=0.0007$) in the Tg mice.

The cumulative frequency of the first appearance of the H-Ig in the sera of the mice is shown in Fig. 4. The Tg mice developed H-Ig significantly later ($P<0.001$) than the LM mice. At their 50% survival age, 25% of the Tg mice had an H-Ig in the serum, in contrast to 66% of the LM mice.

Up to the age of approximately 80 weeks, the LM mice showed an enhanced increase of H-Ig components as compared to the Tg mice. In this period, the increase of MG in Tg mice was also enhanced as compared to the time-period after 80 weeks. After this age, the slopes of the curves were essentially parallel, meaning that from this age on there was an equal influence of age on the appearance of H-Ig in Tg and LM mice.

Table 3. Cumulative frequency of MG-categories in aging Tg and LM mice.

Category of MG	Tg (N=58) n / % [a]	LM (N=68) n / %
I. Multiple myeloma	1 (1.7)	1 (1.5)
II. Benign MG	6 (10.3) [b]	26 (38.2)
III. Transient MG	5 (8.6)	11 (16.2)
Unclassifiable	10 (17.2)	8 (11.8)
Total MG	22 (37.9) [c]	46 (67.6)

a) Number and percentage of mice that developed MG of the designated category.
b) Two-sided P-value = 0.0007.
c) Two-sided P-value = 0.0016.

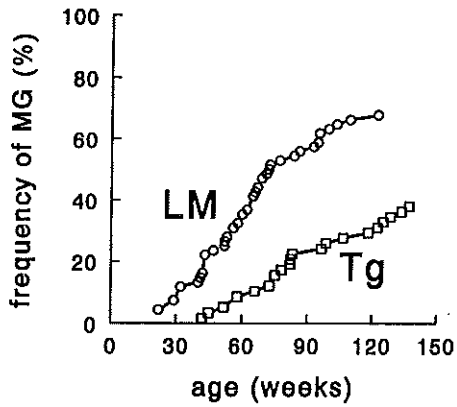

Figure 4. Cumulative representation of the incidence of all H-Ig components in Tg (squares) and LM mice (circles).

H-Ig isotype distribution

The H-Ig isotype distribution as determined by immunoblotting (Table 4) did not show statistically significant differences between Tg and LM mice. The mean age of the mice in table 4 was 27 months (range = 22.6 - 28.6 months). At this age 27% of the Tg mice showed an H-Ig compared to 67% of the LM. The Tg mice had developed significantly less H-Ig components (Fisher's Exact two-tailed, P=0.028) at this age than LM mice. It is remarkable that none of the Tg mice developed an H-Ig producing the Tg Igh-6a allotype. A double H-Ig was detected by agar electrophoresis in 6 Tg mice (10.3%) and in 12 LM (17.6%) within the entire follow-up period.

There were H-Ig components that stained for several isotypes in immunoblotting. Because goat anti-mouse antisera were used for detection of the H-Ig on the blots, these H-Ig were carefully checked for specificity for goat IgG. Therefore, after blotting, the PVDF membranes were incubated with biotinylated normal goat IgG (protein G purified), diluted in blocking solution. A subsequent incubation with avidin-peroxidase allowed for staining of the blots with DAB substrate as described. None of these H-Ig showed anti-goat specificity (not shown). Thus a rheumatoid factor-like activity of these H-Ig could not be proved and we designated them as multireactive Ig (MR). In the Tg group, a MR could be detected in two (11.8%) of the mice, whereas in the LM group six (15%) of the mice had a MR.

Lambda light-chain usage was apparently not increased since in both groups only 1 H-Ig gave a positive reaction with ShaM-λ-PO in immunoblot.

Persistent H-Ig of the IgD isotype were detected in low frequencies in both groups [Tg: 5.1% (n=39); LM: 7.7% (n=52); $p>0.05$]. The mice tested for IgD were 7 months younger than the mice tested in table 4.

Table 4. Isotype distribution of H-Ig components in aged μ-κ Tg mice and LM controls.

H-Ig isotype [a]	Tg (N=17) n (%) [b]	LM (N=26) n (%)
IgG1	6 (35.3)	7 (17.5)
IgG2a	2 (11.8)	9 (22.5)
IgG2b	4 (23.5)	6 (15)
IgG3	2 (11.8)	3 (7.5)
IgM (Igh-6b)	1 (5.9)	9 (22.5)
IgM (Igh-6a)	0	- [c]
IgA	0	0
MR [d]	2 (11.8)	6 (15)

a Isotype distribution of H-Ig components was determined by immunoblotting, using GaM isotype-specific peroxidase conjugates.
b Number and percentage of total number of H-Ig components per group.
c LM mice do not have IgM (Igh-6a).
d Multireactive H-Ig, showing multiple staining in immunoblotting.

DISCUSSION

The exact phenotype, localization and B cell lineage origin of the neoplastic precursor cells of MG are unknown. Unlike most B cell chronic lymphocytic leukemias (B-CLL) in humans, there is no constitutive expression of CD5 on the cell surface of neoplastic B cells from MG, making it difficult to speculate about the cell lineage origin of these B cell proliferative disorders. Data on this, however, are needed to discriminate early stages of benign and malignant B cell proliferative disorders and may be crucial for the timely efficient treatment of the malignant forms. In this respect the possible

origin of MG from either B-1 or B-2 cells is of interest. While B-CLL and some cases of Waldenström's macroglobulinemia were shown to develop from the B-1 cell, experiments with mouse multiple myeloma and BMG indicated the possibility that the target cell is a memory cell, possibly of the B-2 cell lineage. Our results demonstrated that both these neoplastic disorders can also stem from the B-1 cell.

Although the transgenic mouse model we used does not reflect a normal immune system, it still has some advantages that are crucial for this study. Firstly, the B-1 cell population could be studied without being dependent on cell surface markers. The expression of markers specific for the B-1 lineage (CD5 and CD11b) might be affected by the growth deregulating processes that occur during neoplastic transformation. Furthermore, B cell proliferative disorders do not express these markers at the end-stage of their differentiation.

The presence of an Ig transgene can result in downregulation of the Tg expression or even in total shut down of Tg transcription [16,17]. This would result in normalization of the serum Ig isotype levels at old age. This was not the case in this animal model, in which the mice had a stable level of Igh-6a during their entire life-span. Effects of aging on the B cell system as reflected by the isotype levels were approximately the same in Tg and LM mice. Even after more than two years normal Ig levels in the sera of the Tg mice were not reached and the relative humoral immunodeficiency remained present in the old mice.

During aging, the T cell compartment also shows diminished capacities and this, acting together with the already inflicted B cell compartment, could very well have an influence on age-associated pathology in Tg mice. However, histological examination of about half of the LM and Tg mice did not reveal specific lesions which could be ascribed to the Tg status. Type and incidence of lesions were similar to that of historical data [9], with the exception of a clearly higher incidence of acute inflammatory lesions in both groups, due to conventional housing of the mice, and of the absence of histiocytic sarcomas in Tg mice.

The observed difference between the life-spans of male Tg and LM mice was marginal considering that antibody responses to T-cell independent antigens, many of which are bacterially related, were almost absent and the response to T-cell dependent antigens was retarded [13] in the Sp6-B6 mice. Furthermore, the degenerate specificity of the Sp6 antibody [18] did not result in manifest autoimmune diseases, reflecting ample compensatory mechanisms and a functional T cell system. From the histopathological examination of necropsy material it can be concluded that the presence of the Ig transgene itself is not a stimulating factor for neoplastic transformation in general.

The interpretation of the electrophoresis and immunoblotting data was

not complicated by a monoclonal band of Tg IgM, probably due to the secretion of "mixed" IgM pentamers, as was suggested earlier [13,15]. Although the Tg IgM-kappa is principally a monoclonal Ig, the introduction of the Ig transgene in B cells resulted, at least in a subpopulation of B cells, in the appearance of double-Ig producers. Various, probably random, amounts of endogenous and Tg IgM molecules in the secreted IgM pentamers, together with the possibility for combination of the Tg μ-chain with endogenous light-chains, prevented the appearance of a monoclonal band in serum electrophoresis and rather resulted in a more diffuse electrophoretic pattern.

We purified Tg IgM (Igh-6a) from pooled Tg sera by affinity chromatography and compared the differentiated carbohydrate residue content with purified IgM from LM pooled sera and purified IgM paraproteins of both allotypes (unpublished data). Differences in carbohydrate content between the samples were minimal (analysis performed by Dr.M.Tomana, University of Alabama, Birmingham, AL). Therefore the absence of a consistent H-Ig of Tg origin in serum agar electrophoresis could not be attributed to differences in post-translational modification of the transgenic and endogenous μ-chains.

Others have shown that the B cell compartment responsible for endogenous Ig production is extensively activated in these mice [14,19]. In the Sp6-Tg mice the Ig specificity development was clearly shifted to the population of B cells that is still able to form a near-to-normal Ig repertoire [20], in this case the B-1 cells. Still, the transgenes expressing B cells are functioning properly after encountering the relevant antigen TNP [21].

Certain specific conditions may be necessary to allow for excessive proliferation of B cell clones, such as an extensive antigenic activation or, like in this model, a relative B-2 cell immunodeficiency that leads to compensatory activation of the B-1 cells. Because the development of MG of B-2 cell origin seems to be suppressed by the presence of an Ig transgene coding for an irrelevant specificity, it can be reasoned that antigenic specificity and activation of a B cell might be a competent contributing factor in MG development. In this respect it would be interesting to know whether Sp6-Tg mice, after repeated immunization with TNP-conjugates, develop IgM paraproteins with TNP specificity.

In Tg mice about 10% of splenic B cells express endogenous Ig [7]. During aging the frequency of MG in Tg mice is about 50% of that in LM mice. This suggests that there is a five fold higher contribution of endogenous Ig producing B-1 cells to the formation of MG in Tg mice than in LM mice.

It has been shown in non-transgenic mice that peritoneal B-1 cells can become clonally restricted during aging [22]. More studies need to be done to find out whether these B-1 cell clones are the producers of a paraprotein.

The results of this study are in accordance only with some points from a

similar study on MG development in M54 Tg mice [23]. In M54 mice the transgene encodes for a μ-heavy chain only, derived from a NP-specific IgM producing hybridoma. The authors did not find H-Ig of transgenic origin, which is analogous with our observations in the Sp6 Tg mice. In contrast to our findings in Sp6 mice, only transient H-Ig of endogenous isotype were found and all of low concentration. The frequency of those monoclonal components was higher in Tg mice than in LM controls, most of them using lambda light-chains. The differences in results of these two studies can in part be explained by the difference in mouse strains and their genetic background and age, in microbiological status, and in the use of different transgenes. Recently Gueret et al published a study on MG development in C57BL/Xid mice [24]. The Xid mutation resulted in a deficient B-1 cell population. No H-Ig were detected in the Xid-mice against a 50% incidence in control mice. Unfortunately, these mice did not live long enough to develop full-blown MG of neoplastic origin. Still, this observation also suggests a role for the mouse B-1 cell lineage in the pathogenesis of age-related B cell proliferative disorders.

The exact function of the B-1 cells is still unknown. Their localization in the coelomic cavities, together with intestinal IgA plasma cells derived from the B-1 cells, emphasizes their role in mucosal immunity [5,7,25,26,27]. Excessive and prolonged antigenic stimulation is not uncommon in this environment. For example, bacterial cell walls contain LPS-like substances that are potent polyclonal B cell stimulators. It was shown that orally fed LPS was able to stimulate the B-1 cell population but not the B-2 cells [28]. When the B-1 cells become clonally restricted with increasing age [22], in the presence of genetic susceptibility to MG development, an H-Ig may become detectable in the serum.

In conclusion, we have shown that the B-1 lineage contributes to the development of monoclonal gammopathies in mice. Studies of gene abnormalities in the B cell clones involved in MG development and the determination of the H-Ig specificities will be useful in further explaining the origin of these B cell proliferative disorders.

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2.3

CHAPTER 2.3

IgH ALLOTYPE-CONGENIC MOUSE CHIMERAS REVEAL INVOLVEMENT OF THE B-1 CELL LINEAGE IN THE DEVELOPMENT OF BENIGN MONOCLONAL IgM GAMMOPATHY

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SUMMARY

Plasma cell proliferative disorders present themselves by homogenous, monoclonal immunoglobulins (H-Ig) in the serum. Cells of the neoplastic representatives of these monoclonal gammopathies (MG), such as multiple myeloma (MM), Waldenström's macroglobulinemia (WM) and benign monoclonal gammopathy (BMG), are mainly located in the bone marrow, spleen and lymph nodes. Still, it is not clear what the exact phenotype, localization, and cell lineage origin is of the precursor cells of MG. Knowledge of the B-cell lineage origin might be helpful for correct diagnosis and timely efficient treatment of malignant forms of MG.

We used Ig allotype-congenic mouse chimeras to determine the cell lineage origin of MG. Using this model, the H-Ig of aging C57BL mice originating from the B-1 cells in the peritoneal cavity could be discriminated from the bone marrow derived B-2 cell Ig product by allotype difference.

Due to late effects of the total body irradiation (9.5 Gy), the life-span of the chimeras was reduced and consequently the number of H-Ig detected in this study was less than expected. The determination of the IgM-allotypes of H-Ig in aged chimeras revealed that most of these H-Ig originated from B-1 cells. These MG could be classified as BMG and transient MG. In the chimeras (n=166) we detected only one multiple myeloma, with an allotype corresponding with the BM donor.

This study indicates that age-related monoclonal B-cell proliferative disorders in genetically susceptible mice can originate from the BM as well as from the peritoneal cavity, involving the B-1 cell population.

INTRODUCTION

Monoclonal B cell proliferative disorders at the differentiation stage of a plasma cell synthesize monoclonal immunoglobulins that are detectable in the serum as homogenous Ig components (H-Ig). Therefore they are designated monoclonal gammopathies (MG). Using biological and pathogenetic characteristics, Radl divided MG into four categories [1,2]. Clinically the most important MG are those of the neoplastic categories. They develop due to intrinsic B cell disorders, resulting in either a benign MG (BMG) or in a malignant plasma cell neoplasia, such as multiple myeloma (MM), (extra-) medullary plasmacytoma, or Waldenström's macroglobulinemia. The development of these B cell proliferative disorders is age-related. Monoclonal gammopathies of other categories are mainly the result of imbalances between B and T cell functions, such as during immunosuppressive treatment, or as a result of a homogenous antibody response after excessive antigenic stimulation. Influences of genetic background, T cell function impairment and antigenic stimulation on the development of MG have been reported earlier in humans as well as in laboratory animals [2,3,4]. The C57BL/KaLwRij mouse strain proved to be a suitable model for studying MG, since aging mice of this strain develop spontaneously MG of all categories [5,6].

The phenotype of the neoplastic MG precursor cells is not known. In mice two B cell lineages can be distinguished [7,8]. The B-1 cells constitute a self-replenishing population of B cells in the peritoneal and thoracic cavities. The conventional B cells are bone marrow derived, and are called B-2 cells. Unlike mature B cells, plasma cells do not express the lineage markers CD5 or CD11b that are characteristic of the B-1 lineage. The precise function of the B-1 cells is not known, but they may play a role in mucosal defence and in various autoimmune phenomena [9]. It has been shown in mice that with aging the B-1 cell population becomes increasingly clonally restricted [10]. This phenomenon might also account for a relatively high chance of neoplastic transformation within these cells. Therefore they form a pool of potential precursor cells of MG.

We used allotype congenic mouse chimeras to study the B cell lineage origin of MG in aging C57BL mice. A long-term follow-up study was performed in lethally irradiated mice that were reconstituted with bone marrow (BM) cells and IgH allotype congenic peritoneal wash cells. The cells from the peritoneal washes provided the precursors of the B-1 cell population. The BM cells gave rise to the B-2 cells [11]. The cellular origin of the H-Ig that developed at old age in these chimeras could thus be determined by Ig-allotyping of the H-Ig component in the serum.

We were able to show that some of the H-Ig that were detected in the old chimeras, were derived from peritoneal B cells. These MG could only have developed from the B-1 cell lineage, since the peritoneal wash cells in adult mice do not provide for B-2 stem cells.

MATERIALS AND METHODS

Mice and cell transfer

C57BL/6 (Igh^b) and C57BL.Igh^a mice from the breeding stock of the Department of Immunology of the Erasmus University in Rotterdam were maintained under clean conventional conditions. Recipient mice received a single dose of 9.5 Gy total body irradiation, 24 hours prior to cell transfer. Only female recipient mice (aged 5 months) were used. The donor mice were of the same age. Peritoneal cells were washed out of the peritoneal cavities (PerC) of the donor mice with 10 ml ice-cold balanced salt solution (BSS). BM cells were prepared by flushing the femur and tibia with BSS. Transfer of PerC cells was performed by i.p. injection of $3x10^6$ PerC cells in 250 μl BSS. Transfer of BM cells was performed by i.v. injection of $2x10^6$ BM cells in 0.5 ml BSS.

We used three groups of irradiated recipient mice: one group of 63 C57BL.Igh^a mice received only Igh^b BM cells; 57 C57BL.Igh^a mice received Igh^a BM cells and Igh^b PerC cells; 46 C57BL/6 mice received Igh^b BM cells and Igh^a PerC cells. Groups of 33 unirradiated aging mice of both strains were used as control mice. The mice were allowed to live out their life-spans. Seriously ill or moribund mice were submitted to necropsy according to a standard protocol [12].

Antibodies

Purified polyclonal goat anti-mouse (7S fractions) IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA (Nordic Immunological Laboratories, Tilburg, The Netherlands) were used in the ELISA as capture antibodies. Peroxidase conjugates of the same antibodies were used in the immunoblots for determining the isotype of the H-Ig. Detection of IgM-allotypes by immunoblotting was done with biotin conjugated RS3.1 mouse anti-mouse-Igh6a and MB86 mouse anti-mouse-Igh6b (kindly donated by Dr.A.Coutinho). Biotinylated mouse anti-mouse Igh1b, clone 145-3.1 (Nordic Immunological Laboratories) was used to determine $IgG2a^b$ allotype positive H-Ig. Because the total IgG2a (a and b allotype together) was also determined, H-Ig that were negative in the Igh1b detecting immunoblotting experiments had to be of Igh1a allotype.

Detection and characterization of H-Ig

Every three months a small blood sample was taken from the tail veins of the mice. The undiluted serum was used for high resolution serum electrophoresis in agar 0.9% (Difco, Detroit, MI) as described previously [13]. The H-Ig detection limit of this technique was 100 μg per ml. To determine the heavy-chain and light-chain isotypes of the H-Ig we used immunoblotting on PVDF membranes (Immobilon; Millipore, Bedfort, MA) as described previously [13]. The same method was used for allotype detection of H-Ig. Since B-1 cells

produce relatively large amounts of IgM, we optimized H-Ig allotype detection of Igh6a and Igh6b (IgM allotypes). Additionally, the Igh1b ($IgG2a^b$) allotype was determined.

ELISA

Briefly, 96-well round-bottom microtiter plates (Greiner, Alphen a/d Rijn, The Netherlands) were coated with purified goat anti-mouse isotype antibodies (10 μg/ml in PBS, 50 μl per well) overnight at 4 °C. After blocking the residual sites with PBS-Tween-20/5% BSA, the serum samples were added in appropriate dilutions and incubated at room temperature for 2 hours. After washing the plates with PBS-Tween the plates were incubated with alkaline-phosphatase (AP) conjugated goat anti-mouse IgG, -A, and -M (KPL, Gaithersburg, MD, USA) for one hour at room temperature. After washing and final incubation with substrate solution (p-nitrophenylphosphate 1mg/ml in a 1M diethanolamine, 0.05 M $MgCl_2$ buffer, pH 9.8) the absorbance was read by 405 nm using a BioRad Model 3550 Microplate Reader (BioRad, Veenendaal, The Netherlands). For detection of IgM allotypes, the RS3.1 mouse anti-mouse Igh6a (a kind gift of Dr.A.Coutinho, Institut Pasteur, Paris) and the AF6-78 mouse anti-mouse Igh6b (Pharmingen, San Diego, CA) antibodies were used as a coating. After incubation with appropriate serum dilutions, AP conjugated goat anti-mouse IgM (KPL) allowed for specific IgM detection. The assays were standardized with affinity-purified mouse monoclonal antibodies of appropriate isotype and allotype.

Statistical analysis

Survival-curves were constructed according to the method of Kaplan-Meier and differences were assessed by the logrank test. ELISA data were log-transformed and analyzed by ANOVA. Frequencies were analyzed by Fisher's Exact Test. A P-value smaller than 0.05 was considered statistically significant.

RESULTS

Survival

All groups that received total body irradiation (9.5 Gy) died significantly earlier ($P < .001$) than the two control groups (fig. 1). C57BL/6 recipient mice that received Igh^b-BM cells and Igh^a-PerC cells had the shortest life-span ($P < .001$). C57BL.Igh^a mice that received only Igh^b-BM cells lived shorter than C57BL.Igh^a mice that received Igh^a-BM cells and Igh^b-PerC cells ($P < .001$). The two control groups had equal survival characteristics. Survival of the irradiated mice did not improve markedly when the dosage of radiation was decreased to 8.5 Gy, but a dose of 10 Gy significantly further reduced the life-span of the treated mice (data not shown). The cause of death was not unsuccessful cell transfer, as this would have caused death at a much earlier time point. Necropsies of the mice which died spontaneously or were killed when moribund revealed that the decreased survival was due to severe late renal damage, caused by the irradiation. The renal changes were dominated by

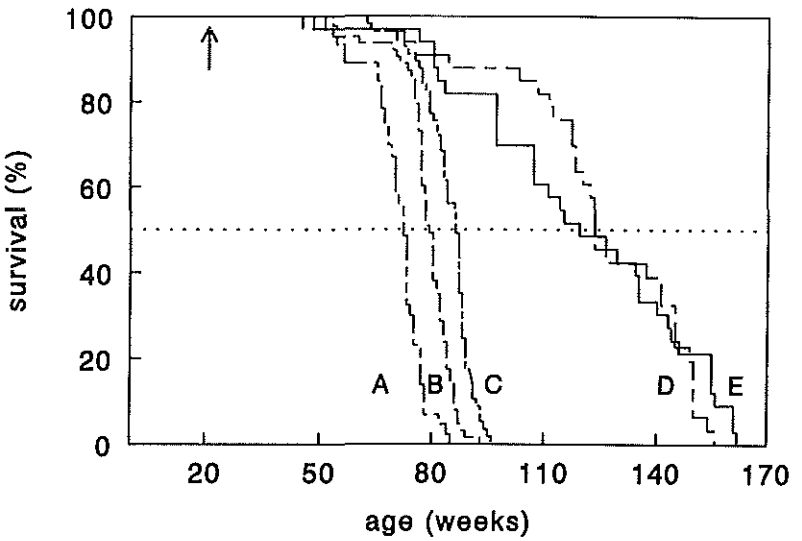


Figure 1. Kaplan-Meier survival curves of the allotype-congenic radiation chimeras and control groups. The age at which the irradiation and cell transfers took place is indicated by an arrow. The horizontal dotted line is the 50% survival reference line. (A) C57BL/6 recipient mice of Igh^b BM cells and Igh^a PerC cells (n=46); (B) C57BL.Igh^a recipient mice of Igh^b BM cells (n=63);(C) C57BL. Igh^a recipient mice of Igh^a BM cells and Igh^b PerC cells (n=57); (D) unirradiated C57BL/6 mice (n=33); (E) unirradiated C57BL.Igh^a mice (n=33).

severe endothelial damage leading to fibrinoid change of many arterioles and small arteries. Additionally, plugging of the lumen of arterioles and glomerular capillaries by fibrin thrombi resulted in extensive mesangiolysis and ischemic glomerular changes.

Similar subendothelial fibrinoid changes were sporadically seen also in small arteries elsewhere in the body. These, however, did not result in severe organ damage as seen in the kidneys. The low incidence of other life-limiting or age-related diseases, such as malignant lymphomas or amyloidosis, indicated that the steep decline of the survival curve was due to a single intercurrent fatal disease, *i.e.* renal damage. Consequently the mice did not live long enough to develop the various categories of MG in an incidence that is seen in untreated aging C57BL mice.

Ig isotype concentrations during aging

Individual serum isotype concentrations are of interest in relation to the isotype distribution of the H-Ig. The dominant isotypes usually represent the majority of the H-Ig. The serum concentrations of Ig isotypes were determined in 12 to 22 mice of each group by a double sandwich ELISA (fig. 2) at four consecutive time points (ranging from 3 months till 12 months after irradiation and cell transfer).

As for the group of C57BL.Igh^a mice that received only BM of Igh^b allotype, most of the Ig isotypes were lower in concentration than in the C57BL.Igh^a control group. Only the total IgM concentration was higher than in the control group ($P < .001$).

The C57BL.Igh^a mice that received BM of Igh^a allotype and PerC cells of Igh^b allotype had lower levels of IgG1 and IgA than the C57BL.Igh^a control mice ($P < .001$), but higher levels of total IgM (significant at the last three time points). The IgG2a and IgG2b levels in this group were significantly lower than in the control group at the first time point, that was 3 months after transfer ($P < .001$). Later in life the levels of these isotypes in this group equalled that of the control group.

The other group of allotype chimeras, C57BL/6 (Igh^b) mice that received Igh^b BM and Igh^a PerC cells, had equal levels of IgG1, IgG2a (except at 3 months: $P < .05$), IgG2b, IgG3 (except at 3 months: $P < .01$), and total IgM compared to the C57BL/6 control group. At 3 and 6 months after cell transfer the IgA levels were lower than in the control group ($P < .01$).

Comparison of the control groups showed that C57BL/6 mice had higher levels of IgG2a ($P < .05$), and slightly higher levels of IgM (significantly so at 6 and 9 months after cell transfer; $P < .05$ and $P < .01$, respectively).

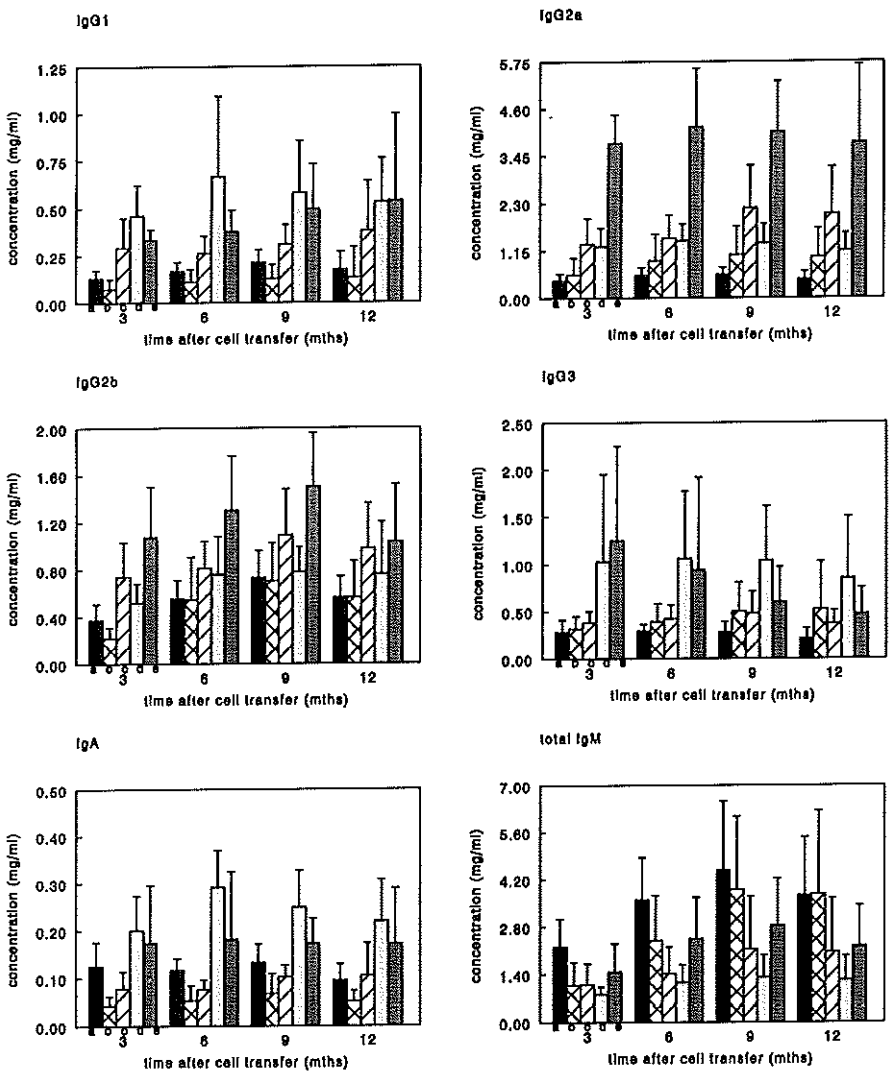
Although satisfying in immunoblotting experiments, the MB86 monoclonal mouse anti-mouse Igh6b antibody did not work out well in the ELISA. We therefore tested the AF6-78 mouse anti-mouse Igh6b (Pharmingen) as a coating antibody (10 μg/ml), which gave reproducible and specific results in ELISA.

The results of the Igh6a and Igh6b ELISA showed that about 25% to 50% of the serum IgM originated from the donor peritoneal B cells in both groups that received BM and PerC cells (fig. 3).

Frequency and classification of H-Ig development

Figure 4 shows the cumulative frequency of H-Ig development in allotype congenic chimeras and control mice. The incidences of H-Ig in unir-

Figure 2. Serum Ig isotype concentrations during aging of allotype-congenic radiation chimeras and age-matched controls. The groups are indicated by (a) C57BL.Igh^a recipient mice of Igh^b BM cells; (b) C57BL.Igh^a recipient mice of Igh^a BM cells and Igh^b PerC cells; (c) C57BL/6 recipient mice of Igh^b BM cells and Igh^a PerC cells; (d) unirradiated C57BL.Igh^a mice; (e) unirradiated C57BL/6 mice.



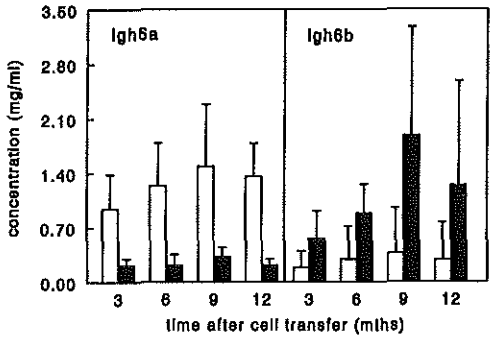


Figure 3. Serum Igh6a and Igh6b (IgM-allotypes) concentrations during aging of allotype-congenic radiation chimeras. The open bars represent the group of C57BL.Igh^a mice that received Igh^a BM cells and Igh^b PerC cells; the black bars represent the group of C57BL/6 mice that received Igh^b BM cells and Igh^a PerC cells.

radiated control mice were consistently higher in the C57BL/6 (Igh^b) mice than in the age-matched C57BL.Igh^a mice, but only after 19 months this difference was statistically significant (two-sided P-value = 0.01). The allotype chimeras approximately followed the frequencies of their original BM allotype. However, 12 months after cell transfer all irradiated groups showed

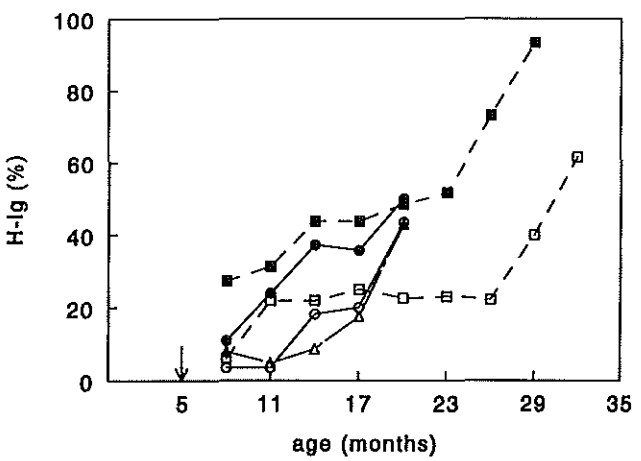


Figure 4. Incidence of H-Ig in the experimental and control groups during aging. (□) C57BL.Igh^a control mice; (■) C57BL/6 control mice; (△) C57BL.Igh^a recipient mice of Igh^b BM cells; (○) C57BL.Igh^a recipient mice of Igh^a BM cells and Igh^b PerC cells; (●) C57BL/6 recipient mice of Igh^b BM cells and Igh^a PerC cells.

an enhanced frequency of H-Ig development as compared to the control mice of the same age. This time-point was just before the initiation of the relatively fast extinction of the irradiated mice, caused by fatal renal damage. We therefore concluded that later than 12 months after lethal irradiation the experimental groups could not be compared any more to the control groups.

Table 1 lists the various serologically determined categories of the H-Ig. The data that are shown here were collected up to one year after cell transfer. C57BL/6 control mice showed a higher incidence ($P<.05$) of total H-Ig than C57BL.Igh^a control mice, but there was no significant difference in the incidences of the individual categories.

The C57BL.Igh^a mice that received only Igh^b BM cells developed less total H-Ig ($P=.0033$), BMG ($P=.004$) and multiple H-Ig ($P=.05$) than the C57BL/6 control group.

The C57BL.Igh^a mice that received Igh^a BM and Igh^b PerC cells showed the same incidences of H-Ig categories as did the C57BL.Igh^a control group. The C57BL/6 mice that received Igh^b BM and Igh^a PerC developed less BMG ($P=.02$) and multiple H-Ig ($P=.03$) than the C57BL/6 control group.

Isotype distribution of the H-Ig

The isotype distribution of the H-Ig in the aged groups is shown in table 2. Comparison with the control groups showed a higher incidence of H-Ig of the IgG2a isotype in the C57BL/6 mice ($P=0.05$). This observation is in accordance with the higher serum levels of IgG2a in this group (fig 2). In analogy, the C57BL.Igh^a mice that received Igh^b BM cells developed more H-Ig of the IgM isotype than the control groups (13.8% vs 3% and 0%), but these numbers were just not significantly different. Overall, the incidences of H-Ig of the IgM isotype were higher in the irradiated groups than in the control groups.

Allotype distribution of the H-Ig

Most H-Ig in C57BL/6 mice were of the IgG2a isotype. A substantial part of the weak H-Ig were IgM. Therefore we determined the frequencies of Igh1 and Igh6 a- and b-allotypes of the H-Ig one year after irradiation and cell transfer (Table 2). At this time point the majority of the mice was still alive and the incidence of H-Ig had reached a maximum in the irradiated mice. The presence of both allotypes of IgM in every recipient mouse during their entire life span indicated successful transfer of BM cells as well as of PerC cells.

We did not find H-Ig of the IgG2a isotype that originated from the PerC cell transfer. The pronounced H-Ig of Ig1a allotype that was found in a single

Table 1. Classification of the H-Ig in aging allotype congenic C57BL chimeras. The follow-up time was one year after transfer of cells.

	Recipient mouse strain			Unmanipulated controls	
	C57BL.Igh^a		C57BL/6	C57BL.Igh^a	C57BL/6
Transferred cells	Igh^b-BM	Igh^a-BM + Igh^b-PerC	Igh^b-BM + Igh^a-PerC	none	none
Total number of mice	63	57	46	33	33
Mice with H-Ig (%) [a]	16 (25.4) [b]	16 (28.1)	25 (54.4)	10 (30.3) [g]	19(57.6)
I. Multiple myeloma	0 (0)	1 (6.3)	0 (0)	0 (0)	0 (0)
II. Benign MG	1 (6.3) [c]	7 (43.8)	4 (16) [e]	4 (40)	10(52.6)
III. Transient MG	10 (62.5)	3 (18.8)	7 (28)	5 (50)	7 (36.8)
Unclassifiable	8 (50)	5 (31.3)	14 (56)	2 (20)	4 (21.1)
Multiple H-Ig	1 (6.3) [d]	2 (12.5)	4 (16) [f]	0 (0)	7 (36.8)

a) The number of mice with H-Ig is the 100% reference value for the percentages of the subsequent categories of MG. Cumulative percentages of all categories of MG can be more than 100% due to the possible presence of (multiple) transient MG and the occurrence of other MG in the same mouse; b) $P=0.0033$; c) $P=0.004$; d) $P=0.05$; e) $P=0.02$; f) $P=0.03$; g) $P<.05$; all P-values were calculated in comparison to the C57BL/6 control group.

Table 2. Isotype distribution of H-Ig components in the sera of aged allotype chimeras. The sera were taken at the age of 17 months ± 2 weeks, i.e. one year after lethal irradiation and cell transfer.

	Recipient mouse strain			Unmanipulated controls	
	C57BL.Igh^a		C57BL/6	C57BL.Igh^a	C57BL/6
Transferred cells	Igh^b-BM	Igh^a-BM + Igh^b-PerC	Igh^b-BM + Igh^a-PerC	none	none
Number of tested mice	58	51	21	33	33
Isotype					
IgG1	1 (1.7) [a)]	3 (5.9)	0	1 (3.0)	2 (6.0)
IgG2b	1 (1.7)	1 (2.0)	0	4 (12.1)	2 (6.0)
IgG3	2 (3.4)	2 (3.9)	2 (9.5)	2 (6.0)	1 (3.0)
IgA	0	0	0	0	1 (3.0)
IgG2a ^{a} (Igh1a)	0	1 (2.0)	0	1 (3.0)	-
IgG2a ^{b} (Igh1b)	1 (1.7)	0	1 (4.8)	-	7 (21.2)
IgM ^{a} (Igh6a)	7 (12.1)	2 (3.9)	3 (14.3)	1 (3.0)	-
IgM ^{b} (Igh6b)	1 (1.7)	6 (11.8)	1 (4.8)	-	0

a) absolute and relative number (% between brackets) of H-Ig components

mouse in the C57BL.Igh^a group that received Igh^a BM and Igh^b PerC cells turned out to be a multiple myeloma. Because of its Igh^a allotype, this multiple myeloma could be identified as originating from B-2 cells in the BM.

In the irradiated groups H-Ig of Igh6a and Igh6b were found, indicating that H-Ig of the IgM isotype could originate from BM cells as well as from PerC cells, with a higher incidence of the latter. The C57BL.Igh^a mice that received only Igh^b BM cells were still able to develop H-Ig of Igh6a and Igh6b allotypes.

DISCUSSION

Malignant representatives of age-associated MG, multiple myeloma and Waldenström's macroglobulinemia, consist of expanding plasma cell clones that produce large quantities of H-Ig (paraprotein). These malignant plasma cells expand in the BM, spleen and/or lymph nodes. However, this category of MG is much less common than the BMG, with a 100 times higher incidence in C57BL mice, and the transient MG. Although it is sometimes possible to find the relatively small benign plasma cell clone in the BM, it is not clear where the excessive monoclonal B cell proliferation originated, what the exact phenotype is of the B cells and what the cell lineage origin is. Information about these parameters might enable us to discriminate in an early phase between malignant and benign MG and could therefore be necessary for correct diagnosis and timely treatment.

In mice it has been shown that roughly two populations of B cells can be distinguished by their phenotype and localization [7, 8, 11, 14]. In adult mice, the B-1 cells are exclusively derived from precursor cells in the peritoneal cavity, probably residing in the omentum [15, 16]. The B-2 cells originate from the BM. Based on this dichotomy we generated different types of IgH allotype chimeras [11] for long-term follow-up of the Ig spectrum. This enabled us to determine the cell lineage origin of the MG by detection of the allotype of the H-Ig component.

While a somewhat decreased median survival time due to an increased incidence of a variety of tumors and of non-neoplastic lesions, among which some late renal damage, was envisaged due to the irradiation of the mice [17, 18], the severely reduced survival as observed in our experimental groups was unexpected. Also the histopathological aspect of the renal lesions was extraordinary. Usually after total body irradiation (TBI) late renal damage is limited to mainly the glomeruli; lesions of tubuli and especially of arterioles and small arteries are rare [18, 19]. Strain differences in survival time after TBI have been described [18] and it could be that the abnormal subendothelial

fibrin transudation and the formation of fibrin thrombi is due to an exceptionally high radiosensitivity of the substrains used in our experiments.

Consequently, the number of H-Ig detected in this study was less than expected. Table 1 shows that one year after lethal irradiation and cell transfer the chimeras had developed about the same number of the various MG categories as the control groups. However, the two control groups lived much longer after this time point than the chimeras and developed still more H-Ig, as is shown in figure 3. The Ig isotype levels in the sera of the chimeras were slightly affected.

The two mouse strains used in these experiments did not develop equal numbers of MG, as appears from table 1. The C57BL.Igh^a mice, whether in the control group or in the allotype-chimera groups, had less H-Ig than their Igh^b counterparts, the C57BL/6 mice. This difference between the two strains was not extended to the individual frequencies of the various MG categories. However, the time point of our observations, *i.e.* one year after irradiation, might be responsible for the latter, as mentioned before. The allotype related difference in development of MG was observed previously in the individual mouse strains [20], as well as in C57BL/KaLwRij and CBA/BrARij radiation chimeras [21] and in (C57BL/LiARij x CBA/BrARij) F1 mice [22].

The determination of the IgM-allotypes of H-Ig in aged chimeras revealed that most of these H-Ig originated from PerC cells (11.8% - 14.3% of total H-Ig). This holds even true for the group of C57BL.Igh^a mice that received only BM cells of Igh^b allotype: in this cohort we detected 7 H-Ig of Igh6a allotype. This was only possible when some of the original Igh^a precursor cells survived the irradiation. Whether these precursor cells resided in the PerC or in the BM is uncertain. Before starting the experiment we confirmed in a pilot study that the dose of 9.5 Gy was lethal to the mice. However, it has been shown that B-1 precursor cells are more radioresistant than their B-2 cell counterparts [14]. This means that there might have been some viable Igh^a B-1 precursor cells left in the PerC of this group of mice after irradiation. These B-1 cell precursors could reconstitute the PerC of these mice, because they did not receive unirradiated, and thus viable, peritoneal wash cells.

The frequency of PerC derived IgM positive H-Ig was inversely related to the serum concentration of the PerC derived IgM allotype (fig. 3). This phenomenon might reflect the clonal restriction of the PerC derived B-1 cells, which increases with age [10].

Besides producing large quantities of IgM natural antibodies [23], B-1 cells are known to provide the lamina propria of the gut with IgA producing plasma cells [24]. It is possible to detect IgA allotypes in the serum; however,

none of the chimeras in this study developed an H-Ig of IgA allotype. The fact that we did not detect IgG2a positive H-Ig derived from the peritoneal cells reflects the small amount of IgG2a produced by these B-1 cells.

Our observation that monoclonal IgM producing MG can originate from B-1 cells does not answer the question whether these excessive proliferations are induced by antigen or whether they are merely a result of the restricted "natural" repertoire of the B-1 cells at old age [10]. We have shown previously that the B-1 cell lineage contributes to the development of MG in genetically susceptible mice [25]. Because we detected only benign IgM producing MG of B-1 cell origin, it is speculative that malignant IgM producing MG, as in Waldenström's macroglobulinemia, can originate from the B-1 cell population in C57BL mice. Whether human Waldenström's macroglobulinemia, (extra-)medullary plasmacytoma, BMG, and transient MG can be manifestations of plasma cell clones of B-1 cell origin, remains to be investigated.

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3

CHAPTER 3

FREQUENCY OF CLONAL DOMINANCE IN THE SPECIFIC ANTIBODY RESPONSE TO DNP-HSA IN CBA AND C57BL MICE REFLECTS THEIR SUSCEPTIBILITY TO AGE-ASSOCIATED DEVELOPMENT OF MONOCLONAL GAMMOPATHIES *

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SUMMARY

The effects of age, genetic background and neonatal thymectomy on the levels and the heterogeneity of the specific antibody response were investigated in an experimental mouse model. Both intact and neonatally thymectomized (NTx) C57BL/KaLwRij (C57BL) and CBA/BrARij (CBA) mice were immunized at the age of 3 ("young") or 22 months ("old"). Highly sensitive antigen-specific immunoblotting techniques (ABL), in combination with agar-electrophoresis and isoelectric focusing (IEF), were used to investigate total specific antibody levels, the number of responding antigen-specific clonotypes, and the dominance of responding B cell clones in the antibody response against dinitrophenylated human serum albumin. After immunization, the specific antibody levels progressively increased in all experimental groups with the exception of old C57BL mice. All mice responded with a specific polyclonal heterogenous response. In addition, some mice showed a clonal dominance of antibody-producing cells, as is reflected in the appearance of distinct homogenous antibody components (H-Ab) in the sera. This clonal dominance was scarce in CBA mice but frequent in C57BL mice. Age at time of immunization and NTx had little if any additive effect on the incidence of H-Ab in either mouse strain. All dominant clones showed different electrophoretic mobility, indicating the proliferation of various clonotypes and not a strain-specific dominance of one clone. In old C57BL mice the specific antibody response was more restricted in heterogeneity as is illustrated by more visible spectrotype bands in IEF and subsequent ABL.

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Hence, in old C57BL mice smaller amounts of specific antibodies were produced by fewer clones. Still the incidence of H-Ab in this group was the same as in the group of young C57BL mice. This indicates that at old age the responding B cell clones are more prone to becoming clonally dominant in C57BL mice. This tendency correlates with the high incidence of spontaneously developing monoclonal gammopathies in aging C57BL mice.

INTRODUCTION

The diversity of antibody responses strongly depends on the available B-cell repertoire in the adult individual. The selection of responding clonotypes is therefore not only antigen dependent, but also under genetic control with a major role for the variable heavy- and light-chain immunoglobulin (Ig) genes, followed by the ability to express the corresponding clonotypes before and after antigenic stimulation [1]. Furthermore, T-B cell interactions affect the quantity and quality of the antibody response.

Long-term effects of these modifying factors become more pronounced during aging [2]. Although the overall Ig levels do not decrease with age, a progressively increasing variation of the Ig levels is a characteristic finding in aging humans as well as in many laboratory animals [3,4]. The occurrence of less heterogenous antibodies with respect to isotype, affinity and idiotype at old age were described previously [5-8]. Electrophoretic and immunodiffusion techniques applied on sera of very old people demonstrated a common deviation of the Ig spectrum, namely an Ig pattern of restricted heterogeneity, often accompanied by transient or persistent homogenous Ig components (H-Ig) [9]. These conditions are clinically classified as monoclonal gammopathies (MG). Three categories of MG are age-associated: 1) B cell malignancy, 2) B cell benign neoplasia, benign MG, and 3) transient MG reflecting an immunodeficiency due to T < B cell imbalance [10].

It is not exactly known why and how these B cell proliferative disorders develop but it has been demonstrated that the clonotypes responsible for the development of H-Ig could have been selected by antigen at a much earlier time in the life of man [11] as well as of mice from the C57BL/KaLwRij strain. Although antigen-specific antibody levels declined gradually with aging in DNP-HSA immunized mice, they still developed age-associated MG with antibody activity to DNP [12,13]. In other words, some memory B cells eventually evolved for yet unknown reasons into clones of a sufficient size to make the H-Ig detectable in the sera, or became a target for an oncogenic event.

We studied the capacity of the immune system to recruit the available

antigen-specific clonotypes directly after immunization. The phenomenon of clonal dominance in antigen-specific antibody formation was of special interest because it can illustrate the intrinsic characteristics of a certain mouse strain to allow for 'overshooting' of B cell clones. If this relation exists, it might help to explain the susceptibility of the different mouse strains to develop an age-associated MG.

Here we investigated the effects of aging, genetic background, and impaired T cell system (using neonatal thymectomy) on the antibody response to DNP-HSA, with special attention to antibody levels, heterogeneity of the antibody response, and the appearance of H-Ab. We used two different inbred mouse strains: the C57BL/KaLwRij, which has a high incidence of spontaneously developing age-related MG, and the CBA/BrARij, which in contrast shows a low incidence of these B cell proliferative disorders [14]. Sensitive immunoblotting techniques allowed for the detection of antigen specific homogenous IgM, IgG and IgA of about 0.005 μg/ml.

MATERIALS AND METHODS

Mice

C57BL/KaLwRij and CBA/BrARij mice were bred and maintained under conventional conditions in the mouse colonies of the REP Institutes TNO in Rijswijk (presently TNO-PG, Dept. of Immunological and Infectious Diseases, Leiden, The Netherlands). All mice received pelleted food (Hope Farms, Woerden, The Netherlands) and acidified water (pH 3-4) *ad libitum*.

Experimental groups

Eight experimental groups were selected, each consisting of 25 mice at the start of the experiment: Two mouse strains, C57BL/KaLwRij (C57BL) and CBA/BrARij (CBA) mice; two age groups, young-adult mice immunized at the age of 2-3 months ("young") and mice immunized at the age of 22 months ("old"); they were used either intact or neonatally thymectomized ("NTx") according to Roubinian as described previously [15,16]. The mice were allowed to live out their life-spans. The 50% survival was 27 and 29 months for intact C57BL and CBA mice, respectively, and 25 months for mice of the NTx groups of both strains. Postmortem microscopic examination of serial sections of the anterior mediastinum of NTx mice revealed complete removal of thymic tissues.

Antigens

A hapten-carrier complex of DNP-HSA was chosen as immunogen. DNP is a hapten with a limited number of epitopes and the immune response to DNP-HSA should thus result in a relatively limited number of clones producing antibodies to DNP. Human serum albumin

(Nordic, Tilburg, The Netherlands) was conjugated to a level of ± 12 DNP molecules per protein molecule according to Good *et al.* [17] and stored in small aliquots at -20°C until use.

Immunization procedure

Mice of the experimental groups received an intraperitoneal (i.p.) injection once a month (four times in total) containing 50 μg DNP-HSA in 50 μl phosphate-buffered saline (PBS) and 50 μl Freund's adjuvant (complete for the first two immunizations and incomplete for the latter two). Control mice, 25 for each experimental group, received only 50 μl PBS and 50 μl Freund's adjuvant. Prior to the first, two weeks after each consecutive immunization, and regularly each third month thereafter until death, small blood samples were taken from their tail veins and the sera were immediately analyzed and/or stored at -20°C for later use. One young and four old NTx C57BL mice, and five old NTx CBA mice died of natural causes during the immunization period, but none of the mice died due to the immunization procedure.

Preparation of transfer membranes

Because polyvinylidene difluoride membranes (Immobilon PVDF; 0.45 μm pore size, Millipore, Etten-Leur, The Netherlands) are hydrophobic, they were pre-wetted first with methanol and then with distilled water. Subsequently, they were incubated with 30 μg/ml DNP-HSA in a 0.5 M $NaHCO_3$ buffer solution (pH 8.0) per cm^2, for at least 2 hours at room temperature [18]. Since DNP is light sensitive, membranes were coated in the dark. To prevent nonspecific binding to the PVDF membrane, unoccupied protein binding sites were blocked with 1% bovine serum albumin (Boseral DEM; Organon Technika, Oss, The Netherlands) in PBS, containing 0.05% Tween-20 (PBS-BSA-T) during 1 h at room temperature. Any undesired detachment of the antigen during subsequent treatment was prevented by "fixating" the membrane with a 2.5% glutardialdehyde solution in a 0.2 M sodium acetate buffer (pH 5.0) for 15 min.

As a negative control for non-specific binding of proteins to the membrane, a serum pool of non-immunized C57BL/KaLwRij mice was used. Since this normal mouse serum (NMS) had a natural antibody activity against DNP (NMS^{DNP}), the NMS was purified by affinity-chromatography using a DNP-binding column (NMS^{DNP-}). After blotting, reactivity of NMS^{DNP-} could be demonstrated only below a 1/200 dilution, reflecting non-specific binding of proteins (data not shown). Thus, antibody activity of serum diluted 1/200 and in higher dilutions was considered specific.

Quantification of the antigen-specific antibody response

The specific antibody response was quantitated by an antigen-specific dot-immunobinding assay (ADIBA), modified from Hawkes *et al.* [19] and Jol-Van der Zijde *et al.* [20]. The antigen-coated Immobilon PVDF membrane was rinsed with PBS, transferred to a dot-blot filtration apparatus (Bio-Rad, Richmond, CA) and washed with 150 μl 0.05% Tween 20 in PBS (PBS-T) per application well. Sera from the experimental animals were serially diluted two-fold (range 1/40,000 to 1/320,000) in PBS-BSA-T and 50 μl of sample was spotted onto the membrane. When the samples had completely soaked into the membrane (30 min without vacuum), they were washed under vacuum with 150 μl PBS-T per well. The blot was then removed from the apparatus and shortly rinsed again, followed by incubation of peroxidase-conjugated rabbit-anti-mouse-Ig (RaM-Ig-PO; Dakopatts, Glostrup, Denmark) and subsequent coloring reaction to reveal the antigen-specific antibodies that bound to the

membrane.

Semi-quantitative determination of the antibody levels was performed with a reflectance densitometer (TR-944, MacBeth, Newburgh, NY) and expressed as relative OD values based on comparison with a standard curve obtained with a serum pool of selected young C57BL mice which were repeatedly immunized with DNP-HSA and reached high levels of corresponding antibodies.

Wieme electrophoresis (W)

High resolution agar gel electrophoresis according to Wieme [21] was modified and carried out as described previously [22]. Each slit was filled with 3 μl of serum, diluted 1/500 and 1/1000 in PBS. The detection limit of H-Ig in sera after total protein staining of the gels was 0.1 mg/ml. When performing immunoblotting techniques, this limit was considerably lower [22].

Isoelectric focusing (IEF)

IEF was performed in a 0.5 mm thick 0.8% agarose gel, pH 3-10 (Agarose IEF, Pharmacia-LKB, Uppsala, Sweden), as described in detail elsewhere [22]. Each slot of the application strip was filled with 5 μl of serum sample, diluted 1/200 in PBS.

Antigen-specific immunoblotting (ABL)

After W or IEF, antigen-specific antibodies were transferred from the gel onto the antigen-coated membrane by diffusion under pressure. The membrane was rinsed in PBS, laid on the gel and covered with three layers of wet and seven layers of dry Whatman No. 3 MM chromatography paper. A weight of 100 g was placed on a glass plate on top of the paper stack. After 30 min, the blot was removed from the gel and rinsed shortly with PBS. The detection limit of antigen specific H-Ig using this technique was 0.005 μg per ml, as reported previously by Nooij *et al.* [22].

Specific color reaction

The blots were incubated with peroxidase-labeled polyclonal rabbit anti-mouse-Ig (RaM-Ig-PO, Dakopatts, Glostrup, Denmark), diluted 1/2500, for 2 hours at room temperature. After intensive washing with PBS-T, they were incubated with substrate solution. A specific color reaction was developed for 10 min in the dark with a freshly prepared solution of 0.02% (w/v) 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO) and 0.03% (v/v) hydrogen peroxide in PBS-T. Finally, the blots were rinsed in distilled water to stop the enzymatic reaction, dried and stored in the dark to prevent bleaching.

Statistical methods

Comparison of frequencies between two groups was performed by Student's t-test, if applicable, or the Mann-Whitney-U test. Proportions were compared by Fisher's Exact test or by chi-square analysis, using Yates continuity correction, when the observed frequencies were higher than 5. P values smaller than 0.05 were considered statistically significant.

RESULTS

Levels of DNP-HSA-specific antibodies after successive immunizations

The specific antibody response was quantitated in serum samples taken prior to the first immunization with DNP-HSA and two weeks after each of the consecutive immunizations (Figure 1). As tested by double radial immunodiffusion with DNP-HSA and DNP-ovalbumin conjugates, each responding serum reacted with both antigens, but not with unconjugated HSA. The pre-immune sera from all groups already contained some antibody activity against DNP-HSA, while the negative control (NMS^{DNP-}) did not. Kinetics of the antibody response after immunization were similar for the young CBA, young C57BL and the old CBA mice. After the first immunization, the response was low, but the response rapidly increased after successive immuni-

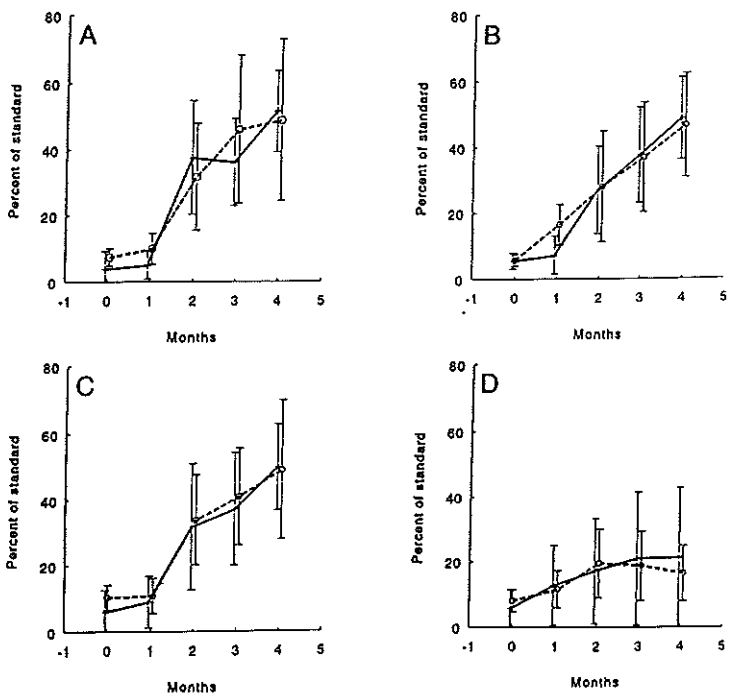

Figure 1. Determination of specific antibody levels by ADIBA after successive immunizations with DNP-HSA. Serum samples were taken prior to the first immunization and two weeks after each of the consecutive immunizations. Results are shown for young CBA (A), young C57BL (B), old CBA (C) and old C57BL (D) mice. Groups of normal intact mice are indicated by a solid line and NTx groups by an intermitting line. Results are in arbitrary units relative to a standard serum pool from mice after repeated immunization with DNP-HSA and are the mean and S.D. of 25 mice per group.

zations. The kinetic pattern of the specific antibody response was aberrant in old C57BL mice as compared to the other groups: mean antibody levels rose gradually, but reached a low-level plateau after the third immunization. Both in intact and in NTx treated old C57BL mice these maximum levels were significantly lower than for young C57BL mice ($P < 0.001$). It should be noted that the standard deviation was significantly higher in the old intact C57BL group than in the old NTx C57BL ($P = 0.01$) or in the young intact C57BL mice ($P < 0.01$), indicating a high individual variation within this group. There were no significant differences in maximum levels of specific antibodies between intact and NTx-treated mice in any of the four experimental groups (Figure 1).

Heterogeneity of the DNP-HSA-specific antibodies as tested by W + ABL

The heterogeneity pattern of DNP-HSA-specific antibodies was determined at regular intervals using W + ABL. Immunizations with DNP-HSA induced a heterogenous antibody response against the antigen in mice of all experimental groups, which was reflected by a smear pattern on the W + ABL blots. The antibody response reached its maximum after the last immunization and gradually subsided during the following months. The electrophoretic patterns in the sera from the C57BL and CBA mice were distinct: sera from the C57BL mice had the maximum of their antibodies in the cathodic gamma-2 region, while this was in the more anodic gamma-1 region for the CBA mice.

In addition to the heterogenous antibody pattern, some sera showed products from antigen-reactive dominant clones, as reflected by the presence of one (example shown in Figure 2) or, less frequently, two DNP-HSA-specific H-Ab components on the W + ABL blots. These H-Ab were of different mobility in different individual mice and had rather variable kinetics: they could be persistent (*i.e.* detectable for more than 6 months) or, in the majority of cases, transient. The fact that the levels of all specific antibodies, including the dominantly produced H-Ab, gradually decreased after the peak response illustrated that these components were regulated in the same way as the other specific antibody-producing clones. Because most of them were not visible on Wieme agar electrophoresis, we can deduce that their concentration was below 100 μg/ml. The frequency of these specific H-Ab components was determined at the peak of the antibody response, *i.e.* after 4 successive immunizations. Results for the sera from the intact mice and the NTx mice are presented in Table 1.

H-Ab in the sera from intact CBA mice were found in low numbers (8%), both in young- and old-immunized animals. In intact C57BL mice, the

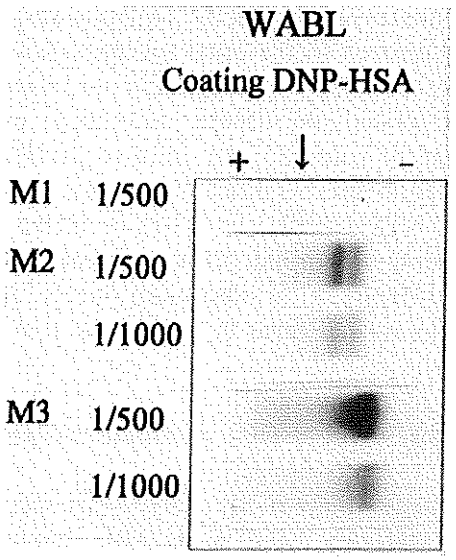

Figure 2. An example of a dominant H-Ab response to DNP-HSA in the serum of a young C57BL mouse (M2), as detected by W + ABL (WABL) on DNP-HSA coated PVDF membranes. A heterogenous response is illustrated by the serum of another C57BL mouse (M3). Preimmune serum of the mouse M2 served as a negative control (M1). The application site is indicated by an arrow, anodic site by +. All sera were tested at dilutions 1/500 and 1/1000.

picture was quite different: in 56% and 48% of the young- and old-immunized animals, respectively, H-Ab could be shown at or around the peak of the antibody response. No new H-Ab arose after the peak response (*i.e.* not until very old age [12]), as was shown by a follow-up of all mice for their entire life-span. In a few old mice, an H-Ab reactive with DNP-HSA could already be demonstrated in preimmune serum.

In NTx CBA mice, antigen-specific H-Ab were found in 16% of the young- and in 30% of the old-immunized animals. In NTx C57BL mice, this frequency was higher: 54% and 52% for the young- and the old-immunized mice, respectively. In the pre-immune sera of NTx mice, H-Ab with activity against DNP-HSA could be demonstrated in three C57BL mice and one CBA mouse to be immunized at old age (21 months), but in none of the animals to be immunized at young age.

The above data show that the incidence of DNP-HSA-specific H-Ab was much higher in the C57BL mice than in the CBA mice. This was measured at the peak of the antibody response where the two strains did not show significant differences in total levels of antigen-specific antibodies, with

Table 1. Homogenous antibody components (H-Ab) with DNP-HSA specificity in pre-immune and immune sera as detected by agar electrophoresis (W) with subsequent antigen-specific immunoblotting (ABL). The immune sera were analyzed at the peak of the antibody response, *i.e.* after 4 successive immunisations.

	CBA		C57BL	
	young	old	young	old
A. Intact mice				
Number of tested mice	25	25	25	25
Mice with pre-immune H-Ab (n (%))	0 (0)	0 (0)	0 (0)	1 (4)
H-Ab after 4 immunizations (n (%))	2 (8)	2 (8)	14 (56)[a]	12(48)[b]
B. Neonatally-thymectomized (NTx) mice				
Number of tested mice	25	20	24	21
Mice with pre-immune H-Ab (n (%))	0 (0)	1 (5)	0 (0)	3 (14)
H-Ab after 4 immunizations (n (%))	4 (16)	6 (30)	13 (54)[c]	11(52)[d]

a Two-sided P-value = 0.0006 as given by Fisher's exact test when compared to the group of young intact CBA mice.

b Two-sided P-value = 0.0036 as given by Fisher's exact test when compared to the group of old intact CBA mice.

c Two-sided P-value = 0.0072 as given by Fisher's exact test when compared to the group of young thymectomized CBA mice.

d Two-sided P-value = 0.2 as given by Fisher's exact test when compared to the group of old thymectomized CBA mice.

exception of the old C57BL group. This group responded with significantly lower levels of anti-DNP antibodies. Nevertheless they developed an equal incidence of DNP-HSA-specific H-Ig, as did the young C57BL group.

Neonatal thymectomy did not result in a clear change in the incidence of DNP-HSA-specific H-Ab, although the NTx CBA groups showed a tendency to develop relatively more of these components in the serum than the intact CBA mice (16% to 30% vs. 8%). The generally highly significant differences between intact CBA and C57BL mice disappeared in the old thymectomized mice (Table 1).

Heterogeneity of DNP-HSA-specific antibodies as tested by IEF + ABL

The heterogeneity of the antibody response against DNP-HSA was examined further in sera taken at the peak of the response from the groups of intact, unthymectomized mice. They were tested for spectrotype bands at optimal dilutions by isoelectric focusing. For young C57BL, young CBA and old CBA mice, these dilutions were 1/250, 1/500 and 1/1000. Since the antibody response was quantitatively much lower in the old C57BL mice, optimal dilutions for this group were 1/10, 1/30 and 1/100. Individual spectrotypes in sera from each experimental group are illustrated in Fig. 3. On average, the anti-DNP antibodies in the sera from C57BL mice, making up the spectrotype pattern, had an electric charge in a more basic pH range than those of CBA mice. In the sera illustrated, no H-Ab were detected using W + ABL, only a heterogenous antibody response was demonstrated (data not shown). Unlike the W + ABL technique, the heterogenous antibody pattern after IEF + ABL was represented by a pattern of small discrete bands on a smear background. In Table 2 the mean number of detectable spectrotype bands for each experimental group is presented. The number of bands was determined in 11-23 sera taken at the peak of the antibody response against DNP-HSA. Young CBA and young C57BL mice showed on average the same number of bands. The difference between young and old CBA was not statistically significant (P=0.0547). The spectrotype of old C57BL mice showed significantly more bands than old and young CBA and young C57BL mice (Figure 3 and Table 2).

Table 2. Comparison of antigen-specific spectrotype bands in immune sera of intact mice as detected by IEF + ABL. *

Experimental group	bands [a]			two-sided P-value
Young CBA (n=23)	15.0 ± 0.7	n.s.		
Young C57BL (n=11)	13.3 ± 0.8			p=.0547
Old CBA (n=23)	17.1 ± 0.8	p<.001	p<.001	
Old C57BL (n=13)	26.6 ± 1.6			

* *Note:* The sera used in this experiment did not show H-Ab components.

[a] The number of bands (mean ± SE) as determined in sera taken at the peak of the antibody response against DNP-HSA.

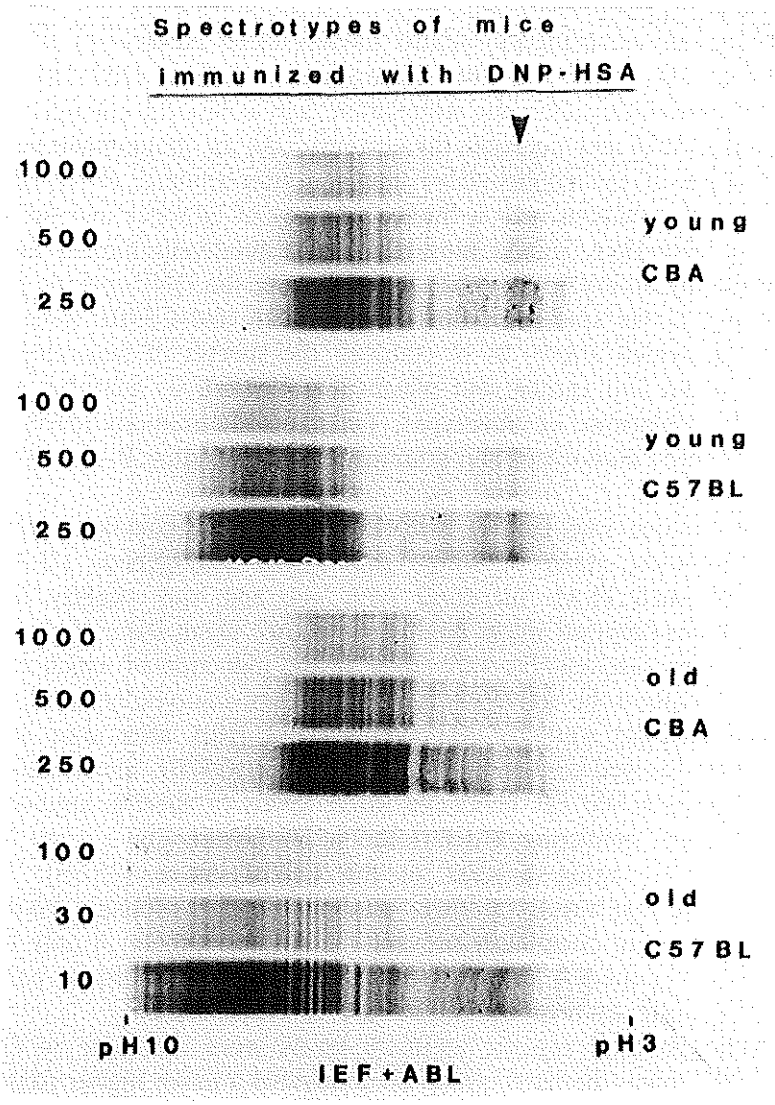

Figure 3. Illustration of antigen-specific spectrotypes in sera from 4 individual mice, each of a different experimental group. All mice had been immunized with DNP-HSA. Analysis was performed by IEF + ABL (pH 3-10) of sera taken at the peak of the antibody response. The application site is indicated by an arrow. For each experimental group, 3 serum dilutions were chosen that were optimal for counting the number of antigen-specific spectrotype bands. Note that sera of old C57BL mice were far less diluted than sera of the other groups. The application site is indicated by an arrow.

DISCUSSION

Using an experimental mouse model, three parameters were under study which may influence the heterogeneity of the DNP-HSA-specific antibody response after antigenic stimulation: age at time of immunization, the genetic background and neonatal thymectomy. As an immunogen, a hapten-carrier complex of dinitrophenylated human serum albumin (DNP-HSA) was chosen. DNP is a hapten with a limited number of epitopes and the immune response to DNP-HSA should thus result in a relatively limited number of clones producing antibodies to DNP. Furthermore, the DNP-specific precursor cell frequency in the spleens of non-immune CBA and C57BL mice is about the same [1], which allows us to compare the spectra of generated specific antibodies in the two strains, but which does not tell us anything about the total available repertoire of clonotypes. Total specific antibody levels, number of antigen-specific clonotypes and dominance of responding B cell clones were studied in detail.

Pre-immune sera from a few old mice already contained DNP-HSA-reactive antibodies, some of which even were homogenous (H-Ab). The frequency of such H-Ab increased with age and was slightly higher in neonatally thymectomized mice. Nothing is known about their origin. It might be that they are cross-reactive antibodies, in the induction of which regulatory T cells are thought to be active via the idiotype-anti-idiotype network [23,24].

The specific antibody response to DNP-HSA after four successive immunizations showed similar kinetics in CBA and C57BL mice. Only C57BL mice immunized at old age generated lower levels of specific antibodies than the other groups, reflecting a deterioration of the immune system due to a more pronounced aging process in the C57BL mice [25,26]. Neonatal thymectomy did not result in lower antibody levels, probably because this intervention does not completely prevent T cell development; it only results in a moderately impaired T cell system in adult life. In addition, part of the DNP-specific antibody response might be T-cell independent.

Although total specific antibody levels were the same in CBA and young C57BL mice, the latter group demonstrated a much higher frequency of H-Ab at the peak of the antibody response regardless of NTx treatment. On the other hand, old C57BL mice responded with lower levels of antibodies to DNP-HSA, but developed the same incidence of H-Ab as did the young C57BL mice. This illustrates that the detection of dominant clones in the antibody response to DNP-HSA was not dependent on the total serum anti-DNP antibody levels in these two strains. The electrophoretic mobility of the detected H-Ab components differred from mouse to mouse, indicating the proliferation of different clones and not a strain-dependent dominance of one

clone. This peculiar homogenous response of C57BL mice has already been noted by Takiguchi *et al.* [27] and adds new insight to earlier observations of high incidence of H-Ig, regardless their specificity [25].

The sensitive IEF + ABL immunoblotting technique (detection limit for H-Ab 670 ng/ml; [22]) was used to visualize the products of specific antibody-producing clones and to gain an insight into the heterogeneity of their response. The evaluation of the analyses is not easy. The banding pattern (spectrotype), using an appropriate pI range and proper dilutions, consists of a smear with superimposed tiny bands. It should be kept in mind that none of the sera used for this analysis contained an H-Ab component, as was demonstrated by W + ABL, a technique with a higher sensitivity for H-Ab than IEF + ABL [22]. It can therefore be safely assumed that the banding pattern found here does not reflect one or more H-Ab in the strict sense of the word, but the products of several "more prominent" clones. For the CBA and the young C57BL mice the same dilutions of the tested sera could be used to generate comparable conditions. However, since the antibody response was quantitatively much lower in the old C57BL mice than in the other groups (see ADIBA analysis), a different set of dilutions had to be used to yield a comparable banding pattern in the sera from this group. When sera from the old C57BL mice were analyzed at the optimal dilutions as found for the CBA mice, no smear or banding pattern was seen at all. The other way round, when sera from the CBA mice were analyzed at the optimal dilutions as found for the C57BL mice, only a very dark smear without any banding was visible (data not shown).

We believe it is legitimate to use the number of bands to gain an insight in the heterogeneity of the antigen-specific antibody response [18]. The more bands are detected on top of the smear, the less heterogenous is the response and the more of the prominent clones become visible. The age at time of immunization had a profound effect on the number of spectrotype bands as seen in the old C57BL mice. Thus not only the total generated amount of specific antibodies was diminished in old C57BL mice, but the specific antibody response was also more restricted in heterogeneity as illustrated by more visible spectrotype bands in IEF and subsequent ABL. Hence, in old C57BL mice smaller amounts of specific antibodies were produced by fewer clones. Still the incidence of H-Ab, a marker of clonal dominance in the specific antibody response, in this group was the same as in the group of young C57BL mice. This indicates that at old age the responding B cell clones are more susceptible to become clonally dominant in C57BL mice.

Young CBA mice resembled very much young C57BL mice in their response to DNP-HSA. They showed similar total specific antibody levels as

well as an equal number of spectrotype bands. The only difference between these groups was a higher incidence of H-Ab in young C57BL mice. This observation stresses the importance of other factors than an impaired T cell system or age at time of immunization in the development of clonal antibody responses, which was also illustrated previously by the fact that clonality of the antibody response was not dependent on the presence of antigen-specific T-helper cells [28]. Probably strain dependent, genetically defined factors account for the high tendency of C57BL mice to develop clonal dominance in antigen specific antibody responses. This tendency correlates with the high incidence of spontaneously developing monoclonal gammopathies in aging C57BL mice [14,25]. Nooij *et al.* were able to find MG with antibody-specificity for the antigen (DNP-HSA) used for immunization at young age. Deducing from spectrotype characteristics, the majority of these spontaneously appearing H-Ig at old age were "new" but some obviously originated from previously expanded dominant clones [12].

Thus, although the mechanism accounting for the high frequency of clonal dominance at the peak of the antibody response in C57BL mice might also be responsible for the high tendency to develop a deregulated clonal B cell proliferation that results in MG, this mechanism is not restricted to certain clonotypes. It rather might be a random process, hitting one of the 'overshooting' B cell clones that respond to the corresponding antigen. The results of this study show that this mechanism is already present at young age in C57BL mice, but is almost absent in CBA mice.

It is not likely that the development of a B cell clone into a benign or malignant MG is triggered by antigen alone, but external and internal antigens may contribute to this process by starting the development of clonal expansions of memory-B cells that may escape control of the immune system. Although the T cell system is not an important factor in the initiation of clonality of the antibody response, it certainly plays an important role in the progression into bigger clones and subsequent development of MG [16,29-31]. The biological significance of dominant clones in antibody responses, observed in some animals but not in others within the same inbred strain, is speculative and remains to be elucidated.

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4

CHAPTER 4

TENFOLD INCREASED INCIDENCE OF SPONTANEOUS MULTIPLE MYELOMA IN LONG-TERM IMMUNOSUPPRESSED AGING C57BL/KaLwRij MICE *

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SUMMARY

Persons undergoing maintenance immunosuppressive treatment (MIST) were shown to be at increased risk for the development of early malignancies, often of cells of the immune system. Very little is known about the late effects of MIST. Some clinical studies indicated an age-related increase in the incidence of plasma cell disorders, in particular in that of multiple myeloma (MM). In the present study the influence of MIST on the development of monoclonal B-cell proliferative disorders, monoclonal gammopathies (MG), was studied in an animal model, the C57BL/KaLwRij mouse. This strain is known for its susceptibility to develop with aging MG similar to those in humans. Two widely used immunosuppressive treatment protocols in humans (azathioprine/prednisolone and Cyclosporin A/prednisolone) were tested in young and adult mice. Both regimens were shown to increase tenfold the incidence of spontaneous MM. Unexpectedly, the same high incidence of MM and in addition the development of a life-shortening lymphoblastic lymphoma were found in a high frequency in the control group that received Cremophor EL only, *i.e.* the solvent of Cyclosporin A. Repeated experiments with an other lot of Cremophor showed a sixfold increased frequency of MM but no lymphoblastic lymphoma. With respect to the life-span and the incidence of hemopoietic neoplasms the least harmful drugs for MIST appeared to be azathioprine/prednisolone. The results of the experiments in this C57BL/KaLwRij mouse model give a warning for increased incidence of MM in susceptible aging individuals and address a question whether Cremophore EL is a safe solvent for Cyclosporin A.

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INTRODUCTION

The number of individuals undergoing maintenance immunosuppressive treatment (MIST) for long periods of time (*i.e.* recipients of organ transplants, some patients with autoimmune disorders) grows steadily. Short term evaluation studies suggest that they are at markedly increased risk for the development of malignancies, often of lymphoid origin [1-4]. The majority of these post-transplant lymphoproliferative diseases are B-cell non-Hodgkin lymphomas and are usually EBV related. Malignancies of B cells in a more mature stage are rare [5]. Little is known about the late effects of MIST. In some older studies the late effects of selected immunosuppressants on immunocompetence were studied [6-8]; however, any information on the late effects of more recently introduced immunosuppressants is scarce [9]. Our own studies performed in man [10] indicated that age-related immunodeficiency, which gradually develops in individuals with genetically determined different speed, may substantially be accelerated and potentiated by MIST. We hypothesize that MIST will eventually lead in susceptible individuals to a more frequent development of benign and malignant proliferative disorders, mainly of B-cells.

The aim of this study was to test this hypothesis. Because a follow-up investigation needs to involve the whole life-span of an individual a suitable animal model is necessary. The aging mice of the C57BL/KaLwRij strain offer an appropriate experimental model for such a study because these mice spontaneously develop in a high frequency monoclonal B-cell proliferative disorders that are similar to those in humans [11-13]. Two widely used immunosuppressive treatment protocols: azathioprine/prednisolone and Cyclosporin A (CsA)/prednisolone were tested in young as well as in late adult mice. Both regimens were shown to increase the frequency of spontaneous multiple myeloma (MM) in mice of this strain. Control experiments using Cremophore EL, the solvent of CsA, showed the same incidence of MM and indicated strong adverse effects of this preparation.

MATERIALS AND METHODS

Mice

SPF derived female C57BL/KaLwRij mice from the colony of the TNO Institute for Experimental Gerontology in Rijswijk (presently TNO-Prevention and Health, Leiden, The Netherlands) were maintained under clean conventional conditions. Detailed information on husbandry, health status, survival data, and age-related pathology of the strain has been published previously [14]. A complete necropsy was done on mice within two hours of death or

immediately following euthanasia of moribund animals. Histological examination of representative samples of all relevant tissues was performed according to a standard protocol. Ultrastructural examination by electron microscopy was performed on bone marrow samples in mice suspected of having multiple myeloma.

Young adult (4 to 5 months old) and late adult (14 months old) mice of the C57BL/KaLwRij strain were first immunized with a purified human IgG1-lambda paraprotein (KAT). Each mouse received intraperitoneally 50 μg of the protein dissolved in 0.1 ml phosphate buffered saline (PBS) and emulsified with 0.1 ml of complete Freunds adjuvant and boosted after 4 weeks with the same amount of protein dissolved in incomplete Freunds adjuvant/PBS (1:1). The immunization was used to obtain an additional marker for the detection of some H-Ig that were expected to develop with aging. Immunization by itself, using protein antigens and Freund's adjuvant or adjuvant alone, does not lead to the increased incidence of multiple myeloma as shown in our previous experiments [11,15].

One month later, the mice were submitted to MIST by intraperitoneal route because administration *per os* did not guarantee an equal intake of the drugs. The mice received azathioprine (The Wellcome Foundation Ltd., London, UK) and prednisolone (prednisolone sodiumsuccinate, N.V. Organon, Oss, The Netherlands): Group A (young mice: n= 50, adult mice: n= 50), or Cyclosporin A (kindly donated by Sandoz AG, Basel, Switzerland) and prednisolone: Group B (young mice: n= 50, adult mice: n= 47). These drugs were given twice a week in doses corresponding to those used in human MIST as calculated according to Freireich *et al.* [16]. The dosage schedule was: Azathioprine, first five doses 0.9 mg each per mouse and further 0.4 mg/mouse; CsA, first six doses 3.4 mg each per mouse and further 0.72 mg/mouse; prednisolone: first dose of 0.6 mg/mouse, six following doses of 0.3 mg/mouse and further 0.12 mg/mouse, continued over the whole remaining life-spans of the mice. The average total doses received per mouse were: MIST from the age of late adult: 27.3 mg of azathioprine and 9.0 mg of prednisolone, 47.5 mg of CsA and 6.8 mg of prednisolone; MIST started at young age: 53.6 mg of azathioprine and 17.6 mg of prednisolone, 117.4 mg of CsA and 17.9 mg of prednisolone.

Control groups consisted of mice receiving the solvent of the corresponding drug twice a week only, *i.e.*, 0.25 ml PBS for azathioprine (Group D, young mice: n= 28, adult mice: n= 30) and the solvent for CsA, Cremophor EL (Sigma Chemical Company, St. Louis, USA; lot 97F0246), 160 mg in 0.25 ml of 10% ethanol and saline (Group C, young mice: n= 51, adult mice: n= 48).

Due to unexpected results in group C, several additional experiments were performed to test the Cremophor EL influence. They, however, could only be performed with an other, more recent Cremophor EL, lot 70H0361. Mice of group CR1 (n= 15) received, intraperitoneally, a dose of Cremophore EL (in 0.25 ml) four times higher (650 mg) than those of group C in the first experiment. The same dose was given to mice of the group CR2 (n= 15) but once a week. Groups CR3 (n= 15) and CR4 (n= 15) represented the same conditions as group C in the first experiment; however, only mice of group CR4 were immunized with the human IgG1-lambda paraprotein before starting MIST. Control mice were injected intraperitoneally with 0.25 ml PBS/10% ethanol solution, either once a month (group K1) or once a week (group K2). All mice in the second experiment were approximately 6 months of age at the start of the experiment.

Detection of homogenous immunoglobulin components

Blood samples were taken every 2 months and the sera were tested for the presence of homogenous immunoglobulin components (H-Ig) by high resolution electrophoresis and

immunoelectrophoresis [17]. The appearance of H-Ig in the sera of individual mice was tentatively classified according to the following criteria [12]: (1) H-Ig which progressively develop within 2 months and reach levels above 5 mg/ml. This pattern is typical for MM spontaneously appearing in this mouse strain at the frequency of about 0.5%. (2) Distinct H-Ig with a concentration below 4 mg/ml, which persist for at least 6 months and can be detected till the death of the animals. This pattern is typical for benign monoclonal gammopathy (BMG), a benign neoplasia of B cells, which is frequent in the C57BL/Ka mice. (3) Transient H-Ig components, usually of a low concentration, appearing for a limited period of time. These H-Ig were described as resulting from insufficient control of T-cell function in various immunodeficiencies with preserved B-cell function (including the immunodeficiency due to aging of the immune system). (4) H-Ig components which appeared shortly before the death of the mice were considered unclassifiable.

The potential specificity of H-Ig to the human IgG1-lambda protein used for the immunization of the mice was tested by antigen specific immunoblotting as described previously [17].

Statistical analysis

Survival curves were estimated by the method of Kaplan-Meier with differences assessed by the logrank test. Proportions were compared by chi-square analysis, using Yates continuity correction, or Fisher's exact test when a number was smaller than 5. P-values smaller than 0.05 were considered statistically significant.

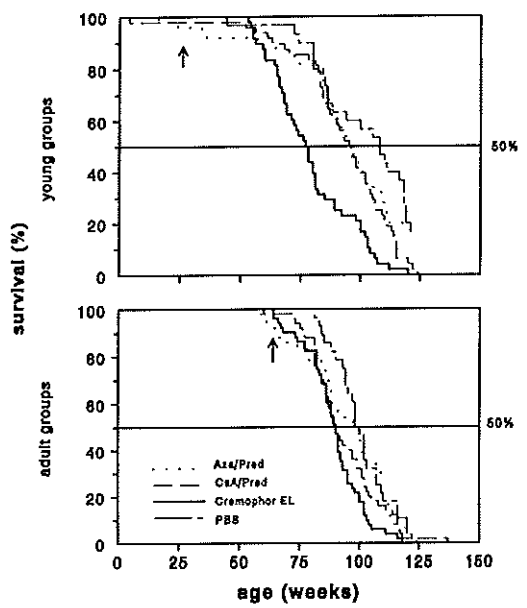

Figure 1. Survival curves of experimental and control mice. Start of immunosuppressive treatment is indicated by arrows. The upper and lower graphs represent the groups of young adult and the late groups of adult mice, respectively. A 50% survival line is shown in both graphs. Experiments with young groups A and D were terminated at 121 weeks.

Table 1. Survival analysis of C57BL/KaLwRij mice submitted to two maintenance immunosuppressive treatments.

	Mice (n)	χ^2	Logrank P-value	Hazard ratio
Group A:				
azathioprine/prednisolone [a]	young (50)	0.2023	$P > 0.2$	1.10
	old (50)	0.4685	$P > 0.2$	1.17
Group B:				
Cyclosporin A/prednisolone	young (47)	15.4782 [b]	$P < 0.001$	0.47
		4.2164 [a]	$P < 0.01$	1.55
	old (50)	2.0002 [b]	$0.1 < P < 0.2$	0.77
		2.7998 [a]	$0.05 < P < 0.1$	1.45
Group C:				
Cremophor EL/10% ethanol [a]	young (48)	25.06	$P < 0.001$	2.75
	old (51)	11.8466	$P < 0.001$	2.08

[a] Compared to PBS-control group D;
[b] Compared to Cremophore EL/10% ethanol control group C.

RESULTS

Survival

The life-span of mice in which MIST was started at the age of 6 months was not significantly different from the life-span of mice in which the same treatment-protocol was started at late adult age (data not shown). The treatment-protocol of group A, azathioprine and prednisolone, affected the life-span neither of young nor of adult mice as compared to the PBS-control group D (Table 1). The young immunosuppressed mice in group B had a shorter life-span compared to the PBS treated control group. While assessing differences between the CsA/prednisolone treated group B and the Cremophor EL treated group C, it became apparent that young mice in the control group C had a significantly shortened life-span as compared to young mice treated with the immunosuppressants. This was not the case in adult mice, but both young and adult mice treated with Cremophor EL had a significantly shortened life-span as compared to group D ($P < 0.001$) (Fig.1).

Because of these adverse effects of Cremophor EL in group C,

additional experiments were performed, although with a different batch of Cremophor EL. Only when given a 4 times higher dose of Cremophore once a week (group CR2), the life-span of the mice was significantly shortened ($P < 0.001$; Hazard Ratio 2.76). None of the other groups of the second experiment showed differences in survival compared to PBS/10% ethanol control groups. No differences were detected in life-spans of the PBS-control group D of the first experiment and the PBS/ethanol control groups K1 and K2 in the second experiment (data not shown).

Homogenous immunoglobulin components

The frequency curve of H-Ig (Fig.2) and its slope of the control mice receiving only PBS did not differ much from those in untreated normal C57BL/Ka mice as seen in numerous previous experiments [12]. The H-Ig frequency curve of mice receiving azathioprine followed more or less that of its control group, being only slightly higher in the beginning of the treatment. The frequencies of H-Ig in group C and even more in the CsA/prednisolone treated group B were clearly higher than that of group D for several months after initiation of the treatment. At the end of the observation period, which is in fact determined by the natural ending of the lives of the animals, the frequencies of H-Ig were similar in all groups, reaching values above 70%. The characterization of the H-Ig components (Table 2) showed that the pattern of MM as detected in high resolution serum electrophoresis was seen in 5% of the mice in each of the groups A, B, and C, which was a ten times higher incidence than was expected ($P < 0.0001$, one-proportion frequency analysis). Also transient small H-Ig components were most frequently seen in these three groups. No difference in the incidences of BMG was found. In group A the incidence of unclassified H-Ig was significantly lower than in the control group D. Over all, no differences were detected in H-Ig incidence and characteristics between groups B and C.

In the additional experiments on the effect of Cremophor EL, heavy H-Ig components with the characteristics of MM were detected in one case in group CR1 and one in group CR4. Thus the incidence of MM in the later experiments in all Cremophore groups together was 3%.

H-Ig isotypes and specificity

Testing the isotypes of H-Ig revealed that 11 of the 17 MM were of the IgG2b isotype. Of the remaining 6 MM, 4, 1 and 1 belonged to the IgG2a, IgG1 and IgG3 subclasses, respectively. All of them were of the kappa light chain type. The most frequent isotype of the H-Ig in the BMG group was

Table 2. Frequency of monoclonal gammopathies in C57BL/KaLwRij mice submitted to two maintenance immunosuppressive treatments.

Experimental group	n[a]	H-Ig (%) +	H-Ig (%) -	MM[b]	BMG[b]	Transient H-Ig[b]	Unclassified H-Ig[b]
A: azathioprine/prednisolone	95	80	20	5[c]	37	26[d]	12[g]
B: CsA/prednisolone	97	89	11	5[c]	41	23[e]	20
C: Cremophore EL/10% ethanol	97	89	11	5[c]	36	25[f]	23
D: PBS	53	87	13	0	51	6	30

[a] Number of mice suitable for evaluation.
[b] Percentage of mice within this category of monoclonal gammopathy.
[c] $P<0.0001$ as tested in a one proportion frequency analysis, using the expected frequency of 0.5% for MM.
[d] Yates corrected P value = 0.004 when compared to group D.
[e] Yates corrected P value = 0.015 when compared to group D.
[f] Yates corrected P value = 0.007 when compared to group D.
[g] Yates corrected P value = 0.001 when compared to group D.

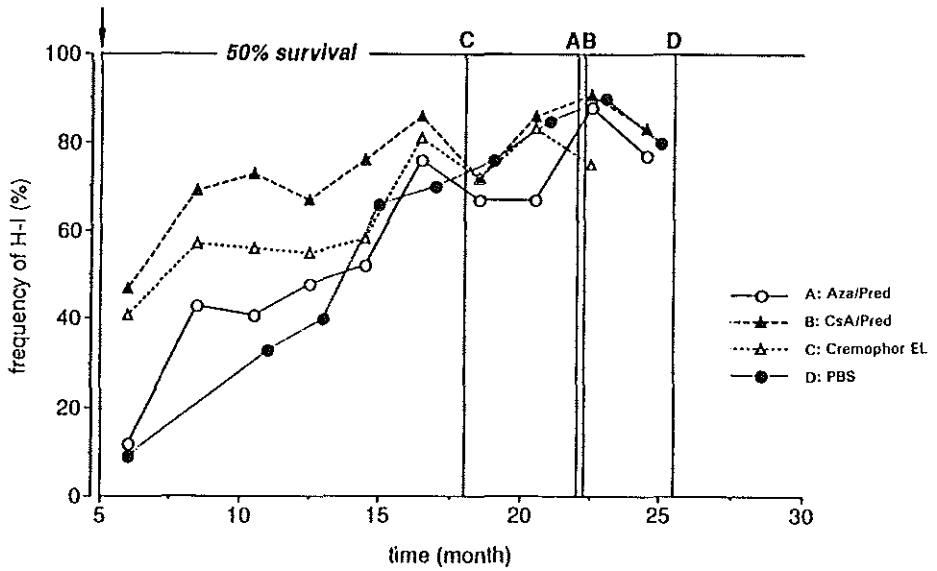

Figure 2. Frequency curves of H-Ig in mice treated from the age of 5 months with Azathioprine/prednisolone (A), CsA/prednisolone (B), Cremophor EL (C), and PBS (D) in relation to the age (in months) of the mice. The 50% survival value of mice of the different groups is indicated by the vertical lines. The arrow indicates the time at which the immunosuppressive treatments were started.

IgG2a. H-Ig of the transient type were of various isotypes, including IgM. There was no clear-cut H-Ig of the IgA isotype found in any of the groups.

Tests for the antibody specificity of individual H-Ig components to the antigen used for immunization revealed that one of 114 cases of BMG and one of 15 cases of MM had a positive anti-human IgG activity. The latter was a mouse (BL-59) from group A, treated with azathioprine/prednisolone. At the age of 22 months, this mouse developed an IgG2b-kappa paraprotein that reached within the remaining 5 months of life a concentration far above 10 mg/ml (Fig 3). The epitope on human IgG recognized by this mouse myeloma protein (as tested by a number of human IgG paraproteins of different subclasses and light chain types) was shown to belong to a common determinant on the gamma chain. Similar specificity was also found when the BMG H-Ig was tested. In none of the cases a specificity to IgG1 subclass, to lambda light chain type, or to the KAT-protein idiotype was detected.

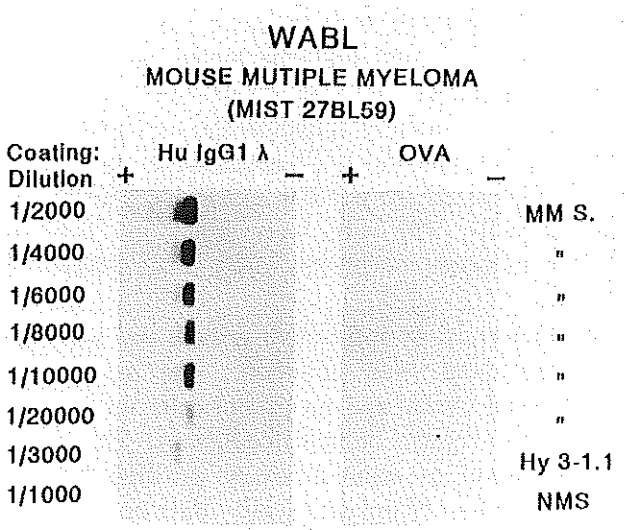

Figure 3. Demonstration of the antibody activity to human IgG of the myeloma protein (BL59) by antigen-specific immunoblotting. The membrane on the left side was coated with the purified human IgG1-lambda myeloma protein (KAT) used for immunization of the mice prior to the start of immunosuppressive treatment; as a control, coating with ovalbumin was used on the right side. Serum of the BL59 mouse (MM S.) at the age of 27 months was tested in dilutions from 1/2000 up to 1/20000. Normal mouse serum (dil. 1/1000) and ascites of a hybridoma 3-1.1 protein (± 5 mg/ml) with activity to a human IgG1 epitope (dil.1/3000) were used as negative and positive controls, respectively. Anode and katode are indicated by + and - symbols.

Histopathological examination

Hematopoietic neoplasms were common in all experimental groups (Table 3), whereas other neoplasms were rare and randomly distributed over the experimental groups. The most common neoplasm was the follicular center cell lymphoma (FCCL), a slowly progressive malignancy of B cells [14]. In mice treated from young age with CsA/prednisolone (group B), the incidence of FCCL was significantly higher as compared to group C, but was not different when compared to the PBS control group D.

Lymphoblastic lymphoma (LL) occurred significantly more in the group that had received Cremophore EL, but was rare in the other treatment groups. This unexpected high incidence of LL in group C was another reason to start the additional experiment. There were no LL found in mice of this latter experiment.

Histiocytic sarcomas (HS) were moderately frequent in all groups.

Twelve mice had more than one hematopoietic tumor: four mice had FCCL and HS, six mice had FCCL and LL, one mouse had LL and HS, and one mouse had FCCL, LL and HS.

FCCL was most frequently found in the mesenteric lymph node, but other lymph nodes, spleen and Peyer's patches were also commonly affected. The FCCL was characterized by nodular expansive growth and contained a mixed population of cells. The cells were small and large lymphoid cells mixed with variable numbers of plasma cells, macrophages, multinucleated cells and granulocytes.

LL was characterized by infiltrative growth and was usually leukemic. Bone marrow involvement was common. The neoplasm was present throughout the peritoneal cavity and neoplastic cells infiltrated the abdominal viscera from the serosal surface or hematogenously. The neoplasm consisted of homogenous population of non-cohesively growing lymphoid cells with numerous mitotic figures. Neoplastic cells did not contain cytoplasmic immunoglobulin as demonstrated by immunoperoxidase histochemistry on paraffin sections (not shown).

HS was primarily present in the liver, lung, lymph nodes and uterus. The tumor consisted of ovoid or spindle shaped histiocytic cells. Multinucleated cells and erythrophagocytosis were common.

MM was characterized on light microscopy by the presence of multiple foci of pleomorphic plasmacytoid cells without distortion of the normal architecture of the bone marrow or osteolysis. These were most commonly found in the pelvic bones and in the distal femur and proximal tibia. Ultrastructural examination demonstrated typical plasmacytoid cells with abundant rough endoplasmic reticulum and a well developed Golgi-apparatus.

DISCUSSION

Short term studies evaluating the effects of MIST on the development of neoplasias showed an increased risk of the treated persons for malignancies, often of lymphoid cells [1-5]. Only little is known about the late effects of long-lasting MIST. Literature review [5] and preliminary data from our studies on kidney recipients [10] indicated increased frequencies of MM and BMG that were related more to the higher age than to the duration of MIST. This led to the hypothesis that MIST accelerates and potentiates the development of an age-related immunodeficiency and eventually leads in susceptible individuals to benign and malignant neoplasias mainly of the B cells. This hypothesis seems to be corroborated by the present experiments for at least the increased incidence of MM. A tenfold increase in the incidence of

Table 3. Incidence of hematopoietic neoplasms in C57BL/Ka mice submitted to two maintenance immunosuppressive treatments.

	Experimental groups			
	A	B	C	D
Neoplasm / age of mice	**(Aza/pred)** No.(%) [a]	**(CsA/pred)** No.(%)	**(Cremophore EL)** No.(%)	**(PBS)** No.(%)
Follicular center cell lymphoma (FCCL)				
young [b]	4/16 (25)	10/16 (63) [c]	2/16 (13) [d]	4/7 (50)
old	8/16 (50)	9/16 (56)	7/16 (44)	3/8 (43)
total	12/32 (38)	19/32 (59) [e]	9/32 (28)	7/15 (47)
Lymphoblastic lymphoma (LL)				
young	0/16 (0)	1/16 (6) [f]	9/16 (56)	1/7 (13)
old	2/16 (13)	1/16 (6) [g]	8/16 (50) [h]	0/8 (0)
total	2/32 (6)	2/32 (6) [i]	17/32 (53) [j]	1/15 (7)
Histiocytic sarcoma (HS)				
young	1/16 (6)	1/16 (6)	4/16 (25)	1/7 (14)
old	2/16 (13)	1/16 (6)	4/16 (25)	0/8 (0)
total	3/32 (9)	2/32 (12)	8/32 (25)	1/15 (7)

a) Number of mice with neoplasm/total number of mice in which postmortem examination was performed; b) Age at start of maintenance immunosuppression; c) Yates corrected P value = 0.011 when compared to group C; d) Two-sided P value = 0.045 as given by Fisher's Exact test when compared to group D; e) Yates corrected P value = 0.023 when compared to group C; f) Yates corrected P value = 0.008 when compared to group C; g) Two-sided P value = 0.015 as given by Fisher's Exact test when compared to group C; h) Two-sided P value = 0.022 as given by Fisher's Exact test when compared to group D; i) Yates corrected P value = 0.0001 when compared to group C; j) Two-sided P value = 0.006 as given by Fisher's Exact test when compared to group D.

MM was observed in both treatment regimens. The incidence of BMG, a benign B-cell neoplasia, was not increased. However, BMG is a typical phenomenon of old age and therefore, the shortened survival of the mice in some of the experimental groups may have influenced the results.

The frequency distribution of isotypes within H-Ig of the different categories was comparable with that seen in other experiments [11,12]. While the most frequent isotype of H-Ig in BMG was IgG2a, in MM, typically, the IgG2b isotype was clearly dominant. An interesting finding was the anti-IgG specificity of one MM and one BMG to the protein used for immunization prior to the MIST initiation. Both paraproteins recognized a common determinant on the human IgG1-lambda protein. This indicated that both BMG and MM developed from B-cell clones responding to a specific antigenic stimulation, even long before the disorder developed. As also our other previous experiments indicated [11,15], it may be the memory B cell that becomes target for oncogenic events.

As far as the other malignancies are concerned, there was no significant difference in the occurrence of HS among the four groups. Young mice in group C had the lowest incidence of FCCL, even less than control group D. Since FCCL is a disease of old age, the shortened survival of these mice can explain this finding. Although malignant LL was detected in a few mice of the MIST treated groups and once in the PBS-control group D, 53% of mice ($P<0.006$, Table 3) in the Cremophore control group C had histologically confirmed LL.

The effect of Cremophor EL unexpectedly complicated the whole study. Due to its highly carcinogenic effect (MM, LL) in the first experiment, it was further tested in additional experiments. There, the incidence of MM was found to be 3%, but that of LL was not increased. The second lot of Cremophore EL diminished the survival probability significantly only in high doses (experiment 2, data not shown). The only plausible explanation for our findings leading to the development of malignant LL in a high frequency and causing the shortened survival of the mice, would be the presence of an unknown contaminant in the first lot of Cremophor EL with carcinogenic properties. Our search for some additional information on the previous lot of Cremophore EL remained unsuccessful. It may well be that the effect of Cremophore EL in the first experiment was an example of (co-) clastogenic effects of Cremophore, which is the enhancement of genotoxicity by Cremophore in the presence of a carcinogenic substance [19]. The mechanism by which Cremophore, a derivative of Castor-oil and ethylenoxide, enhances carcinogenicity is not known. It is used as a solvent for hydrophobic drugs, such as CsA and paclitaxel. It possibly facilitates carcinogenic substances to cross the cell membranes. The incomplete reversal of multiple drug resistancy

by Cremophore may also be a mechanism by which this solvent induces cytotoxicity or even malignant transformation [20]. Our observation stresses the importance of well defined control groups. While Cremophore is being used as a solvent for intravenous application only for a short period in the beginning of MIST, it itself or its derivatives used for peroral treatment may not be innocent solvents, especially when carcinogenic contaminants could be present.

In this study using a mouse model, azathioprine/prednisolone were shown to be the least harmful drugs for MIST when considering life-span and incidence of hematopoietic neoplasms. Only the incidence of MM was increased, but this was also the case in the CsA/prednisolone treated group. In this latter group, it is not clear whether Cremophore contributed to the increased incidence of MM and this should further be studied. In humans, the question should be addressed whether the genetically determined increased susceptibility for the development of MM could be predicted and, consequently, whether any long-term immunosuppression protocols could be avoided or adapted to minimize the risks.

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CHAPTER 5

IMMUNOGLOBULIN V_H GENE SEQUENCE ANALYSIS OF SPONTANEOUS MURINE IMMUNOGLOBULIN-SECRETING B-CELL TUMORS WITH CLINICAL FEATURES OF HUMAN DISEASE

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SUMMARY

The 5T series of multiple myelomas (MM) and Waldenström's macroglobulinemia-like lymphomas (WM), which developed spontaneously in ageing mice of the C57BL/KaLwRij strain, shows clinical and biological features that closely resemble their corresponding human diseases. In order to compare the patterns of somatic mutation in V_H genes of mouse tumors with those of human counterparts, we have determined and analyzed sequences of immunoglobulin (Ig) V_H genes of five cases of murine MM, two of WM and one of biclonal benign monoclonal gammopathy (BMG). Four of five MM and 2/2 WM cases used V_H genes of the large J558 family; 1 MM used a gene of the VGAM3.8 family and both clones of the BMG used genes of the 36-60 family. N-region insertions were observed in all cases, but D-segment genes were only identified from 6/9 cases, which were all from the D-SP family and translated in reading frame 3. Compared to human MM, in which the V_H genes have been found to be consistently hypermutated (mean% $\pm$ SD = 8.8 $\pm$ 3.2), the degree of somatic mutation in the murine tumors was significantly lower (mean% $\pm$ SD = 2.9 $\pm$ 2.3). There was no significant evidence of clustering of replacement mutations in complementarity-determining regions (CDR), a feature considered to be characteristic of antigen selected sequences.

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However, one clone of the biclonal BMG case showed intraclonal variation, a feature described in some cases of human BMG. These results indicate that murine V_H genes in mature tumors differ from human counterparts in the level and distribution of somatic mutations, but support the concept that BMG may be distinct from MM.

INTRODUCTION

Monoclonal B cell proliferative disorders are a common feature in the elderly, both in humans and in laboratory animals [1]. Benign and malignant forms are able to produce large quantities of monoclonal immunoglobulins, which are detectable in the serum. Multiple myeloma (MM) is the most common malignant plasma cell tumor, producing IgG, IgA, IgD or IgE. However, there is a benign counterpart of MM that also involves plasma cells, but with stable levels of the monoclonal immunoglobulin and no clinical characteristics of malignant disease. This benign form has been designated as BMG or monoclonal gammopathy of undetermined significance (MGUS), the latter term being used on the grounds that only time will show whether a monoclonal disorder is truly benign or will develop into malignant disease. In fact, the rate of conversion from MGUS to MM is approximately 15% within a median time of 9.6 years [2]. In humans, Waldenström's macroglobulinemia (WM) is a relatively rare low grade lymphoma involving lymphoplasmacytoid cells that secrete monoclonal IgM. The course of disease tends to be influenced by the molecular nature of the IgM, with hyperviscosity or cryoglobulinaemia as common features.

In human disease, immunoglobulin (Ig) V gene sequences of various B-cell tumors have been investigated extensively. Recent data indicate that the malignant cell of MM is a B cell that has passed the germinal centre and undergone somatic hypermutation [3-5]. Both Ig V_H and V_L genes are extensively mutated and do not show intraclonal variation, indicating that the malignant plasma cells are no longer able to accumulate further mutations. Furthermore, there is clustering of replacement mutations in CDR regions in V_H genes in 25% of cases [5,6], consistent with a role for antigen in selecting V_H sequences. Clustering has also been found in V_L in some cases [6]. The lack of intraclonal variation might be a distinctive feature of MM, since data from human MGUS studies demonstrated that a proportion of cases of MGUS showed intraclonal variation, indicating that the tumor cell may still be under the influence of a mutation mechanism [7]. There have been fewer studies of

WM, but the limited sequence data on V_H and V_L genes show evidence for somatic mutation and intraclonal homogenity [8].

The 5T series of MM and WM, which developed spontaneously from ageing mice of the C57BL/KaLwRij strain, shows clinical and biological features that closely resemble those of the corresponding human diseases and could be used as experimental models [9,10]. Studies on the influences of T-cell function, antigenic stimulation, and genetic background on monoclonal gammopathy (MG) development have been described previously [11,12]. In this paper, we report sequences of Ig V_H genes for 5 MM, 2 WM and 1 biclonal BMG. The sequences are analyzed in comparison with human disease counterparts.

MATERIALS AND METHODS

Cells

Spontaneously developed mouse MM and WM lines have been maintained and propagated by intravenous transfer of bone marrow or spleen cells for several generations in syngeneic C57BL/KaLwRij recipients. Their cytogenetic abnormalities are relatively stable, as reported previously [13]. In this study, we used spleen cells of mice in terminal phase with high levels of serum monoclonal proteins, as detected by serum electrophoresis. The following established tumor lines were studied: 5T2, an IgG2aκ-secreting myeloma; 5T7, an IgG2bκ-secreting smouldering myeloma; 5T13 and 5T33, both IgG2bκ-secreting, aggressive myelomas; 5T14, an IgG1κ-secreting, moderately aggressive myeloma; 5T10 and 5T16, both aggressive, IgMκ-secreting WM.

The biclonal BMG arose in a long-term immunosuppressed old C57BL/KaLwRij mouse [11]. Both paraproteins, IgG2aκ and IgG2bκ, were detectable in the serum for more than 6 months without clinical signs of progression, and can therefore be considered as BMG rather than MGUS. Bone marrow cells were flushed out of the femura and tibiae of the mouse and subsequently used in this study.

cDNA synthesis and amplification

Total mRNA was extracted from the cells by RNAzol B (Cinna Biotex Labs Inc, Houston, TX). Single-stranded cDNA was synthesized using oligo (dT) primer in a Reverse Transcription system (RT) (Promega, Madison, WI). The Ig V_H genes were amplified by polymerase chain reaction (PCR) using Taq DNA polymerase and a 5' universal FR1 V_H primer, or a mixture of 5' primers specific for each of the V_H leader sequences in combination with a mix of J_H primers, or an appropriate 3' constant region primer (Table 1). The conditions of the PCR amplification after initial denaturation of the cDNA at 94 °C for 5 min, comprised 30 cycles of 1 min at 94 °C, followed by 1 min at 60 °C, decreasing in the first 5 cycles with 1 °C each cycle to 55 °C, and a final 1 min at 72 °C. The PCR products were run in a 1.5% agarose gel, and the bands were cut out and purified using the Geneclean kit (Bio 101 Inc, Vista, CA). The purified products were ligated into pGEM-T vector (Promega). The ligation mixture was used to transform JM109 competent cells. Plasmid DNA was prepared from over-

Table 1. Primers used in mouse V_H gene amplification.

VH FR1 consensus:	5'-AGG TSM ARC TGC AGS AGT CWG G-3'
L-J558:	5'-ATG GRA TGG ASC TGG RTC TTT-3'
L-VGAM:	5'-ATG GAA TGG CTG TGG AA-3'
L-IIc:	5'-ATG AAA TKC AGC TGG RTY AT-3'
L-Va:	5'-ATG ATR GTG YTR AKT CTT YTG-3'
MHALT1:	5'-TGG RAT GSA GCT GKG TMA TSC TC-3'
MHALT2:	5'-ATG RAC TTC GGG YTG AGC TKG G-3'
MHALT3:	5'-ATG GCT GTC TTG GGG CTG CTC-3'
JH1:	5'-TGA GGA GAC GGT GAC CGT GGT CCC-3'
JH2:	5'-TGA GGA GAC TGT GAG AGT GGT GCC-3'
JH3:	5'-TGC AGA GAC AGT GAC CAG AGT CCC-3'
JH4:	5'-TGA GGA GAC GGT GAC TGA GGT TCC-3'
5T13 FR1:	5'-GGC TGA GCT TGT GAA GCC TGG-3'
5T13 CDR1:	5'-ACC AGC TAC TGG ATG CAC TGG-3'
VH heptamer:	5'-ATG TGG TTR CAA CRC TGT GTC-3'
$C\gamma$:	5'-CAC AGG RRC CAG TGG ATA GAC-3'
$C\mu$:	5'-GCT CTC GCA GGA GAC GAG GGG GA-3'

IUPAC-IUB codes for nucleotides are used.

night cultures using QIAprep Spin Plasmid kit (QIAGEN GmbH, Hilden, Germany). At least 6 clones were sequenced for each case.

Screening of 5T13 V_H-related germ line gene

C57BL genomic DNA was amplified by PCR using 5'-primers based on 5T13 V_H FR1 and CDR1 region sequences together with a 3'-primer based round the heptamer recombination sequence (Table 1). The PCR conditions were as above except that the final annealing temperature was 50 °C. The PCR products were purified, cloned and sequenced.

Sequencing and sequence assignments

The nucleotide sequences of amplified V_H genes were determined by the dideoxynucleotide chain termination reaction using the Sequenase™ kit (United States Biochemicals Corp, Cleveland, OH) or the T7 Sequencing kit ™ (Pharmacia Biotech Inc.). Both T7 and SP6 promoter primers (Promega) were used for sequencing. Sequence alignment analysis was carried out by searching the Entrez databases of the National Center for Biotechnology Information (NCBI) using the BLAST program [14].

RESULTS

Five MM, two WM and one BMG were studied. The disease phenotypes and V_H gene usage are summarized in Table 2. Comparison of the sequences with the NCBI's Entrez database was made, and the most homologous germ line genes were considered as the germ line donors. In some cases, sequences were aligned to rearranged genes for best match, since the closest germ line genes were far less homologous and probably not the actual germ line counterparts.

MM V_H sequence analysis

V_H gene sequences. The V_H gene sequences derived from the 5 MM cases are shown in Figure 1. 5T2 used a gene from the VGAM3.8 family, but the other 4 cases all used distinct V_H genes derived from the largest J558 family (for a review of mouse V_H gene family, see ref. 15). The best germ line match for 5T2 is VGK7 [16]. There were six nucleotide substitutions, of which three were silent and three replacement. 5T7 was almost identical to the germ line gene S1.2 V_H [17], with one silent substitution in FR2 and one replacement substitution in CDR2, changing Ser to Asn. 5T33 was highly homologous to an expressed V_H gene from a monoclonal antibody directed against carcinoma-associated antigen 17-1A [18]. One nucleotide difference was found in FR3, resulting in an amino acid change. Although 5T33 was aligned against an expressed gene, the fact that 5T33 V_H and 17-1A V_H were highly homologous strongly indicates that they were derived from a common germ line gene. 5T13 V_H and 5T14 V_H were less well matched to known germ line genes. The closest germ line match of 5T14 found in the Entrez database was the V102.1 gene [19]. 5T14 had nine nucleotide differences, introducing one silent and seven replacement mutations.

The known germ line gene in the database to which 5T13 V_H was most homologous was VH124 [20]. Compared to VH124, 5T13 V_H had 14 nucleotide substitutions that resulted in 11 replacement amino acid changes. This low percentage homology between 5T13-derived V_H gene and the closest germ line gene could be due either to somatic mutation of a germ line gene, or to the presence of a novel germ line gene yet to be identified. Therefore, the corresponding germ line gene of 5T13 V_H was investigated further by screening PCR products amplified from genomic DNA isolated from a C57BL mouse using two 5T13-specific 5'-primers and the consensus heptamer 3'-primer. The first 5T13-specific primer is located in FR1, and the second in CDR1. Both primers had two mismatches with VH124 (Table 1 and Figure 1). In total, 11 clones amplified with FR1 primer and 8 clones with CDR1

Table 2. Analysis of MM-, WM-, and BMG-derived V_H gene sequences.

Proliferative disorder	Isotype	V_H family	D_H	J_H	Most homologous V_H genes	% homology
MM 5T2	IgG2a-k	VGAM3.8	D-SP2.5	J_H 3	VGK3	97.5
MM 5T7	IgG2b-k	J558	-	J_H 2	S1.2VH*	99.6
MM 5T13	IgG2b-k	J558	D-SP2.3	J_H 3	VH124*	92.3
MM 5T14	IgG1-k	J558	-	J_H 4	V102.1*	96.6
MM 5T33	IgG2b-k	J558	D-SP2.5	J_H 2	17-1AVH**	99.3
WM 5T10	IgM-k	J558	D-SP2.2	J_H 1	VH205.12*	95.9
WM 5T16	IgM-k	J558	D-SP2.2	J_H 2	MUSIHCVRA**	98.5
BMG 4.1Ca	IgG2a-k	36-60	D-SP2.2	J_H 2	6D6VH**	95.6
BMG 4.1Cb	IgG2b-k	36-60	-	J_H 3	VHMRB9**	98.9

* The best matched germ line V_H genes in the Entrez database.
** The best matched rearranged V_H genes in the Entrez database.

primer were sequenced. Of the 11 FR1 clones, 9 were identical to VH124, the others were different and had even lower homology to 5T13 V_H. Of the 8 CDR1 clones, 4 were identical to VH124, one differred from VH124 by two nucleotides, and the remaining 3 clones were different and less homologous as compared to 5T13 V_H. VH124 was therefore considered to be the genuine donor of 5T13 V_H.

The distribution of the somatic mutations in MM-derived V_H genes is shown in Table 3. Analysis of the distribution of somatic mutations in each sequence was carried out by the method of Chang and Casali [21]. In this method, each V gene sequence is analyzed codon by codon for significance of deviation from the germ line sequence. A modification of the binomial distribution model is then used to calculate whether the probability (p) of an excess (in CDR) or scarcity (in FR) of replacement mutations resulted by chance alone. 5T7 and 5T33 were not included, as the numbers of mutations were too small. In all 3 cases, the observed and expected numbers of mutations were not significantly ($p > 0.05$) different in either FR or CDR, indicating no evidence for antigen selection in the V_H sequences.

CDR3 and J_H sequences. The CDR3 of 5T2 was composed of DSP 2.5 D segment and J_H3 gene. Both D and J segments were used in germ line configuration. N-region nucleotide insertions, either G or C, were observed at both V_H-D and D-J_H junctions. The CDR3 of 5T7 was short with 6 amino acids and the J_H2 gene was utilised. The nucleotides between V_H and J_H could be derived either entirely from N additions or contributed partially by a D segment too short to be identified with certainty. The CDR3 sequence of 5T14 was also short, and again there was no clearly identifiable D gene. In this case, however, it was more likely that the V_H was rearranged directly to the J_H4 gene, and the 6 nucleotides between V_H and J_H were derived entirely from N-region insertions, since the nucleotides are exclusively C and G, which have been found to be predominant in N regions [22]. The CDR3 of 5T13 was composed of the DSP 2.3 D segment and J_H3 gene. There were N insertions at both V_H-D and D-J_H junctions. The CDR3 of 5T33 V_H was formed by the DSP 2.5 D segment and J_H2 gene. N additions were only seen at the V_H-D junction. In all cases, there were no mutations in J_H segments, and N-region nucleotide insertions were predominantly G and C. In the 3 cases where the D segments were identifiable, reading frame 3 was used exclusively.

WM V_H sequence analysis

V_H gene sequences . Two WM cases, 5T10 and 5T16, were investigated.

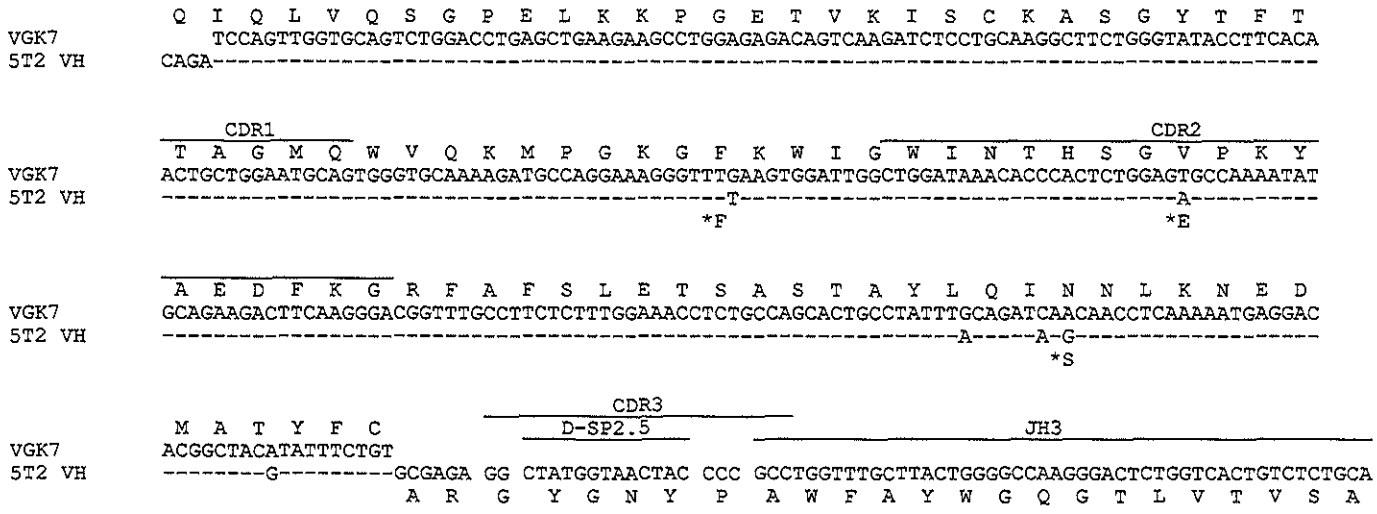

Figure 1. Nucleotide and deduced amino acid sequences of V_H genes derived from MM. Comparisons were made with the closest germ line or expressed V_H genes. Dashes represent identity with the representative germ line sequence. Replacement amino acids are starred.

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                E  V  Q  L  Q  Q  S  G  P  E  L  V  K  P  G  A  S  V  K  I  S  C  K  A  S  G  Y  T  F  T
S1.2VH          GAGGTCCAGCTGCAACAATCTGGACCTGAGCTGGTAAAGCCTGGGGCTTCAGTGAAGATATCCTGTAAGGCTTCTGGATACACGTTCACT
5T7 VH          ----------------------------------------------------------------------------------------

                     CDR1                                                                          CDR2
                D  Y  Y  M  K  W  V  K  Q  S  P  G  K  S  L  E  W  I  G  D  I  N  P  N  N  G  G  T  S  Y
S1.2VH          GACTACTACATGAACTGGGTGAAGCAGAGCCATGGAAAGAGCCTTGAGTGGATTGGAGATATTAATCCTAACAATGGCGGTACTAGCTAC
5T7 VH          --------------------------------A-------------------------------------------------------A----
                                                                                                      *N

                N  Q  K  F  K  G  K  A  T  L  T  V  D  K  S  S  S  T  A  Y  M  Q  L  N  S  L  T  S  E  D
S1.2VH          AACCAGAAGTTCAAGGGCAAGGCCACATTGACTGTAGACAAGTCCTCCAGCACAGCCTACATGGAGCTCCGCAGCCTGACATCTGAGGAC
5T7 VH          ----------------------------------------------------------------------------------------

                                                CDR3
                S  A  V  Y  Y  C  A  R  D                                  JH2
S1.2VH          TCTGCAGTCTATTACTGTGCAAGAGAT
5T7 VH          -------------------------- GAAAGGTT CTTTGACTACTGGGCCCAAGGCACCACTCTCACAGTCTCCTCA
                                           E  R  F   F  D  Y  W  A  Q  G  T  T  L  T  V  S  S
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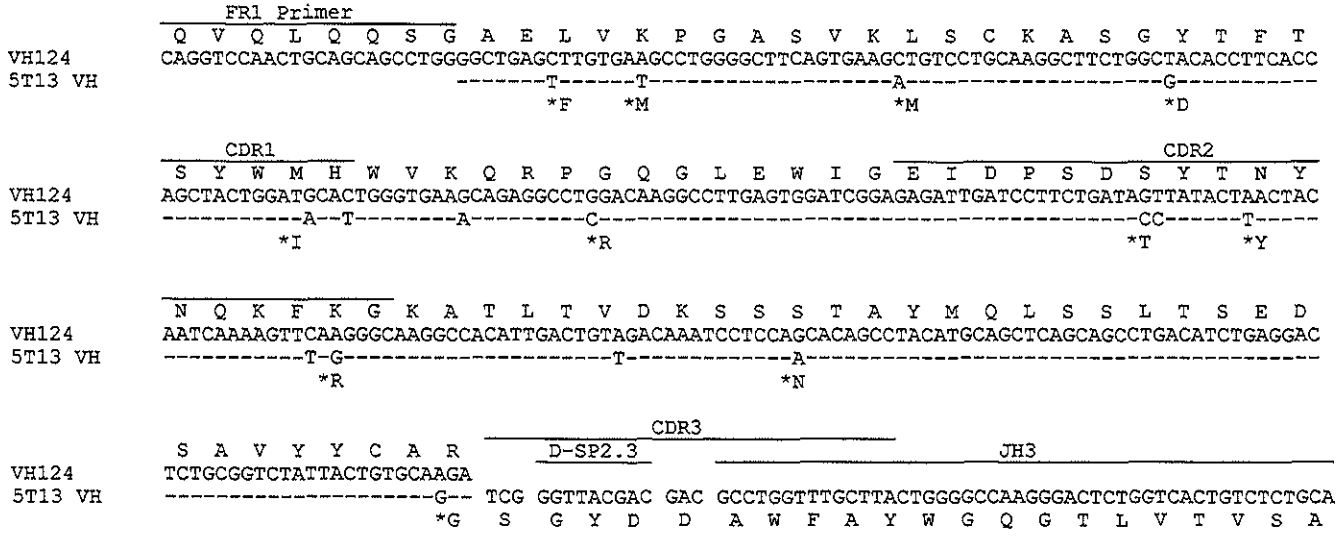
Figure 1. (Continued).



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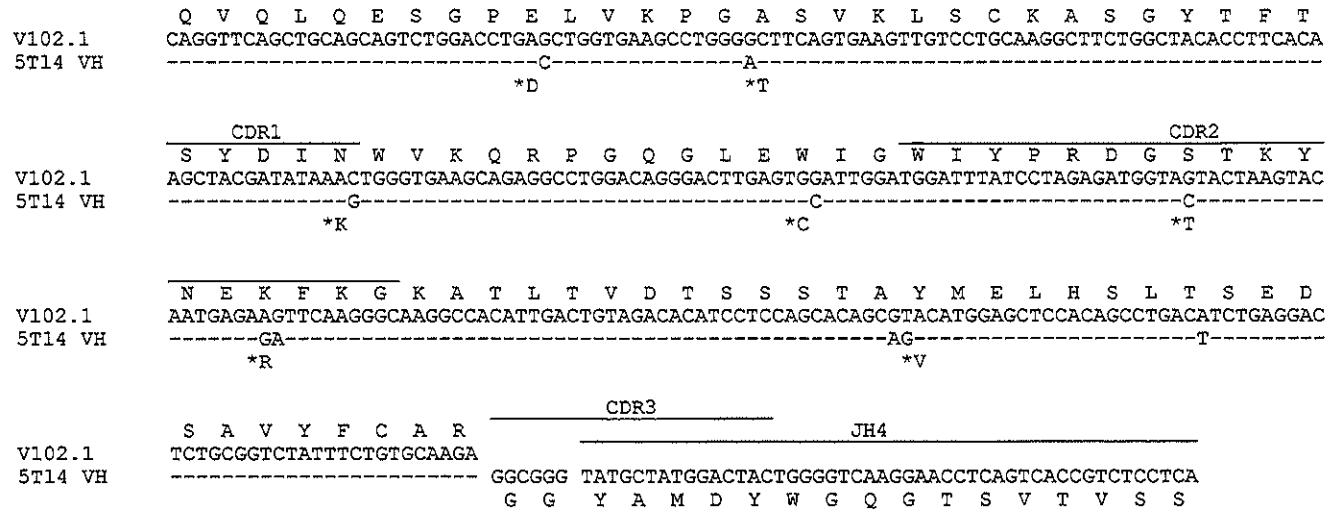

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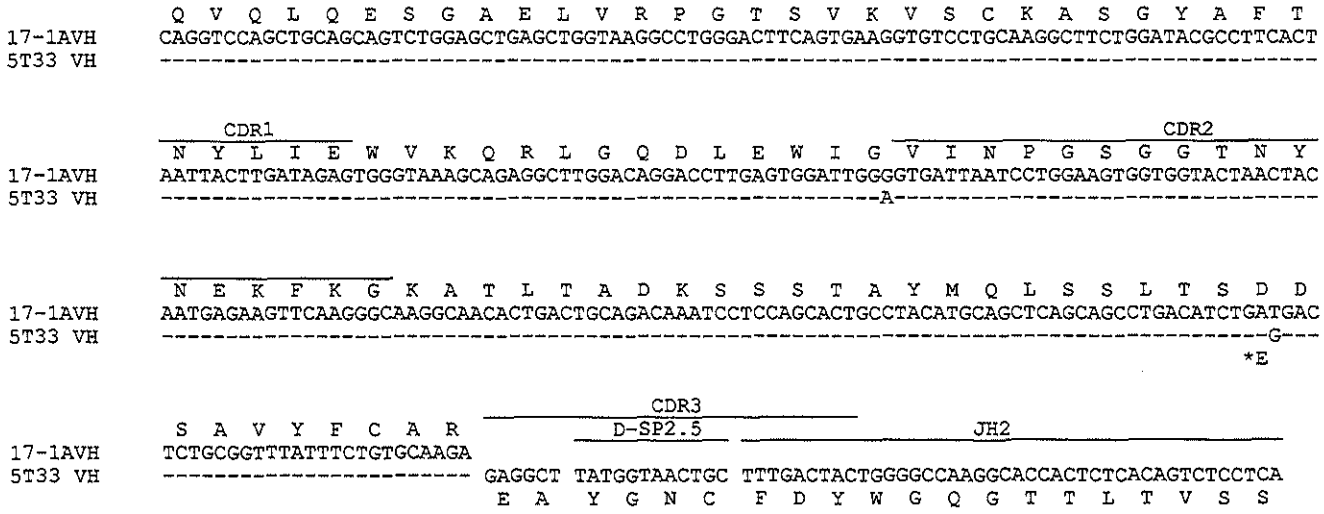

Figure 1. (Continued).

Table 3. Distribution of mutations in MM-, MW-, and BMG-derived V_H genes.

		observed			expected	
Tumor	Region	R	S	R:S	R:S	*p*
5T2	FR	2	4	0.5	3.4	0.08
	CDR	1	0	-	3.6	0.39
5T13	FR	6	3	2	2.9	0.06
	CDR	4	3	1.3	5.4	0.21
5T14	FR	4	2	2	3.0	0.13
	CDR	3	1	3	4.8	0.18
5T10	FR	5	1	5	3.1	0.17
	CDR	3	2	1.5	4.9	0.22
5T16	FR	3	1	3	3.4	0.16
	CDR	0	0	-	3.8	0.43
41C-A	FR	5	2	2	2.9	0.10
	CDR	5	1	5	4.2	0.07
41C-B	FR	2	0	-	2.9	0.36
	CDR	2	0	-	4.7	0.15

The expected theoretical inherent R:S mutation ratio and the probability (*p*) that observed R mutations resulted from chance only were calculated according to Chang and Casali [21].

Again, genes from the J558 family were used in both cases. The V_H sequences aligned to the best matched sequences in the Entrez database are shown in Figure 2. 5T10 was 96% homologous to the germ line gene VH205.12 [23]. 5T10 had 11 nucleotide substitutions, two were silent mutations and nine replacement. 5T16 was closest to an expressed V_H sequence derived from a hybridoma producing anti-human procollagenase antibody [24]. There were four nucleotide differences, resulting in 3 amino acid changes.

The distribution of the somatic mutations in WM-derived V_H genes was also analyzed according to Chang and Casali (Table 3). Again, there was no significant evidence of antigen selection in either case (Table 3).

CDR3 and J_H sequences. 5T10 had a long CDR3 sequence that was composed of the D-SP2.2 D segment and J_H1 gene. The stretch of nucleotides

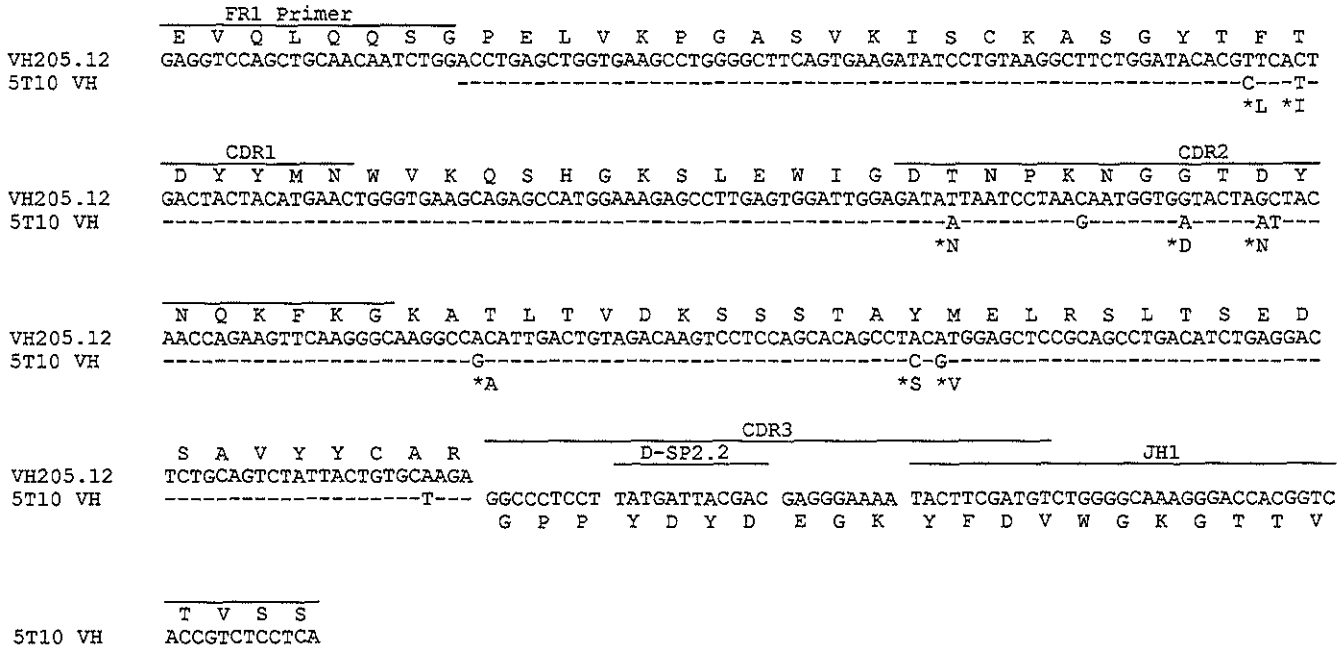

Figure 2. Nucleotide and deduced amino acid sequences of V_H genes derived from WM-like lymphomas. Comparisons were made with the closest germ line or expressed V_H genes. Dashes represent identity with the representative germ line sequence. Replacement amino acids are starred.

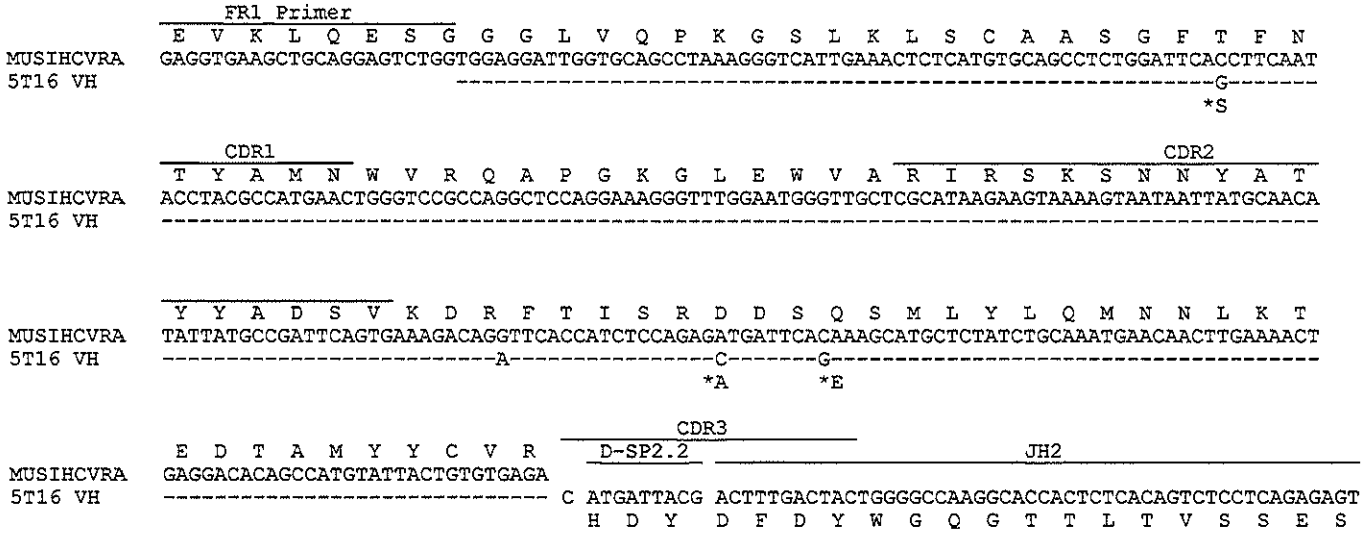

Figure 2. (Continued).

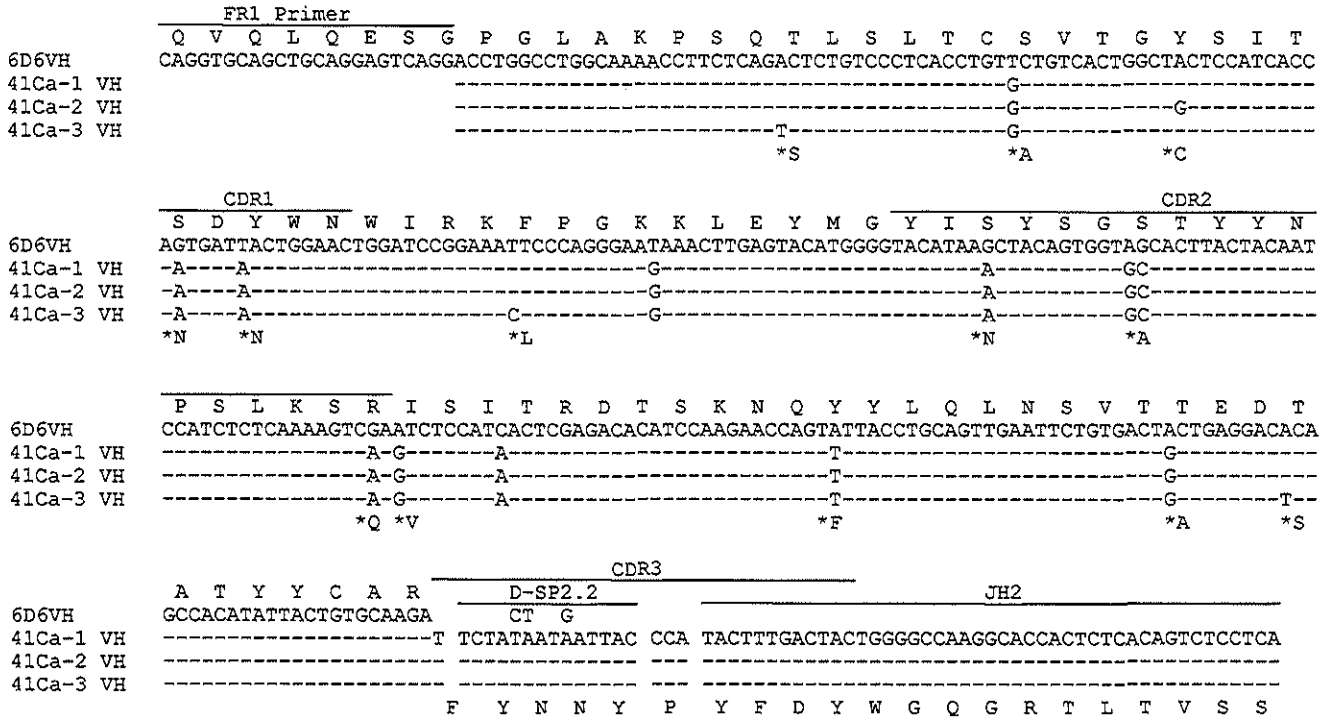

Figure 3. Nucleotide and deduced amino acid sequences of V_H genes derived from mouse BMG. Comparisons were made with the closest germ line or expressed V_H genes. Dashes represent identity with the representative germ line sequence. Replacement amino acids are starred.

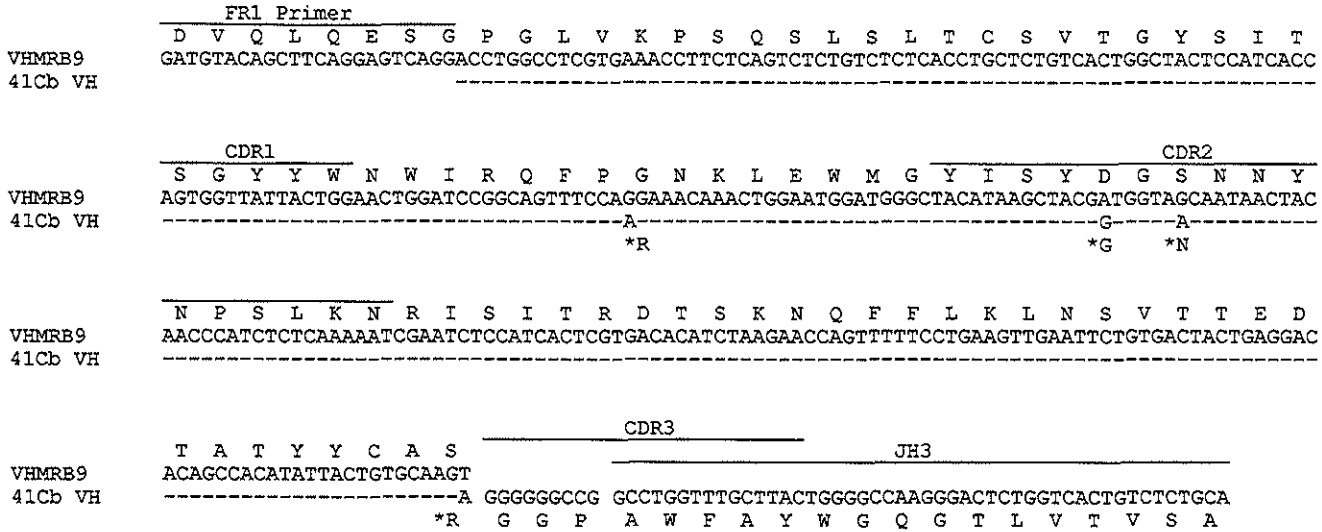

Figure 3. (Continued).

between V and D and D and J could not be assigned to any D segments, and were presumably derived from N additions. By contrast, the CDR3 sequence of 5T16 was very short and formed by D-SP2.2 and J_H 2.

BMG V_H sequence analysis

The BMG had two paraprotein bands, and V_H gene analysis also showed that there were two distinct groups of sequences, both derived from the small 36-60 family (Figure 3). In the first group, 41Ca, a number of sequences were closely similar to each other with an identical V-D-J junction sequence, indicating that they originated from the same clone. It is unlikely that the nucleotide heterogeneity was introduced by Taq DNA polymerase, since it was not seen in the other cases, and the Taq DNA polymerase error rate in our system is much lower ($< 1/5000$ bases). All intraclonal nucleotide variations led to change in amino acid sequence. The best matched gene found in the database for this group of sequences was a gene used in an anti-MPO antibody (Gilbert *et al.*, unpublished data, accession Z37144). 41Ca clones were different from this gene by between 12 to 15 nucleotides, which were located in FR as well as in CDR. The D segment used matched equally well to D-SP2.2 and D-SP2.9, both with 1 silent and 2 replacement nucleotide changes. The D segment was rearranged to J_H2 gene in germ line configuration. The second clone, 41Cb, was most homologous to the gene VHMRB9, which was used in an anti-histone antibody [25]. There were three nucleotide differences, one in FR2 and two in CDR2, all resulting in amino acid changes. No intraclonal variation was observed. The CDR3 was short, with no identifiable D segment. The J_H3 germ line gene was used. The nucleotides between V and J were exclusively G and C, probably derived entirely from N additions.

In both cases, there was no evidence of antigen selection, since no significant difference was found in the observed and theoretically expected numbers of mutations in either FR or CDR.

DISCUSSION

We have determined and analyzed Ig V_H gene sequences derived from five cases of MM, two of WM and one of biclonal BMG. In contrast to corresponding human diseases, in which V_H genes have consistently been found to be extensively mutated with a mean% $\pm$ SD of 8.8 $\pm$ 3.2 [5], the rate of somatic mutation in the mouse tumors studied here was significantly lower (two-sample *t* test, $p < 0.01$), with a mean% $\pm$ SD of 2.9 $\pm$ 2.3. In the

five mouse MM, 5T2, 5T7 and 5T33 had only a limited number of mutations. In contrast, 5T13 and 5T14 were considerably different from the closest germ line genes, with 11 and 7 amino acid replacement mutations, respectively. Although mouse Ig V_H repertoire has not been fully sequenced and seems to be more complicated, with estimates varying between 100 to 1000 members [15], we could not find a better-matched germ line donor for 5T13 by sequencing PCR products amplified with 5T13-specific primers. We believe therefore that the low homology between 5T13 V_H and VH124 is due to somatic hypermutation.

Recent studies indicate that in humans the neoplastic cell of MM is a B cell that has passed the germinal centre and undergone somatic hypermutation [3-5]. Both Ig V_H and V_L genes are extensively mutated and in a proportion of cases (~25%) there is evidence for antigen selection, as significant clustering of replacement mutations has been observed in CDR regions in either V_H or V_L [6,7]. In the mouse, however, the origin of MM cells is less clear, since the mutation rate of V_H genes varies from case to case. 5T13 and 5T14, which have numerous nucleotide substitutions, may originate from B cells that have been exposed to the hypermutation mechanism in the germinal centre, possibly memory cells. However, there is no clear evidence for antigen selection, as the mutations were not clustered in the CDR regions. In contrast, 5T2, 5T7 and 5T33 have only a small number of mutations. It is possible therefore that they originated from naive cells that had not undergone somatic mutation process. On the other hand, it is generally believed that the rate of somatic mutation of mouse V genes is lower, especially in old animals [26]. It can not be ruled out that the precursor cells of 5T7 and 5T33 were at the same differentiation stage as 5T13 and 5T14, but failed to accumulate further mutations. It is interesting to note that in mouse B-cell lymphomas, BCL1 [27], 38C13 and A31 [28], the V_H genes have just one (BCL1) and no (38C13 and A31) somatic mutation [our unpublished data].

Compared to human MM, the CDR3 regions of mouse MM are less complex. Unlike human MM, no D-D fusion and D segments in reverse orientation were observed and the D segments identified were all in germ line configuration. There was evidence of N-region additions in all the murine tumors investigated; however, it is difficult to compare the degree of N additions to that of the human tumors because of the complexity of the D segment usage of the latter. Interestingly, in 2 murine MM cases the CDR3 regions seemed to be contributed mostly by N insertions with no apparent D segments involved. However, the possibility that very short or mutated D segments were used could not be ruled out.

Similar variations in the somatic mutation rate of V_H genes were also observed in the two cases of WM and the biclonal BMG. The two BMG

clones both used V_H genes from the much smaller 36-60 family. The clone 41Ca was more mutated and intraclonal nucleotide variations were observed, suggesting that the tumor cells may still be undergoing somatic mutation. In this aspect, mouse BMG is similar to the human counterpart, as intraclonal variation has been observed in a number of human cases [7]. The degree of somatic mutation in the clone 41Cb was much lower with only 3 nucleotide substitutions. Similar low frequency of somatic mutation in one case of BMG has been observed previously [29].

The 5T series of MM and WM-like lymphomas is, in many aspects, closely similar to corresponding human diseases. However, the rate and pattern of somatic mutation of V_H genes in some cases are quite different. Obviously, the findings have implications in using the 5T series of mouse tumors as experimental models, especially in anti-idiotypic therapy studies, where it might be anticipated that the number of idiotypic determinants arising from somatic mutations and genetic complexity in CDR3 will be more limited than in the human disease counterparts.

Acknowledgments

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General discussion

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CHAPTER 6

GENERAL DISCUSSION

6.1 B-cell subsets and their involvement in monoclonal gammopathies

The studies described in this thesis attempt to give insight into some basic pathogenetic aspects of the development of monoclonal gammopathies (MG). Chapter 2 shows that the recently acknowledged subset of B-cells in the coelomic cavities, the B-1 cells, can be responsible for MG.

In the μ,κ-transgenic C57BL/6 (Sp6) mice two populations of B-cells have been identified that differed in the expression of transgenic (Tg) and endogenous IgM [1]. The majority of the peripheral B-cells expressed exclusively the Tg IgM of μ^a allotype. However, a small proportion of B-cells showed endogenous, *i.e.* non-transgenic, IgM expression of μ^b allotype, usually concomitant with Tg IgM. This B-cell subset possessed a B-1 phenotype: expression of CD11b, CD5, and low levels of IgD. In addition, they were especially enriched in the peritoneal cavity, and they could not fully reconstitute endogenous IgM-positive B-cells in irradiated recipient mice. We therefore assumed that monoclonal B-cell proliferative disorders arising from the B-1 cell population in these aging Tg mice would result in a homogenous immunoglobulin component (H-Ig) of endogenous isotype. A major advantage of this study was that we were able to detect B-1 cell derived H-Ig without prior disruption of the *in vivo* experimental model.

In fact, all H-Ig that we detected in the Tg mice (n=58) were of endogenous origin: one multiple myeloma (MM), six benign monoclonal gammopathies (BMG), five transient MG, and 10 unclassifiable H-Ig (Chapter 2.2). Consequently, these MG were all of B-1 origin. The aging Tg mice showed half as many MG as their normal littermate (LM) counterparts.

From these results we can certainly not conclude that all mouse MG are derived from the B-1 cell population. In this transgenic mouse model the B-1 cell population is the only B-cell subset that can shape the actual antibody repertoire, resulting in extensive activation of this B cell compartment [2,3]. The frequency of the B-1 cell derived H-Ig in the aging Tg mice is therefore probably not similar to the frequency of those in normal LM mice, but exaggerated due to this activation. The B-2 cells, on the other hand, were functioning properly after encounter of the relevant antigen TNP [4]. The μ,κ immunoglobulin (Ig) transgene encoded for this specificity. Since the Tg mice

were not immunized with TNP-conjugates, the B-2 cell compartment was in a relatively resting state. We can hypothesize from the results of this study that without extensive (antigenic) activation no B-2 cell derived MG will occur in this mouse model.

This is in accordance with some previous studies on the role of antigen in the development of MG. It has been shown that repeated immunization of mice at young age resulted at old age in a number of MG with H-Ig specific for the antigen used in the immunization procedure [5,6,7]. Results of studies on the accumulation of somatic mutations in the immunoglobulin heavy-chain variable region (Ig V_H) gene complementarity-determining regions (CDR) also reveal a role for antigen in the process of a B-cell clone becoming larger and persistent and giving rise to MG [8,9,10]. Although antigen is likely to be a major contributing factor in the development of MG, it is not known what antigens caused the excessive monoclonal B-1 cell proliferations in the Sp6 Tg mouse model. Almost all infectious diseases and other pathological conditions have been excluded as causative factors in this study, suggesting that the stimuli that initiated the development of the monoclonal B-cell proliferative disorders might very well be physiological ones. Candidate antigens probably come from the murine intestinal flora. Antibodies produced by B-1 cells have been shown to bind microbial antigens, such as phosphatidylcholine, phosphorylcholine, dextran, pneumococcal polysaccharide, lipopolysaccharide, and others [reviewed in 11]. Furthermore, in anti-red blood cell autoantibody transgenic mice it was shown that bacterial colonization of the murine gut was responsible for the expansion of the B-1 cells [12].

In the second study we used immunoglobulin heavy-chain (IgH) allotype-congenic mouse chimeras to study the same experimental question: do MG originate from both B-cell lineages or not? Again, a long-term study was performed. The C57BL mice under investigation were lethally irradiated and reconstituted with bone marrow (BM) cells and IgH allotype congenic peritoneal wash cells. The cells from the peritoneal washes provided for the precursors of the B-1 cells, while the BM cells gave rise to the B-2 cells [13-15]. The cell lineage origin of the H-Ig that developed at old age could thus be determined by Ig-allotyping of the H-Ig component in the serum.

A great disappointment in the outcome of this study was the markedly decreased survival time of the irradiated mice. Histopathological examination of necropsy material showed that this was not caused by any infectious disease due to an impaired immune system, nor by radiation induced tumors. It appeared that severe renal damage, as a late effect of irradiation, resulted in a protein loosing disorder of the affected mice, leading to their relatively early death. The steep decline in the survival curves indicated that the mice were

severely ill for only a short time. Clinically their deaths could hardly be predicted, since the only early sign of disease was a generalized edema, followed in a few days to almost two weeks by marked discomfort and fast progression of the illness. A subsequent study with allotype-congenic mouse chimeras that received a 1 Gy lower radiation dose (8.5 Gy) than the first groups did not improve the survival times significantly and was therefore left out of the study.

As a consequence, fewer MG could be studied, most of them were BMG and transient MG, and only one MM. This MM could be identified as having the BM derived allotype of the IgG2a isotype. Determination of the IgM-allotypes of the H-Ig in the aging chimeras revealed that most of these H-Ig originated from the peritoneal B-cells and were therefore of B-1 cell origin.

Our experiments, presented in chapters 2.2 and 2.3, showed that B-1 cells can be responsible for age-related MG in a susceptible mouse strain. The MG were mainly transient and benign (BMG) of character. Only one malignant MG of B-1 cell origin, a IgG2b-kappa producing MM, was detected in a μ,κ-transgenic mouse. The B-1 cell derived MG that we detected in the allotype-congenic chimeras produced IgM, the isotype that is secreted in large amounts by B-1 cells. This is in contrast to the findings in the μ,κ-transgenic mice in which we detected almost no H-Ig of IgM isotype. It is speculative that this is the result of the extensive activation of the B-1 compartment in the Tg mice, leading to a relatively high rate of class-switch of the B-1 cells.

There are also other experimental approaches that might be employed to study the differential involvement of the B-1 and B-2 cells in the development of MG. Xid mice, in which the B-1 cells are selectively depleted, can be used as recipients for IgH allotype congenic peritoneal wash cells without the in our model necessary total body irradiation. It was shown by Gueret *et al.* [16] that C57BL/Xid mice had a low incidence of H-Ig compared with the 50% incidence in the control mice. Unfortunately, these mice did not live long enough to develop full-blown MG of neoplastic origin. Selective and permanent depletion of the B-1 cells can also be accomplished by intraperitoneal injections of anti-IgM antibodies [17]. Mice treated this way can function as recipients of allotype congenic B-1 cells. Several other treatment protocols of mice, similar to the former one, can be used, e.g. intraperitoneal injections of anti-IL-10 [18] or anti-mouse CD19 [19] antibodies. Once the peritoneal B-1 cells are depleted and restored by donated B-1 cells, the original B-1 precursor cells will be inhibited by a feedback mechanism [20].

B-1 cells in the peritoneal cavity (PerC) have been recognized as a

separate B-cell compartment, with a distinct phenotype and localization, restricted Ig repertoire, and self-replenishing properties [21-26]. B-1 cells *in vitro* [27] and *in vivo* [28] grow preferentially on peritoneal stromal cells, indicating that the microenvironment contributes to the growth, survival and anatomical distribution of peritoneal B-1 cells. Still a matter of debate is whether the B-1 cells are a different lineage or a subset of B-cells that expresses a B-1 cell phenotype after activation by antigen [29-32]. Although we think that the cell transfer experiments propose the strongest argument of the different B-cell lineages [21-26,29,33], definitive proof is lacking, mainly because a unique identifier of these cells still has to be found. Progress is being made, however, as appears from the different transcription factor induction in B-1 cells [34], the non-constitutive expression of apoptosis-associated Fas receptor on B-1 cells [35], and especially the constitutive activation of STAT3 (signal transducer and activator of transcription-3) in normal B-1 cells [36]. The latter study showed that prolonged stimulation of conventional B-2 cells with anti-Ig, a treatment sufficient to induce CD5 expression, did not result in sustained STAT3 activation, suggesting that STAT3 is a specific nuclear marker for B-1 cells.

Aging affects the levels and clonality of the B-1 cells in mice: numbers of CD5+ cells increase with aging, together with oligoclonal expansions of these cells. The clones could be detected at the cellular level in the peritoneal cavity and spleen, later in lymph nodes, peripheral blood and BM [37]. There are indications that the frequencies of CD5+ B cells in the PerC and the spleen are in part under separate genetic control [38]. At the subcellular level, clonal expansions of B-cells could be detected in old mice, using a PCR-based technique [39]. The latter study did not focus on the B-1/B-2 dichotomy of the B-cell compartment. None of these studies did investigate and reveal Ig spectrum abnormalities as a result of the clonal expansions.

A role for B-1 cells in human lymphoproliferative diseases has been established in B-cell chronic lymphocytic leukemia (B-CLL), although impressive differences exist between normal and malignant CD5+ B-cells [40]. Furthermore, CD5 expression has been found in small lymphocytic lymphoma, and lymphocytic lymphoma of intermediate differentiation or mantle zone lymphoma [41]. CD5+ immunoregulatory B-cells have been found in the peripheral blood and spleen from MM patients [42,43]. These cells inhibited the Ig production in a pokeweed mitogen driven assay. In some cases the CD5+ CD19+ or CD11b+ B-cells may have been a part of the malignant process [44,45,46], while other studies showed a reduced number of CD5+ B-cells in MM patients [47]. In patients with Waldenström's macroglobulinemia (WM) monoclonal CD5+CD11b+ B-cells have been

found in the peripheral blood [48]. Although there is as yet no functional evidence that clearly defines these monoclonal B-cells as a malignant part of the WM, their monoclonality and morphology suggested indeed malignancy. When using phenotypical features of malignant B-cells to define their normal counterparts, it is necessary to have information as to whether the expression of certain antigens is induced by the malignancy itself. As yet there is no such information on the regulation of CD5 or CD11b expression on B-cells. Furthermore, the use of the expression of these two antigens to define a normal B-cell in humans as originating from the B-1 cell lineage is an equally dangerous undertaking. As discussed before, expression of these antigens on B-cells outside the PerC is not sufficient evidence to place these B-cells into the group of B-1 cells, since the expression of these markers can be induced in B-2 cells as well. In the human situation it is hardly possible to investigate the peritoneal resident B-cells. This makes it even harder to find a definitive lineage marker and to investigate the normal physiology of this B-cell subset. However, a study on peritoneal lymphocytes from patients starting continuous ambulatory peritoneal dialysis and from women undergoing bilateral tubal ligation suggested that the phenotype of human peritoneal B cells is similar to that of B-1 cells in mice [49].

Because of the self-replenishing properties and the tendency to become clonally restricted with aging, the B-1 cells might be prone to a higher chance of malignant transformation. Among the age-related MG, some could be of B-1 cell origin. Knowledge of the B-cell lineage origin might be helpful for correct diagnosis and timely efficient treatment of malignant forms of MG.

6.2 Is clonal dominance in antibody responses the basis for development of monoclonal gammopathies?

To elucidate the process that takes place prior to the development of a stable monoclonal B-cell clone, we started the experiments that are described in Chapter 3. We studied the effects of age at time of immunization, the genetic background, and an impaired T-cell system on the levels and the heterogeneity of the specific antibody response to dinitrophenylated human serum albumin (DNP-HSA). This heterogeneity can be envisaged as the capacity of the immune system to recruit the available antigen-specific clonotypes directly after immunization. An exaggerated response of one or a few clones could result in the so called clonal dominance. With the use of sensitive immunoblotting techniques, these H-Ig, as products of the dominant clones, can be detected in the serum.

In this study we used two inbred mouse strains: the C57BL/KaLwRij

(C57BL), which has a high incidence of spontaneously developing MG, and the CBA/BrARij (CBA), which in contrast shows a low incidence of these B-cell proliferative disorders. Similarly, clonal dominance was scarce in CBA mice, but frequent in C57BL mice. This observation could not be related to the number of spectrotypes in the mice: young intact members of both strains had an equal number of spectrotypes. Furthermore, non-immunized mice of both strains have been shown to possess equal numbers of DNP-specific B-cells in the spleen [50], which ruled out the number of available responding B-cell clones as an explanation for the results from this study.

In the C57BL mice immunized with DNP-HSA at old age, smaller amounts of specific antibodies were produced by fewer clones, while the incidence of DNP-HSA-specific H-Ig (produced by the dominant clones) was as high as in C57BL mice immunized with DNP-HSA at young age. This indicated that at old age the responding B-cell clones are more prone to become dominant in C57BL mice. Fewer responding clones in these experiments meant that more spectrotypes could be detected in less diluted serum in the isoelectric focussing (IEF) procedure.

Neonatal thymectomy had a little effect on the levels and the heterogeneity of the antibody response in our experiments, probably because the T-cell system was only moderately impaired and/or a part of the DNP-specific antibody response could have been T-cell independent. However, Benjamini *et al.* [51] concluded from their study that clonality of the antibody response is neither due to a restricted B-cell repertoire, nor to a reduced function of antigen specific T_H cells. The clones will be randomly selected for by the antigen from a larger pool of available B-cell clones, and these responding clones eventually become immuno-dominant, thus establishing the clonal characteristics of the response. This response was shown to be what they called "locked-in" for the life of the immunized individual, meaning that non-responsiveness after the first set of immunizations was maintained after the second set of immunizations. In analogy also positive responsiveness was maintained after a subsequent set of immunizations. In other words: the development of clonal dominance was intrinsic to the B-cells. Specificity maturation was not determined in this study. This mechanism can not explain the restriction in the specific antibody responses since it was found that clonal dominance was present even before significant amounts of specific antibodies were found in the sera. Some animals showed clonal dominance even before immunization [52, and our data]. The inability of the study of Benjamini *et al.* [51] to show T-cell influence might be an antigen based problem or a specific trait of the involved inbred mouse strain, in this case the C3H.SW mouse. Furthermore, the size of the individual responding clones and number of spectrotypes were not established. Both parameters might give insight into the

effect of an impaired T-cell system. However, this study and our data together strongly suggest that the T-cell system might not be the most important factor in the initiation of clonality of the antibody response, but it certainly plays an important role in the progression into bigger clones and subsequent development of MG [53-57].

Our observation of the tendency to respond in a specific antibody response with a restricted set of B-cell clones parallels the genetic susceptibility to develop spontaneously MG in a high frequency in C57BL mice. The importance of this observation is extended by a study from Nooij *et al.* [6], in which it was established that age-associated MG could produce an H-Ig with antibody specificity to the antigen (again DNP-HSA) used for immunization at young age. While most of the MG that developed in the CBA mice were transient and of a low concentration, the majority of MG in C57BL mice had characteristics of a BMG. Interestingly, some of the clonal products had the same mobility and similar spectrotypes as dominant clonal products at the peak of the antibody response. This strongly indicates a close relationship between expanded dominant clones after immunization and BMG appearing later in life. In the same study a clear effect of neonatal thymectomy was found in the old CBA mice, resulting in a higher frequency of MG. This emphasizes the conclusion at the end of the former paragraph.

Thus, clonal dominance in antibody responses is a genetically determined trait of the B-cell compartment. Control over dominant clones is exerted by immuno-regulatory factors, such as the T-cell system. Imbalances in this control mechanism allow for the preferential outgrowth of the dominant B-cell clones and precede the development of MG in mice. As yet there are no studies available that suggest a similar mechanism in humans.

6.3 A long-term impaired T-cell system: submissive for the development of malignant monoclonal gammopathies

The occurrence of H-Ig in the serum of immunosuppressed patients is not uncommon [58-60]. Most of these H-Ig, about 75%, are of transient nature, but persistent MG are certainly present [58,61-63]. The incidence of lymphoproliferative disorders of B-cell origin that do not produce an H-Ig (or only rarely in a low concentration) is greatly enhanced in immunosuppressed patients [64,65]. These B-cell lymphoproliferative disorders are non-Hodgkin lymphomas (NHL), associated with Epstein-Barr virus (EBV) reactivation. However, as yet there are only few observations of a *malignant* MG developing in immunosuppressed patients. They can be divided into

extramedullary plasmacytomas [66-68] and multiple myelomas [63,69-71]. In some of these malignancies an EBV association has been established. Because of this low incidence there is no information as to whether an extramedullary plasmacytoma frequently ends up as an MM in these patients. Analogously, other causes of immunosuppression have been shown to increase the incidence of B-cell NHL and paraproteinemias, e.g. in patients with HIV-infection [72-77].

Our animal model was employed to perform a long-term investigation of the effects of maintenance immunosuppressive treatment on the development of transient and persistent MG (Chapter 4). Although the proportion of mice with H-Ig in the immunosuppressed groups was equal to the proportion of mice with H-Ig in the PBS control group, remarkable differences were observed between the incidences of the individual categories of MG. The transient MG reached a four to five times higher incidence in the mice of the immunosuppressed groups as compared to the mice of the PBS control group. No differences were detected in the incidences of BMG, but a remarkable ten times higher incidence of MM occurred in the immunosuppressed groups.

This is an important observation with regard to the etiology and pathogenesis of the individual categories of MG. Would it be that transiently present mono- or oligoclonal B-cell proliferations, responding to certain antigenic stimulations, were a foregoing event in the development of a BMG, we should have expected to find an equal increase in the incidence of BMG similar as in the incidence of transient H-Ig. This we did not find. The incidence of MM that we observed in our experiments was increased to more or less the same order as transient H-Ig. It is tempting to conclude from these data that MM results directly from a transformed "overshooting" B-cell clone and not from a persistent, but non-progressive, BMG. The long-term immunosuppression probably resulted in the outgrowth of usually small and undetectable antigen responding B-cell clones. These normally functioning dominant clones could subsequently be detected in the serum by their H-Ig product. The accumulation in time of multiple genetic aberrations might than have transformed these normally occurring B-cell clones into malignant MG like MM. The exact nature of these transforming processes is as yet unknown.

As mentioned before, the incidence of BMG was not significantly changed in the immunosuppressed groups treated with either azathioprine/prednisolone or cyclosporin /prednisolone as compared to the PBS control group. To interpret this observation adequately, we should bear in mind that the cumulative frequencies of H-Ig components, as given in Chapter 4, table 2, were collected after the death of the mice. When we

analyze the data from table 2 together with the data from figure 2, which depicts the frequency of H-Ig in relation to the age of the mice, we can conclude that the total number of MG, and more specifically of BMG, was the same in all groups. However, the mice receiving immunosuppressive treatment showed an earlier onset of the MG in general. This finding is in accordance with a study in immunosuppressed patients after renal transplantation [58] where a correlation was found of the frequency of H-Ig and the age of the patient. This earlier onset of the development of the various categories of MG was also found in athymic nude C57BL mice during aging [56]. These studies strongly indicate that an intact T-cell system is required for a normal heterogenous Ig spectrum. Impairment of the T-cell system results in an earlier onset of age-related MG, and increases the incidences of malignant forms of MG, especially MM, and of transient MG.

Furthermore, tests for the antibody specificity of individual H-Ig to the antigen used for immunization (a human myeloma IgG1-λ) revealed that one of the 114 cases of BMG and one of the 15 cases of MM had a positive anti-human IgG1 activity. This observation is in accordance with previous studies on antigen specificity of H-Ig in MG [5-7]. Moreover, studies in HIV-infected patients showed that monoclonal Ig detected in the serum of some of these patients were specific for parts of the HIV [73,74,76].

6.4 Immunoglobulin V_H gene sequence analysis of spontaneous murine monoclonal gammopathies

Although all MG are excessive plasma cell proliferations, the progenitor cells might be less differentiated B-cells that circulate in the peripheral blood [78-80]. These progenitors might even be different for the individual MG categories, including B-1 cells in the peritoneal cavity as precursors for certain types of extramedullary plasmacytomas and part of the BMG. The presence of shared idiotypes between IgM-expressing pre-B lymphocytes and the cells of a myeloma clone suggested the existence of such a progenitor cell [81]. However, others showed that anti-idiotypic antibodies in MM can be cross-reactive to other, different, Ig in the same patient [82]. The third complementarity-determining region (CDR3) sequences and somatic mutations of V-region Ig genes are more potent clonal markers of malignant B-cells and plasma cells. This method can help to identify the normal counterparts of these cells [9,83].

For MM, numerous studies in humans have shown that the malignant plasma cell has undergone extensive somatic mutation [8-10]. Analysis of both V_H and V_L gene sequences revealed no intraclonal variation, indicating that the

malignant cells were no longer able to accumulate further mutations. Furthermore, the clustering of replacement mutations in CDR regions of the V_H genes is consistent with a role for antigen in selecting V_H sequences. After encounter, or several encounters, of antigen in the germinal center the B-cell accumulates somatic mutations [10]. Somewhere between the germinal center and the BM a final event transformed the antigen specific B-cell into a malignant plasma cell that is immortal, generating an MM or a WM. Although thusfar sequence data indicate a common pathway for MM and WM, it is not known why the progenitor cells of MM, in contrast to those in WM, can still make an isotype switch: in case of MM, IgM-positive progenitor cells with identical V_H sequence to the isotype-switched cells have been detected in the BM [84,85].

Our sequence data from spontaneous murine MM, WM, and BMG did not show such extensive somatic mutations in the Ig V_H-regions (Chapter 5). This does not necessarily mean that the progenitor cells of MG in the mouse are different from man. It has been shown that the rate of somatic mutations in mouse V_H genes is lower, especially in old animals [86]. Furthermore, the gene pool that constitutes the V_H-repertoire in mice is estimated to be approximately ten times larger as in humans [87]. Thus, old mice probably can rely more on their repertoire of V_H genes for antigen specific Ig responses and thus do not necessarily employ extensive somatic mutation. Sahota *et al.* demonstrated that if significant clustering of replacement mutations was present in human MM cells, it was either in V_H or in V_L but not in both. Therefore, deductions concerning a role for antigen selection in any B-cell proliferative disorder may require both V_H and V_L sequences for validation [88]. In our study on mouse MG we did not analyze the V_L sequences. Therefore we cannot conclude whether the accumulation of somatic mutations in V genes of mouse MG indeed is smaller than in human MM.

There is otherwise evidence from our own studies that in mouse MG antigenic selection occurs preceding the malignant transformation. In Chapter 4 we mentioned that one MM and one BMG produced an H-Ig with specificity to the antigen used in the immunization procedure (human IgG1-λ). Together with previously published studies [5-7] these results show a strong relationship between the antigen used for immunization at young age and the development of specific H-Ig later in life.

The 5T2 MM tumor produces an antibody with specificity to DNP. The antigenic specificity of the other 5T tumor products is unknown. In humans a large amount of data is available on antibody activity of myeloma proteins [89]. In addition, a recent study of Rettig *et al.* [90] showed that the V_H gene usage in human MM is unique compared to other malignant and non-malignant B-cell populations, and that the physiological process of clonal

deletion resulted in the removal of rearranged V_H genes that are capable of expressing antibodies with anti-self specificity. Assuming that clonal deletion is a process that occurs only during antigen driven stages of B-cell differentiation, this implies that the final oncogenic event in MM occurs in a terminal step of plasmacytic development. This would be in accordance with the data of somatic mutations in human myeloma Ig.

Studies on somatic mutations in V_H-genes of MG of undetermined significance (MGUS) in humans demonstrated that a proportion of these B-cell proliferative disorders showed intraclonal variation [91], indicating that the clone might still be part of a dynamic process that can respond to an antigen. This could be a useful marker to distinguish MM from benign counterparts. Follow-up studies are necessary to show whether these benign clones can develop into malignancies. Although data on murine BMG mostly parallels the data on human MGUS, too few mouse BMG have been analyzed to state that these disorders are comparable at the molecular level. Their biological behaviour, however, strongly suggest similarity.

The use of specific V_H CDR3 region probes can help in the further identification of the progenitor cells of MG in general. Using RT-PCR techniques it is possible to detect small B-cell clones in different organs and to further characterize them and their cellular environment [39,92-94, and Dr Roland Gueret, personal communication]. Such sensitive techniques can reveal T-cell clonalities as well, with frequencies increasing with age [95-97]. A possible link between reduced clonal heterogeneity at the B-cell and T-cell level in the aging individual should be anticipated. This subject has to be investigated further.

6.5 Three-stage hypothesis on the development of benign monoclonal gammopathies

At the end of this discussion we will attempt to review the hypothesis on the stages of development of BMG as has been postulated by Radl in 1979 [98], and add to it new information as is described in this thesis. Radl suggested that the development of BMG is the result of age-related immune dysfunction.

Stage 1: During aging, the function of the cellular immune system becomes impaired. This is illustrated by involution of the thymus and a restricted repertoire of T-cell receptors in aged individuals [95] and the unbalanced development CD4+ and CD8+ T-cell clones [96,97]. The onset, extent and progress of this regulatory T-cell malfunction can be influenced by extrinsic

factors, such as long-term immunosuppressive treatment (chapter 4) and viral infections, as is seen in HIV-infected patients [72-77].

Stage 2: The stage-one condition has a profound effect on normal B-cell responses. Excessive B-cell clonal proliferations will result after antigenic stimulation, due to the improper helper and suppressor T-cell functions. The physiological phenomenon of clonal dominance in antibody responses is now relatively out of control, and a monoclonal Ig product can be detected in the serum. Clonal dominance and its relation to genetic background and the development of age-related MG have been discussed in chapter 3. This second stage is still reversible and the MG are transient in character (category 3 MG; see Chapter 2.1, table 1).

Stage 3: Repeated and prolonged mono- or oligoclonal expansions result in a higher probability for genomic mutation. Part of the susceptibility to this failure of proliferative control is genetically based. Proliferation of the clone has become independent from external signals, because now there is an intrinsic B-cell defect, as was illustrated by the transplantation experiments of BMG cells into congenic recipients. This third stage, BMG (category 2 MG; see Chapter 2.1, table 1), is irreversible. Although inhibition of proliferation can no longer be exerted by normal T-cell functions, the BMG progenitor cells probably have still an intact somatic mutation mechanism (chapter 5). It is not known whether this mechanism in BMG is directed by antigen.

The development of MM and WM may involve some of the steps mentioned above. Certainly the intracellular defects are more complex, and the cells can no longer somatically mutate their Ig V-regions. As was suggested before, these MG probably develop directly from stage 2.

We have not yet fit in into this hypothesis the occurrence of MG derived from the B-1 cell population (chapter 2). There is hardly any information about T-cell influences on this B-cell subset. Still, with aging the B-1 cell population becomes restricted in heterogeneity [37]. Also antigenic stimuli from the gut mucosal surfaces might have an impact on the proliferative responses of this subset. Although transient MG and a few BMG of B-1 cell origin have been detected, it still has to be elucidated whether these B-1 cell clones can evolve into malignant MG in otherwise normal individuals. In humans, certain extramedullary plasmacytomas, WM, and BMG might be the offspring of such a B-1 cell proliferative disorder, apart from numerous transient MG.

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SUMMARY

Monoclonal gammopathies (MG) are B-cell proliferative disorders at the differentiation stage of a plasma cell. They often produce relatively large quantities of monoclonal or homogenous immunoglobulins (H-Ig), that are detectable in the serum. This heterogenous group of monoclonal B-cell proliferative disorders can be classified into several categories of MG. Category 1 comprise the B-cell malignancies that give rise to an H-Ig. The most important representatives of this category are multiple myeloma (MM), Waldenström's macroglobulinemia (WM), and (extra-) medullary plasmacytoma. Category 2 represent B-cell benign neoplasia. This common B-cell proliferative disorder is also known as benign monoclonal gammopathy (BMG). Category 3 comprise MG due to immunodeficiency with T-cell/B-cell imbalance, including primary immunodeficiencies, as well as secondary, such as aging, viral infections, immunosuppressive treatment, and others. Category 4 is represented by the MG due to particular antigenic stimulation, as is seen in response to certain haptens and polysaccharides, and in some autoimmune diseases.

Although a large amount of clinical data is available on MG of neoplastic origin (categories 1 and 2), there is still not much known about the exact etiology of these B-cell proliferative disorders. The possible relationship among the different disorders, the location and stage of differentiation of the B cell at the time of neoplastic transformation, and the causes of this transformation are among the questions that asked for a suitable animal model. It turned out that a particularly useful mouse strain was the C57BL/KaLwRij mouse. Aging mice of this strain spontaneously develop MG of all four categories, with characteristics that are similar to their human counterparts.

Based on cell surface phenotype, anatomical localization, function and origin, B-cells in mice can be divided into two lineages: the predominantly in thoracal and peritoneal cavities localized B-1 cells, expressing the CD11b molecule, often in conjunction with CD5 expression, and the conventional, bone marrow (BM) derived B-2 cells, which express neither CD5 nor CD11b. The B-1 cells comprise a self-replenishing population that becomes increasingly restricted with aging of the individual. This phenomenon might account for a relatively high chance of neoplastic transformation within these cells. Therefore they form a pool of potential precursor cells of MG. Although CD5 expression has been found on the malignant cells of B-CLL in humans and in certain mouse B-cell lymphomas and cell lines, no information was available on the lineage origin of the more mature B-cell proliferative disorders (BMG, WM, MM). Two experimental approaches have been used to investigate this latter question (Chapter 2).

First, a life-long follow-up study of μ,κ-transgenic Sp6 mice was started (Chapter 2.2). The immunoglobulin (Ig) transgene encoded for TNP specificity. Peripheral B-cells in these mice expressed exclusively the transgenic (Tg) IgM of μ^a allotype. Only a small proportion of B-cells showed endogenous μ^b allotype expression. These B-cells possessed the B-1 cell phenotype and characteristics. We therefore assumed that MG arising from the B-1 cell population in these aging Tg mice would result in an H-Ig of endogenous isotype. In fact, all H-Ig that we detected in the Tg mice (one MM, six BMG, five transient MG, ten unclassifiable MG) were of endogenous origin. Consequently, these MG were of B-1 cell origin.

Second, we used IgH allotype-congenic C57BL mouse chimeras in order to be able to evaluate the H-Ig that developed from the B-1 cell lineage separately (Chapter 2.3). The C57BL mice under investigation were lethally irradiated and reconstituted with BM cells and IgH allotype-congenic peritoneal wash cells. The cells from the peritoneal washes provided for the precursors of the B-1 cells, while the BM cells gave rise to the B-2 cells. The cell lineage origin of the H-Ig that developed at old age could thus be determined by Ig-allotyping of the H-Ig component in the serum. Due to late effects of the total body irradiation, the life-span of the chimeras was reduced and consequently the number of H-Ig detected in this study was less than expected. Determination of the IgM-allotypes of H-Ig in aged chimeras revealed that most of these originated from the B-1 cell population. The experiments, presented in Chapters 2.2. and 2.3, showed that B-1 cells can be responsible for age-related MG in a susceptible mouse strain. The MG were mainly of transient and benign (BMG) character, but one malignant MG of B-1 origin (an MM) was detected in a μ,κ-transgenic mouse.

To elucidate the process that takes place prior to the development of a stable monoclonal B-cell clone, we started the experiments that are described in Chapter 3. We investigated the effects of age at time of immunization, genetic background (CBA and C57BL inbred mouse strains) and impaired T-cell system (using neonatal thymectomy) on the levels and the heterogeneity of the specific antibody response to dinitrophenylated human serum albumin (DNP-HSA). This heterogeneity can be envisaged as the capacity of the immune system to recruit the available antigen-specific clonotypes directly after immunization. Using isoelectric focussing (IEF), the specific antibodies can be divided into discrete bands, the so-called spectrotypes or clonotypes. An exaggerated response of one or a few clones could result in so-called clonal dominance. This clonal dominance was scarce in CBA mice, but frequent in C57BL mice. Age at time of immunization and neonatal thymectomy had little if any additive effect on the incidence of homogenous antibody components (H-Ab) in the sera of either mouse strain. In old C57BL

mice smaller amounts of specific antibodies were produced by fewer clones. Still, the incidence of H-Ab in this group was the same as in the group of young C57BL mice. This indicated that at old age the responding B-cell clones are more prone to become dominant clones in C57BL mice. This tendency correlated with the high incidence of spontaneously developing monoclonal gammopathies in aging C57BL mice. In conclusion, clonal dominance in specific antibody responses appears to be a genetically determined trait of the B-cell compartment. Control over dominant clones is exerted by immunoregulatory factors, such as the T-cell system. Imbalances in this control mechanism allow for the outgrowth of dominant B-cell clones and precede the development of MG in mice.

Long-term immunosuppressive treatment has been shown to increase the risk for the development of early B-cell malignancies, especially non-Hodgkin lymphomas. Little is known about the late effects of maintenance immunosuppressive treatment (MIST) on the development of MG. This we studied in the C57BL/KaLwRij mouse model of MG, as is described in Chapter 4. Two widely used immunosuppressive treatment protocols in humans (azathioprine/prednisolone, and Cyclosporin A/prednisolone) were tested in young and adult mice. Both regimens were shown to increase ten-fold the incidence of spontaneous MM. Transient MG reached a four to five times higher incidence in the mice of the immunosuppressed groups as compared to the mice in the PBS control groups. No differences were detected in the incidences of BMG. The proportion of total MG was the same in all groups, however, mice receiving immunosuppressive treatment showed an earlier onset of MG. This study strongly indicated that an intact T-cell system is required for a normal heterogenous Ig spectrum. Furthermore, a long-term impaired T-cell system is submissive for the development of malignant MG.

The 5T series of mouse MM and WM that developed spontaneously in aging mice of the C57BL/KaLwRij strain show clinical and biological features that closely resemble their corresponding human diseases. In order to compare the patterns of somatic mutation in V_H genes of these 5T tumors with those of human counterparts, we determined and analyzed the Ig V_H sequences of five cases of MM, two of WM, and one of a biclonal BMG. This study is reported in Chapter 5. Compared to human MM and WM the degree of somatic mutation in the mouse tumors was significantly lower. There was no significant evidence of clustering of replacement mutations in CDR, a feature considered to be characteristic of antigen-selected sequences. However, one clone of the biclonal BMG case showed intraclonal variation, suggesting that the tumor cells may still be undergoing somatic mutation. In this respect, mouse BMG is similar to the human counterpart.

The continuing search for unique identifiers of malignancy in the

clonal B-cells of MG and the unravelling of the mechanisms that lead to the malignant transformation of the B-cells is necessary for discrimination of benign and malignant MG at an early stage of the disease.

SAMENVATTING

Monoklonale gammapathieën (MG) zijn proliferatiestoornissen van B-cellen in het differentiatiestadium van plasmacellen. Gewoonlijk produceren zij relatief grote hoeveelheden van een monoklonaal of homogeen immunoglobuline (H-Ig), dat detecteerbaar is in het serum. In deze heterogene groep van monoclonale B-cel proliferatiestoornissen kunnen vier categorieën worden onderscheiden. Categorie 1 bestaat uit de B-cel maligniteiten welke een H-Ig produceren. De voornaamste vertegenwoordigers uit deze categorie zijn multiple myeloom (MM), Waldenström's macroglobulinemie en (extra-)medullair plasmacytoom. Categorie 2 omvat de benigne B-cel neoplasie. Deze veel voorkomende B-cel proliferatiestoornis wordt ook wel benigne monoklonale gammapathie (BMG) genoemd. Categorie 3 omvat de MG welke door immunodeficiënties met T/B cel dysbalans worden veroorzaakt, waarbij zowel primaire als secundaire immunodeficiënties betrokken kunnen zijn. Secundaire immunodeficiënties treden bijvoorbeeld op bij veroudering, virale infecties en immunosuppressieve behandeling. Categorie 4 omvat MG welke zijn ontstaan na een bepaalde, vaak langdurige, antigene stimulatie, zoals in reactie op bepaalde haptenen en polysacchariden, en in sommige autoimmuunziekten.

Hoewel er een overweldigende hoeveelheid klinische gegevens beschikbaar is over MG van neoplastische origine (categorieën 1 en 2) is er nog steeds weinig bekend over de etiologie van deze B-cel proliferatiestoornissen. De mogelijke relaties tussen de diverse categorieën MG, de localisatie en het differentiatiestadium van de B-cel ten tijde van de neoplastische transformatie, tezamen met de mogelijke oorzaken van deze transformatie, stimuleerde de ontwikkeling van een geschikt proefdiermodel. Uit eerdere studies bleek dat de C57BL/KaLwRij muis als proefdiermodel voor MG zeer geschikt was. Verouderende muizen van deze inteeltstam ontwikkelen spontaan MG van alle vier categorieën, met eigenschappen die sterk lijken op de MG die bij de mens worden gevonden.

Op basis van het fenotype van de cellen, hun anatomische localisatie, functie en oorsprong, kunnen B-cellen in de muis worden onderscheiden in twee subpopulaties: de voornamelijk in de thoracale en peritoneale holten gelocaliseerde B-1 cellen, die CD11b op de celmembraan tot expressie brengen, vaak samen met CD5 expressie, en de conventionele, uit het beenmerg afkomstige B-2 cellen. Deze B-2 cellen brengen CD11b en CD5 niet tot expressie. De B-1 cellen ontstaan uit unieke voorlopercellen in de peritoneale holte. Het Ig repertoire van de B-1 cel populatie wordt met het toenemen van de leeftijd minder divers. Daarom zouden B-1 cellen mogelijk een grotere kans hebben op het ondergaan van maligne transformatie en

potentiële voorlopercellen voor MG kunnen zijn. Hoewel CD5 expressie is gevonden op de maligne cellen van B-CLL in de mens en op sommige muize B-cel lymfomen en cellijnen, is nog onvoldoende bekend in hoeverre de B-cel proliferatiestoornissen van een rijper differentiatie-stadium (MM, WM, BMG) uit beide B-cel lijnen kunnen voortkomen. Twee experimentele benaderingen werden gekozen om dit laatste te onderzoeken (Hoofdstuk 2).

Ten eerste werd een levenslange follow-up studie gestart van μ,κ-transgene Sp6 muizen (Hoofdstuk 2.2). Het IgM-kappa transgen van deze muizen codeert voor een TNP specificiteit. Perifere B-cellen in deze muizen brengen alleen het transgene IgM van het μ^a allotype tot expressie. Slechts een klein deel van de B-cellen bracht μ^b tot expressie. Deze B-cellen hadden een B-1 cel fenotype en de daarbij behorende karakteristieken. Daarom werd aangenomen dat MG, welke in deze verouderende transgene muizen uit de B-1 cellen zouden ontstaan, een H-Ig zouden produceren van een endogeen isotype. Deze studie resulteerde in de detectie van een groot aantal H-Ig in het serum van de transgene muizen (1 MM, 6 BMG, 5 MG van voorbijgaande aard, 10 niet-klassificeerbare MG). Al deze H-Ig waren van endogene oorsprong en zullen daarom van de B-1 cellen afkomstig zijn geweest.

Ten tweede werd gebruik gemaakt van IgH allotype-congene C57BL chimeren om de van de B-1 cellen afkomstige H-Ig afzonderlijk te kunnen detecteren (Hoofdstuk 2.3). De muizen in deze studie werden letaal bestraald en gereconstitueerd met beenmergcellen en IgH allotype-congene cellen uit peritoneaal spoelsel. De cellen in het peritoneaal spoelsel leverden de voorlopercellen voor de B-1 cellen, terwijl de beenmergcellen voorzagen in de B-2 cellen. De oorsprong van de H-Ig in de verouderende chimeren kon worden vastgesteld aan de hand van Ig-allotypering. Door late bestralingseffecten was de levensduur van de chimeren gereduceerd met als consequentie dat er minder H-Ig konden worden bestudeerd dan waren verwacht. Uit analyse van de IgM-allotypen van de H-Ig in de oude chimeren bleek dat de meeste afkomstig waren van de B-1 cellen. De in de hoofdstukken 2.2. en 2.3 beschreven experimenten laten zien dat B-1 cellen verantwoordelijk zijn voor een deel van de MG in muizen welke gevoelig zijn voor de ontwikkeling van deze categorie van B-cel proliferatiestoornissen. De MG waren voornamelijk van voorbijgaande aard of chronisch-benigne (BMG) van karakter; slechts één maligne MG van B-1 oorsprong werd gevonden (een MM) in een μ,κ-transgene muis.

Om de processen te kunnen bestuderen die plaatsvinden voor het ontstaan van een stabiele monoklonale B-cel kloon, werden de experimenten uitgevoerd zoals beschreven in hoofdstuk 3. Het effect werd bestudeerd van de leeftijd van de muizen ten tijde van immunisatie, de genetische achtergrond (CBA en C57BL inteelt muizenstammen) en een verminderd functionerend T-

cel systeem (middels neonatale thymectomie) op de concentratie en de heterogeniteit van de specifieke antistofvorming op DNP-HSA. Deze heterogeniteit kan worden voorgesteld als het vermogen van het immuunsysteem om de beschikbare antigen-specifieke B-cel klonen te activeren direct na immunisatie. Middels de isoelectrische focussering techniek (IEF) konden de specifieke antistoffen worden gescheiden in een discreet bandenpatroon, de zogenaamde spectrotypen of klonotypen. Een disproportioneel grote reactie van één of enkele klonen resulteert dan in klonale dominantie. Deze klonale dominantie was zeldzaam in CBA muizen, maar frequent in C57BL muizen. De leeftijd op het moment van immunisatie en neonatale thymectomie had nagenoeg geen additief effect op de incidentie van H-Ig in de sera van de muizen van beide stammen. In oude C57BL muizen werden geringere hoeveelheden specifieke antistoffen geproduceerd door minder klonen. Toch was de incidentie van H-Ig in deze groep gelijk aan die in jonge C57BL muizen. Dit duidt erop dat oude C57BL muizen een grotere neiging hebben tot klonale dominantie in de specifieke antistofvorming. Deze tendens correleerde met de hoge incidentie van MG in verouderende C57BL muizen. Concluderend kan worden gesteld dat klonale dominantie in de specifieke antistofvorming een genetisch bepaald kenmerk van B-cellen lijkt te zijn. Controle op deze dominante klonen wordt uitgeoefend door immunoregulatoire systemen, zoals het T-cel systeem. Verstoringen in dit controlesysteem kunnen ervoor zorgen dat dominante B-cel klonen kunnen uitgroeien tot grotere klonen, een stadium dat voorafgaat aan het ontstaan van MG in muizen.

Langdurige immunosuppressieve therapieën verhogen het risico op B-cel maligniteiten, met name van non-Hodgkin lymfomen. Slechts weinig is bekend over de late effekten van immunosuppressieve onderhoudstherapieën op het ontstaan van MG. In hoofdstuk 4 werden twee veelgebruikte immunosuppressieve behandelprotocollen, azathioprine/prednisolon en Cyclosporine A/prednisolon, bestudeerd in zowel jonge als oude C57BL/KaLwRij muizen. Beide behandelmethoden verhoogden de incidentie van MM tienvoudig. MG van voorbijgaande aard bereikten een vier tot vijf maal hogere incidentie in de behandelde groepen ten opzichte van de PBS controle groep. Er werden geen verschillen gevonden in de incidenties van BMG. De incidentie van MG was ongeveer gelijk voor de verschillende behandelde groepen en de controlegroep, maar de muizen in de behandelde groepen lieten eerder een toename zien van MG. De resultaten van deze studie benadrukken dat een intact T-cel systeem noodzakelijk is voor een normaal heterogeen Ig spectrum en dat een langdurig, c.q. levenslang, verminderd functionerend T-cel systeem predisponerend werkt voor het ontstaan van maligne MG.

De 5T muize MM en WM, welke spontaan ontstaan in verouderende

C57BL/KaLwRij muizen, hebben klinische en biologische kenmerken die sterk lijken op de corresponderende humane ziekten. Om de patronen van somatische mutaties in de V_H genen van deze 5T-tumoren te vergelijken met die van de analoge afwijkingen bij de mens, werden de Ig V_H sequenties bepaald en geanalyseerd van 5 MM, van 2 WM, en van 1 biklonale BMG. Dit werd beschreven in hoofdstuk 5. Vergeleken met humane MM en WM is de mate van somatische mutatie in de muize MM en WM significant geringer. Er waren geen duidelijke aanwijzingen voor de groepering van aminozuur volgordeveranderende mutaties in de zogeheten "complementarity-determining regions" (CDR). Zo'n groepering wordt beschouwd als een karakteristiek van antigen-geselecteerde sequenties. Echter, in een kloon van de biklonale BMG bleek intraklonale variatie aannemelijk, hetgeen suggereert dat de tumorcellen nog onder de invloed van het somatische mutatiemechanisme stonden. Wat dit betreft lijkt de muize BMG erg op de humane analoge B-cel proliferatie-stoornis.

Verder onderzoek naar unieke kenmerken van maligniteit in de klonale B-cellen van MG en het ontrafelen van de mechanismen die leiden tot de maligne transformatie van de B-cellen is noodzakelijk voor het onderscheiden van benigne en maligne MG in een vroeg stadium van de ziekte.

ABBREVIATIONS

ABL	-	antigen-specific immunoblotting
ADIBA	-	antigen-specific dot-immunobinding assay
Ag	-	antigen
AP	-	alkaline phosphatase
B-ALL	-	B-cell acute lymphocytic leukemia
B-CLL	-	B-cell chronic lymphocytic leukemia
BM	-	bone marrow
BMG	-	benign monoclonal gammopathy
BSA	-	bovine serum albumin
BSS	-	balanced salt solution
C-region	-	constant region of the immunoglobulin molecule
CDR	-	complementarity determining region(s)
CsA	-	Cyclosporin A
DAB	-	3,3'-diaminobenzidine tetrahydrochloride
D_H	-	D gene segment of the immunoglobulin heavy chain gene cluster
DNP	-	dinitrophenyl
EBV	-	Epstein-Barr virus
FCCL	-	follicular center cell lymphoma
FR	-	framework region(s)
GALT	-	gut-associated lymphoid tissue
GaM	-	goat anti-mouse
H-Ab	-	homogenous antibody
H-chain	-	heavy chain of the immunoglobulin molecule
HS	-	histiocytic sarcoma
HSA	-	human serum albumin
H-Ig	-	homogenous immunoglobulin component(s)
HIV	-	human immunodeficiency virus
IEF	-	isoelectric focussing
Ig	-	immunoglobulin
IgH	-	immunoglobulin heavy chain
IP	-	idiopathic paraproteinemia
J_H	-	J gene segment of the immunoglobulin heavy chain gene cluster
J_L	-	J gene segment of the immunoglobulin light chain gene cluster
L-chain	-	light chain of the immunoglobulin molecule
LL	-	lymphoblastic lymphoma
LM	-	littermate
LPS	-	lipopolysaccharide
MALT	-	mucosa-associated lymphoid tissue
M-Ig	-	monoclonal immunoglobulin component(s)
MIST	-	maintenance immunosuppressive treatment
MG	-	monoclonal gammopathies
MGUS	-	monoclonal gammopathy of undetermined significance
MM	-	multiple myeloma
MR	-	multi reactive immunoglobulin
NHL	-	non-Hodgkin lymphoma

NMS	-	normal mouse serum
NTx	-	neonatal thymectomy
PB	-	peripheral blood
PBS	-	phosphate-buffered saline
PCR	-	polymerase chain reaction
PerC	-	peritoneal cavity
PMA	-	phorbolmyristateacetate
PO	-	peroxidase
PVDF	-	polyvinylidene difluoride
SCID	-	severe combined immunodeficiency
ShaM	-	sheep anti-mouse
STAT3	-	signal transducer and activator of transcription-3
T	-	Tween-20
TBI	-	total body irradiation
Tg	-	transgenic
TNP	-	2,3,6-trinitrophenyl
V_H	-	variable part of Ig heavy chain
V_L	-	variable part of Ig light chain
W	-	Wieme agar-electrophoresis
WABL	-	Wieme agar-electrophoresis and antigen specific immunoblotting
WM	-	Waldenström's macroglobulinemia

DANKWOORD

De kaft van dit boekje doet natuurlijk vermoeden dat dit proefschrift door slechts één auteur is geschreven. Maar zonder de intellectuele en sociale input van vele anderen was het de auteur nooit gelukt om dit tot een goed einde te brengen. Mijn dankbaarheid betreft niet alleen het bereiken van het eindresultaat in de vorm van dit proefschrift, maar juist ook alle (leer)ervaringen die niet in de voorafgaande delen van dit boekje verwoord konden worden.

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