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MULTIPLE MYELOMA

CIP-GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Croese, Jan Willem

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STELLINGEN

1

Het C57BL/KaLwRij muis multipel myeloom (het 5T multipel myeloom) voldoet door de spontane wijze van ontstaan en door de morfologische kenmerken, zoals, bijvoorbeeld, de aanwezigheid van de myeloomcellen in het beenmerg van de verschillende botten en de aanwezigheid van botlaesies, aan de criteria om multipel myeloom genoemd te worden. Door de continue beschikbaarheid van dit muis multipel myeloom in de getransplanteerde vorm is het een geschikt model voor uitgebreide preklinische studies naar deze ziekte.

2

Uit de resultaten van het onderzoek naar het effect van APD op de botdestructie in muizen met het 5T2 multipel myeloom volgt dat, indien men besluit om patiënten met multipel myeloom te behandelen met APD, deze behandeling aangevangen dient te worden vanaf het moment dat de diagnose gesteld is, ongeacht de fase waarin de ziekte verkeert en ongeacht het al of niet aantoonbaar zijn van botdestructie.

3

Voor de behandeling van ziekten, die worden gekenmerkt door een door osteoclasten veroorzaakte verhoogde botresorptie, is van de drie klinisch meest toegepaste bisfosfonaten, EHDP, Cl_2MDP en APD, het laatstgenoemde het middel van eerste keuze omdat het een hoge werkzaamheid heeft in een dosering, die nauwelijks ongewenste bijwerkingen heeft.

4

Klinische studies naar het effect van immuuntherapie van multipel myeloom door middel van toediening van anti-idiotype antilichamen zijn niet uitvoerbaar, zolang geen methode gevonden is om het serumgehalte van het myeloom-immuunglobuline tot werkelijk minimale waarden te verlagen.

5

De beste methode om de sterfte door kanker te meten is de bepaling van het jaarlijkse sterftecijfer van alle vormen van kanker tezamen in een populatie, gecorrigeerd voor de leeftijdsopbouw van de betreffende populatie in een bepaald jaar; toepassing van deze methode laat zien dat we in de strijd tegen kanker aan de verliezende hand zijn.

John C. Bailar III and Elaine Smith (1986): N. Engl. J. Med. 314:1226-1232.

6

Indien kanker zich door middel van klinische symptomen manifesteert, bevindt het zich in een ver voortgeschreden stadium; vroege detectie is daarom bij vele vormen van deze ziekte niet mogelijk omdat iemand zijn/haar arts pas consulteert, indien hij/zij zich ziek voelt.

7

Euthanasie is in principe een aangelegenheid, die zoveel mogelijk in handen dient te blijven van de huisarts; indien er sprake is van euthanasie zal de familie er net zo actief bij betrokken moeten worden als de patiënt.

8

In de onderzoekskamer van de arts in een bejaardenverzorgingstehuis behoren een electrocardiograaf en een defibrillator aanwezig te zijn.

9

Met de tegenwoordige beschikbaarheid van een groot aantal middelen tegen verhoogde bloeddruk met een verschillend werkingsmechanisme behoeft de behandeling van patiënten met hypertensie niet slechts gericht te worden op een vermindering van de morbiditeit en de mortaliteit, doch ook op het welbevinden van de patiënt.

Aram V. Chobanian (1986): N. Engl. J. Med. 314:1701-1702.

10

"De wetenschappelijk onderzoeker wordt aangedreven door de enkele zucht tot in verband brengen, tot vergelijken en kennen der verhoudingen. De hogere betekenis der feiten, de harmonie en schoonheid der verhoudingen, zijn hem, als onderzoeker, onverschillig". Deze uitspraak door Frederik van Eeden opgeschreven in het "voorwoord bij den tweeden druk" in het boek "Van de koele meren des doods" toont aan dat de schrijver minder begrip had voor de wetenschapper dan menig wetenschapper heeft voor de schrijver.

11

Voor het verkrijgen van een nader inzicht in de distributie van myeloomcellen in het lichaam van een myeloom-patiënt zijn immunofluorescentie-studies naar de aanwezigheid van zulke cellen in de verschillende organen noodzakelijk.

12

Uit het geluidsniveau van de moderne popmuziek in gelegenheden waar mensen elkaar plegen te ontmoeten kan men concluderen dat de behoefte om verbaal met elkaar te communiceren niet groot is.

13

Vijftien jaar lang hockeyen in hetzelfde team geeft een vrij aardig inzicht in het proces van veroudering.

14

Het geven van calciumsuppletie aan mensen met osteoporose die er een adequaat voedingspatroon op na houden, is even effectief als het geven van een aspirine aan een influenza-patiënt.

Leiden, 12 november 1987 J.W. Croese

EXPERIMENTAL APPROACHES TO THE TREATMENT OF MULTIPLE MYELOMA

STUDIES IN AN ANIMAL MODEL

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR AAN DE RIJKSUNIVERSITEIT TE LEIDEN, OP GEZAG VAN DE RECTOR MAGNIFICUS DR. J.J.M. BEENAKKER, HOOGLERAAR IN DE FACULTEIT DER WISKUNDE EN NATUURWETENSCHAPPEN, VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN TE VERDEDIGEN OP DONDERDAG 12 NOVEMBER 1987 TE KLOKKE 15.15 UUR

DOOR

JOAN WILLEM CROESE

GEBOREN TE AMSTERDAM IN 1951

1987
DRUKKERIJ J.H. PASMANS B.V., 's-GRAVENHAGE

Promotiecommissie

Promotor:	Prof. Dr. O.L.M. Bijvoet
Co-promotor:	Dr. J. Radl
Referent:	Prof. Dr. R. Benner, Erasmus Universiteit Rotterdam
Overige leden:	Prof. Dr. W. Hijmans
	Prof. Dr. F.J. Cleton

Het onderzoek, dat beschreven is in dit proefschrift, heeft plaatsgevonden in het Instituut voor Experimentele Gerontologie TNO te Rijswijk.

Financiële steun voor dit onderzoek werd gegeven door het Koningin Wilhelmina Fonds (projectnummer: IEG 82-10).

Aan mijn ouders
Ter herinnering aan mijn zusje Tessa

Want zij die b.v. een proefschrift schrijven, dat immers alleen bestemd is om aan het oordeel van enige professoren te worden onderworpen, en die dus de strengste en meest deskundige critici niet vrezen, zijn, dunkt me, meer te beklagen dan te benijden, daar ze zich eindeloos aftobben. Ze voegen toe, veranderen, schrappen, herstellen weer, herzien, werken het weer geheel en al om, laten het graag anderen zien, houden het negen jaar in portefeuille en zijn nooit tevreden met het resultaat. De beloning, die ze er ten slotte voor krijgen - immers de lof van een enkeling - is wel heel duur betaald met al hun zwoegen, zweten en gebrek aan het zoetste, wat er bestaat: de slaap. Voeg hierbij nog dat dit alles gaat ten koste van hun gezondheid, dat ze daardoor humeurig, lelijk, bijziende of zelfs blind worden, tot armoede vervallen, bij ieder uit de gunst zijn, dat ze alle genoegens moeten verzaken, dat ze vóór hun tijd oud zijn, ontijdig sterven en wat dies meer zij.
Doch al deze opofferingen getroosten zij zich gaarne om de goedkeuring weg te dragen van één of twee geleerde boekenwurmen.

Desiderius Erasmus. Uit: De Lof der Zotheid (1509). Vertaald en bewerkt in het Nederlands door Mr. A. Dirkzwager Czn. en A.C. Nielson z.j., [1949]; H.J. Paris, Amsterdam.

CONTENTS

Parts of the work described in chapter IVA and B have been published previously (Cancer 55: 1030-1040; 1985) or are in preparation.
Parts of the work described in chapter IIIA are in press or submitted for publication. Chapter IIIB is in preparation.

CHAPTER I

GENERAL INTRODUCTION

1. Multiple Myeloma: clinical and laboratory features and current status of treatment

a) Incidence

Multiple myeloma (MM) is a neoplasm characterized by an increased number of more or less differentiated malignant plasma cells which are mainly located in the bone marrow. That these cells are monoclonal in origin is inferred from the identical structure of the immunoglobulin molecules they produce. The serum content of the myeloma immunoglobulin increases in proportion to the growth of the tumour mass up to a certain limit, and, therefore, is considered to reflect the tumour load (1,2).

As is the case for most other malignancies, the aetiology of MM is unknown. It is the most common neoplasm of lymphoid origin with an overall annual incidence of three cases per 100,000 persons (3). This varies somewhat between countries, between the two sexes, and among ethnic groups. The annual incidence in the Federal Republic of Germany, for example, amounts to 1.1 new female patient and 1.7 new male patient per 100,000 females and males, respectively. In the United States, the annual incidence is significantly higher than this. This difference may be partly due to a high incidence of MM in the negro population of the United States (4). Approximately ten out of 100,000 negroes will develop MM each year, whereas the annual incidence in whites is 4.3 per 100,000 (5). The reported overall incidence of MM has increased over the last three decades. This is in part due to a refinement of diagnostic procedures resulting in a more frequent and precise diagnosis of MM during the life of the patient. Furthermore, in many countries the improved health care has led to an increase in the mean age of their inhabitants. This has undoubtedly contributed to the increased incidence, since MM is largely a

disease of elderly people. The highest incidence is found in individuals older than 60 years. MM is extremely rare in persons under the age of 40 years (5,6,7).

b) Clinical features

Early, non-specific features of patients with MM are weakness, anaemia, and pain. Many patients suffer from recurrent infections, generally of the respiratory tract, and renal function is ofter impaired. A later and serious complication of MM is skeletal destruction which is associated with bone pain, pathological fractures (mainly of the vertebrae), hypercalcaemia and hypercalciuria. Therefore, X-ray examination of the skeleton is required when a patient is suspected of having MM.

c) Laboratory findings

Serum and/or urine electrophoresis will often reveal the presence of an abnormal homogeneous component in the beta-gamma globulin fraction. Immunoelectrophoresis, immunofixation, or immunoblotting using antisera specific for the different immunoglobulin heavy and light chain isotypes will enable classification of this component. About 80 per cent of patients have MM of the IgG class; IgA myeloma is found in approximately 15 per cent. IgD, IgM, and IgE are rare (3). Immunoelectrophoresis using antisera against the "hidden" determinants of free light chains often reveals the occurrence of free monoclonal lambda or kappa chains, the so-called Bence Jones proteins, in the serum, but especially, in the urine. Some variants of MM produce only Bence Jones proteins. An additional immunochemical finding in the serum from myeloma patients is a reduction in the amount of the normal, non-myeloma immunoglobulins. This hypogammaglobulinaemia contributes to the susceptibility of myeloma patients for infection (8,9,10,11). Serum creatinine and urea concentrations should be determined to detect impairment of renal function. Care must, however, be taken in interpreting creatinine concentrations in elderly subjects. Creatinine reflects lean body mass, which is reduced in old age and especially in cachexia. Severe degrees of renal impairment may then be present with a normal creatinine concentration. In MM, the serum beta-

2-microglobulin concentration is often increased (12). Beta-2-microglobulin is a polypeptide that represents the light chain of class-1 major histocompatibility (MHC) antigens (13). The serum concentration of beta-2-microglobulin is regarded as an important prognostic factor in MM (14,15,16,17). High and low levels correlate well with a high and low tumour burden, respectively. *Intermediate levels are observed in all tumour mass categories (18). Since* beta-2-microglobulin is excreted by the kidneys, the serum level is always increased in patients with impaired renal function (18). Typical for MM is the marked elevation of the erythrocyte sedimentation rate, which is caused by the high serum concentration of the myeloma protein. Bone marrow biopsy or aspiration are routine procedures in the diagnosis MM. To meet the criterion for the diagnosis MM, a bone marrow specimen should contain more than 10 per cent of abnormal plasma cells according to Kyle (19). Furthermore, more than 95 per cent of all immunoglobulin-containing cells in the bone marrow cytocentrifuge slide of a MM patient should have the same isotype as determined by immunofluorescence or immunohistochemical-enzyme staining (20).

d) Clinical course and staging

The course of MM is progressive, although to a variable extent in different patients. Untreated MM will usually lead to death within a year from diagnosis (3). One form of MM (so-called smoldering MM) has a somewhat different course (21). Patients with smoldering MM fulfil the criteria for the diagnosis of MM: ten per cent or more plasma cells are present in their bone marrow, the serum concentration of the myeloma protein is within the range of MM patients, i.e., at least 3 g per 100 ml, and the level of normal polyclonal serum immunoglobulin is reduced. However, this form of MM differs from active MM by the absence of anaemia, hypercalcaemia, and bone lesions. Furthermore, no proliferation of the malignant cells is detectable by measurement of their labelling index (22).

The survival of treated myeloma patients depends on the stage of the disease at detection. A staging system for MM has been developed by Salmon and Durie (23). These investigators classified MM into three stages according to a number of clinical and laboratory parameters which they found to correlate with the immunologically-measured total body myeloma cell number. These

clinical and laboratory parameters are: the haemoglobin concentration, the serum calcium concentration, the degree of bone destruction (as observed by skeletal X-ray examination) and the serum and urine content of the myeloma immunoglobulin. Furthermore, each of the three stages are divided into two subcategories, A and B, according to, respectively, the presence or absence of normal renal function. Patients with MM in stage I have a total body myeloma cell number of less than 0.6×10^{12} cells per square meter of body surface area; stage II corresponds to a number of myeloma cells between 0.6 and 1.2×10^{12}; stage III is characterized by a tumour mass of more than 1.2×10^{12} cells per square meter. The prognosis of patients with stage III myeloma is much worse than that of patients with MM of stage I or II. In addition, patients in the B sub-categories (i.e., those with impaired renal function) have a less favourable prognosis than patients in the A sub-category at the corresponding stage (24,25). Durie and co-workers (24) reported a median survival of 61.2 and 54.5 months in patients with MM of stage I and II, respectively. Patients with stage IIIA MM had a mean survival of 30.1 months while in those with IIIB MM, survival was only 14.7 months.

e) Treatment

Chemotherapy is the treatment of choice (19). Currently available chemotherapeutic approaches have resulted in the induction of a partial or complete remission in a fairly large number of patients, but not in a permanent cure. In the late 1960's, oral treatment with the alkylating agent melphalan (L-phenylalanine mustard) in combination with prednisone came into general use. Melphalan is administered either intermittently or continuously. These treatment regimens produced objective responses in 40 to 60 per cent of the patients. The reported median survival of patients treated in this way varied from 24 to nearly 40 months (reviewed in 19 and 26). Attempts to improve these results have been made with combinations of cytotoxic agents and prednisone. Combination chemotherapy is thought to be more effective than single agent therapy if no cross resistance to the agents used exists. Moreover, a combination of non-overlapping toxic side effects of various drugs in a lower dose would be better tolerated than the toxicity of a higher dose of a single agent (26). Several reports of clinical trials showed improved results of

combination therapy. It should be emphasized, however, that in those trials, no group of patients treated with melphalan and prednisone was included for comparison. In randomized studies, the results of combination therapy were not significantly better. Examples of multiple drug regimens are:

1) CAP, which includes the alkylating agent cyclophosphamide, doxorubicin (Adriamycin), and prednisone;
2) VCAP, i.e., vincristine, cyclophosphamide, doxorubicin, and prednisone;
3) VMCP, including vincristine, the alkylating agents melphalan and cyclophosphamide, and prednisone;
4) VBAP, including vincristine, the alkylating agent BCNU (carmustine), doxorubicin, and prednisone.

The response rates observed after treatment with one of these combinations varied between 46 and 61 per cent and the median survival was between 30 and 32 months (27). Case and his co-workers (28) reported in a study including predominantly patients with MM in stage II (22 per cent) and stage III (74 per cent) that the so-called M-2 combination (BCNU, cyclosphosphamide, vincristine, melphalan, and prednisone) resulted in a response rate of 87 per cent. The median survival of all these patients was 50 months. This is a significant increase relative to the survival of patients treated with the aforementioned combinations. Bergsagel and his co-workers (25), however, carried out a randomized trial in which a group of patients receiving a combination of melphalan, cyclophosphamide, BCNU, and prednisone was compared with a group treated with melphalan and prednisone only. They could not establish that the former combination led to better results. Recently, the result of another study was presented concerning the treatment of MM with the M-2 protocol (29). Only patients with stage II (13 per cent) and stage III (87 per cent) MM were included. An objective response was observed in 90 per cent of the cases. However, most of these responses were only partial and complete remissions were infrequent. In that study, the median survival determined from the initiation of treatment already exceeded four years. Myeloma patients who initially respond to chemotherapy with alkylating agents will eventually become resistent to further treatment with these compounds (19). This is partly due to the development of cross resistance among the different alkylating agents (30). Moreover, some patients with MM do not respond to alkylating agents at all. Combinations of other drugs can induce

remissions in a significant number of such patients. Treatment with carmustine, doxorubicin, and vincristine, given intravenously, in combination with prednisone, resulted in a response in 40 per cent of the resistant patients (19). Seventy per cent of patients with MM refractory to alkylating agents responded to a high dose dexamethasone given in combination with four-day continuous intravenous infusions of vincristine and doxorubicin (31). The latter study included only twenty patients, and the results should be confirmed with larger numbers. One should realize that cytotoxic anticancer drugs cause undesirable and toxic side effects and are of no benefit in inactive disease. Therefore, patients with a stable or hardly progressive disease, and especially those with smoldering MM, should not receive chemotherapy. They need regular follow-up examinations and postponement of treatment until the MM becomes active (21).

Since no real progress has been made in the treatment of MM with cytotoxic chemotherapy in the last two decades, the effects of other treatment modalities have been investigated. Allogeneic bone marrow transplantation has been performed in a number of patients. Before transplantation, the patient is conditioned with cytotoxic chemotherapy and total body irradiation. Application of allogeneic bone marrow transplantation is limited by the problems of graft rejection and graft-versus-host disease. This is not the case for autologous bone marrow transplantation. The patient is treated with cytotoxic chemotherapy to achieve tumour reduction before the collection of bone marrow. The stored bone marrow is re-infused after intensive additional treatment of the patient with high doses of chemotherapy and/or irradiation. Autologous bone marrow transplantation is also complicated by major problems. In spite of intensive treatment, it is very difficult to eradicate the residual myeloma cells in the patient. Furthermore, removal of all residual myeloma cells from the collected bone marrow by chemotherapy causes severe injury to the normal stem cells. Monoclonal antibodies with specificity for myeloma cells and their precursors may be useful for the selective removal of those cells from the stored bone marrow. However, the currently available monoclonal antibodies do not bind to all neoplastic plasma cells (19,32). Recently, the results of treatment of fourteen MM patients with allogeneic bone marrow transplantation were reported (33). Ten out of these fourteen patients were still alive 6 to 34 months after they had received the transplant. Severe acute graft-versus-host

disease was seen in only one patient. These results might be considered promising in view of future treatment of patients with MM refractory to chemotherapy.

Direct cytotoxicity of human recombinant interferon alpha for human myeloma has been observed in in-vitro systems such as the myeloma colony-forming assay according to Hamburger and Salmon (34,35). Phase II studies with recombinant interferon alpha have been completed in patients with MM resistant to conventional chemotherapy. Partial or complete responses were observed in about twenty per cent of the patients (35). The efficacy of the addition of interferon to a melphalan-prednisone regimen is under investigation.

2. Pathological bone resorption in MM

a) Skeletal destruction

One major and characteristic complication of MM is bone disease resulting in diffuse osteoporosis, lytic bone lesions, or both. Typical for MM are round, sharply punched-out bone defects often observed in the skull. These lesions are readily visualized on radiographs. Systemic radiographic studies have revealed that bone lesions are present in nearly 80 per cent of patients with MM (6). Mainly affected are the vertebrae, the ribs, the vault of the skull, the bones of the pelvis, the femora, and the sternum. These effects impair the mechanical properties of bone, and compression fractures of the vertebral bodies are common.

In an autopsy study of 142 myeloma patients (5), the proportion of patients with skeletal destruction appeared even higher (97 per cent) than what had been reported for radiography. The distribution of the different types of skeletal destruction was as follows: lytic lesions only were observed in 51 per cent of the patients; in 35 per cent, lytic lesions were found in combination with diffuse osteoporosis; osteoporosis without lytic lesions occurred in only 11 per cent.

Bone lesions often do not heal during chemotherapy (36,37). Pain from specific osteolytic foci is usually treated by local radiotherapy. Attempts to

reduce the severity of the bone disease with sodium fluoride, calcium carbonate, or androgenic steroids have not been successful (38).

The bone disease in MM is caused by a pathologically increased bone resorption mediated by a factor produced by the neoplastic cells, and several studies to identify the exact nature of this factor have been reported. Cultured lymphoid cell lines from patients with myeloma, Burkitt's lymphoma, and other malignant lymphomas were found to secrete a bone resorption stimulating factor distinct from known stimulators of bone resorption such as parathyroid hormone, 1,25-dihydroxivitamin D3, and prostaglandin E2 (39). The biological and biochemical characteristics of this factor are similar to those of osteoclast-activating factor (OAF), a lymphokine which was detected in the supernatant fluid of human peripheral leukocytes cultured with antigens or mitogens. It was demonstrated that OAF stimulated osteoclastic resorption of fetal bone in vitro (40,41). The supernatant of short-term cultures of human myeloma cells obtained by bone marrow aspiration contained a stimulator of bone resorption that was also similar to OAF (42). In addition, the amount of OAF produced in vitro by myeloma cells from the bone marrow of 33 MM patients correlated with the extent of skeletal destruction in the same patients. The highest OAF production per myeloma cell was measured in bone marrow cells from patients with the most extensive bone disease (43). The conclusion that the increase in osteoclastic bone resorption in MM is due to the production of OAF by the myeloma cells is supported by observations in bone marrow biopsies. In these biopsies, the osteoclasts were mainly located at resorptive bone surfaces adjacent to infiltrations of myeloma cells. This suggests their functional relationship (43).

The recent advances in biotechnology have enabled the purification of a number of lymphokines and monokines in sufficient quantities to characterize their biological activities. Some of these factors have the capacity to stimulate osteoclastic bone resorption in vitro. Examples are lymphotoxin, tumour necrosis factor, and interleukin-1. Purified OAF produced by activated cultures of human peripheral blood mononuclear cells was reported to be identical to interleukin-1 beta (44). OAF probably represents a mixture of different factors which stimulate the resorption of bone both in vitro and in vivo (45).

b) Hypercalcaemia

Twenty to thirty per cent of patients with MM develop hypercalcaemia (46). It occurs in patients with myeloma in an advanced stage. Increased bone resorption is the main cause of hypercalcaemia. However, this is not the only causal factor, since not all patients with a high tumour mass have hypercalcaemia. Impaired renal function may also contribute to this condition (45). Untreated hypercalcaemia in MM or other malignancies has a progressive course. Severe hypercalcaemia is life-threatening. The common symptoms of hypercalcaemia are polydipsia, polyuria, nausea, vomiting and anorexia. A further increase in the serum calcium concentration will lead to the development of more severe symptoms such as confusion, stupor, and eventually coma. Effective treatment of MM results in reduction of the serum calcium concentration, but acute severe hypercalcaemia needs direct intervention. The basis of every treatment of this condition consists of the promotion of a diuresis by intravenous infusions of isotonic saline. This leads to an increase in renal calcium excretion. The treatment with saline can be combined with administration of calcitonin and glucocorticoids in order to inhibit bone resorption (47). Recently, a combination of saline infusions and oral or intravenous administration of the bisphosphonate APD has appeared very effective in inhibiting the excessive bone resorption. This usually results in a decrease of the serum calcium concentration to normal levels within a couple of days (48, 49,50).

The development of an effective strategy for the treatment of bone disease in MM remains a major task since no curative therapy is available for the basic malignant disease. Improvement of the skeletal condition would significantly improve the quality of life of the patients during their illness.

3. The myeloma cell

a) Morphology

MM is generally regarded as a plasma cell malignancy. The similarity in morphology between myeloma cells and plasma cells was first reported by

Wright in 1900 (51). The similarity between myeloma cells and plasma cells is not only morphological but also functional. Just like plasma cells, myeloma cells can synthesize and secrete immunoglobulins. However, differences in morphology between myeloma cells and plasma cells do exist. Myeloma cells often have a larger diameter and they have more abundant nuclear chromatin (52). On the basis of their morphology, myeloma cells are classified into three types according to the degree of differentiation (5,52). The morphology of the differentiated myeloma cell resembles that of a mature, reactive plasma cell: the nucleus has an eccentric position, the nuclear chromatin has the typical "spoke-wheel" pattern, and sometimes typical Russell bodies can be seen in the cytoplasm (52); furthermore, as in normal plasma cells, a typical perinuclear area (Golgi region) is present (5). In contrast, poorly differentiated myeloma of a plasmablastic cell type consists of a markedly pleomorphic cell population with many large and immature looking cells (5,52). These cells frequently occur as bizarre forms. Bi- and even multinucleated variants are seen. The nuclei, which are often centrally positioned, contain clear round nucleoli. Between these two types, an intermediate form of myeloma is recognized, consisting of moderately differentiated cells. The nuclei of these cells have a less eccentric position than those in the differentiated myeloma cells; they vary in size, and often contain several nucleoli.

The characteristic ultrastructural features of myeloma cells reflect the features seen with light microscopy (52). Low magnification electron microscopy may show the eccentric position of the nucleus. Furthermore, the cytoplasm contains an abundant granular endoplasmic reticulum and a well developed Golgi region, which characterize actively protein-producing and -secreting cells. Typical for myeloma cells is the contrast between the high level of development of the cytoplasm and the immature aspect of the nucleus. The nuclear chromatin of a myeloma cell often has a dispersed distribution while the nucleus of a normal plasma cell shows dense chromatin at the periphery. It should be stressed that no single morphological criterion for the malignant nature of the plasma cell exists.

Myeloma cells are mainly located in the bone marrow. Within this bone marrow compartment, three patterns of growth can be distinguished: 1) an interstitial growth pattern, characterized by a diffuse growth of myeloma cells among the remaining bone marrow cells; 2) myelomatous myeloma with distinct

plasma cell aggregates scattered throughout the bone marrow; 3) the packed marrow variant showing a complete, compact infiltration of myeloma cells in the bone marrow (53).

Both, the morphological aspect and the proliferation pattern of the myeloma cells in the bone marrow are of prognostic significance for the patients. In a study on 220 patients (53), those with the highly differentiated form of MM had a median survival time of 32 months, and those with the moderately and less differentiated forms of MM had a median survival time of only 8 and 7 months, respectively (calculated from the time of the first biopsy). When the growth pattern of the myeloma cells within the bone marrow was considered, patients with the interstitial form of MM had a median survival time of 128 months; much worse was the prognosis of patients with myelomatous myeloma and myeloma with a packed marrow growth pattern. These patients had a survival time of 18 and 9 months, respectively.

b) Clonogenic cell

Although MM is a malignant neoplasm of plasmacytes or plasmablasts, there are indications that this malignancy has its origin in a less differentiated precursor cell. The plasma cell is the fully differentiated cell of the B-cell lineage, which originates from a pluripotent stem cell in the bone marrow.

Peripheral blood lymphocytes bearing the idiotype of the circulating myeloma immunoglobulin have been observed in immunofluorescence studies (54,55,56,57). The clonal involvement has even been demonstrated in pre-B-cells (58). Recently, a new cell type has been described and proposed as the clonogenic cell in human MM (59). This novel cell type is characterized by a "dotwise" appearance of its cytoplasmic immunoglobulins. Furthermore, it possess the ability to proliferate, as demonstrated by the presence of surface membrane transferrin receptors, and with labelling experiments with tritiated-thymidine. In the differentiation pathway from B-cell precursor to mature plasmacyte, the "dotwise" cell is assumed to represent a stage in between the B-lymphocyte and the plasma cell.

It is suggested that more than one transforming event is responsible for the development of MM and the other malignancies of the B-cell lineage (60). The initial transforming event might have taken place in a very early

precursor cell of the involved clone, the final transformation would then occur in a cell which had achieved the stage of differentiation reflecting the predominant cell type of the observed malignancy. It is conceivable that the affected precursor cell gives rise to a number of clones and that the final neoplastic event will subsequently occur in cells of two or more of these clones. This might explain the occasional presence of more than one neoplasm of B-cell origin in a single individual.

The immunoglobulin molecules from a single clone have, in addition to an identical isotype and allotype, also the same idiotype. This means that the hypervariable regions within their heavy and light chains are identical. Therefore, the idiotype of a B-cell neoplasm can be regarded as a tumour-specific antigen (61,62,63). The presence of a particular idiotype on a given B-cell neoplasm may be exploited for the development of a therapeutic strategy. It offers a possibility to raise antibodies directed to that idiotype. Several efforts have been made to treat patients with a B-cell lymphoma by intravenous administration of large amounts of murine monoclonal antibodies specific for the idiotype of the lymphoma (63,64). As yet, the results of this approach have been rather disappointing. Only one persistent remission has been reported (64). It is not possible to treat MM by intravenous administration of anti-idiotype antibodies. All injected anti-idiotype antibodies will be neutralized through their binding to the idiotype of the many serum myeloma immunoglobulins before they can reach the myeloma cells.

4. Aim and outline of the study

MM is still an incurable disease. No significant progress has been made in the chemotherapy of MM since the discovery of the relative efficacy of alkylating agents such as melphalan and cyclophosphamide. Moreover, chemotherapy is not harmless. Treatment with alkylating agents increases the risk of developing acute leukaemia (25). However, it has not been excluded that acute leukaemia may also develop as a part of the natural course of MM, and thus not only as a result of chemotherapy.

The severe bone destruction in patients with an advanced myeloma represents an additional therapeutic problem. Frequently, chemotherapy does not

lead to bone repair, even in those patients who have responded to the treatment.

A great deal of the research in the field of MM has been carried out in clinical studies and with material from human origin. Large numbers of patients are required for clinical trials, which necessitates the inclusion of many centres. As a consequence, such studies are of long duration and expensive, and the management is complicated. Therefore, animal models of MM that permit pre-clinical research on the desired scale are of great value.

In the study presented in this thesis, the mouse 5T2 MM was used as the animal model for the human MM. The 5T2 MM originated spontaneously in an aging C57BL/KaLwRij mouse, and has been maintained in vivo by intravenous transfer of bone marrow or spleen cells from donor mice into young recipients of the same inbred strain (65).

The principal aim of the study was to find new, additional possibilities for the treatment of MM. After validation of the 5T2 MM as an appropriate experimental model, it has been used to investigate the efficacy of new treatment modalities. These were directed to the inhibition of the growth of the malignant process with immunological methods, and to prevention and/or reduction of the bone destruction. Accordingly, this thesis contains the following chapters:

In chapter II, the materials and methods used in the different experiments have been described.

Chapter IIIA deals with the morphological, biological, and immunological characteristics of 5T2 MM.

The experiments performed in order to investigate the anti-5T2 MM idiotype immunity and to influence the "take" and the development of 5T2 MM with immunological methods have been described in chapter IIIB.

Chapter IVA gives a brief introduction to the bisphosphonates. In addition, it describes the study performed to find a suitable, non-toxic dose of the amino-bisphosphonate APD for the treatment of 5T2 MM mice.

In chapter IVB, the morphological aspects of the bone destruction which complicates 5T2 MM and the studies on the treatment of the bone lesions with APD have been described in detail.

Finally, in chapter V the results have been summarized, and discussed in the light of their potential applicability in clinical practice.

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CHAPTER II

MATERIALS AND METHODS

1. Strain of mice used in the experiments

Male and female C57BL/KaLwRij mice from the colony of the TNO Institute for Experimental Gerontology were used in all studies. They were derived from specified pathogen free (SPF) conditions (1), and maintained under clean conventional conditions. Detailed information on husbandry, health status, survival data, and age-associated pathology of this strain has been published elsewhere (2).

2. 5T2 MM line

This mouse MM line of C57BL/KaLwRij origin has been propagated by intravenous bone marrow or spleen cell transplantations into young recipients of the same strain (3,4,5). The paraprotein produced by the 5T2 MM clone is an IgG2a-kappa immunoglobulin with an antibody activity directed to dinotrophenyl (DNP) and some nucleotide conjugates (4,6).

3. Intravenous transplantation of 5T2 MM

5T2 MM-bearing donor mice were sacrificed by exsanguination under ether anaesthesia. Bone marrow cells were expelled with a syringe by flushing medium through the shafts of the femora, tibiae, and humeri of the mice. The harvested cells were suspended in Hanks' minimal essential medium (Gibco, Paisley, Scotland) supplemented with 15 mM Hepes (Flow Lab., Irvine, Scotland; pH 7.1, 310 mOsm; abbreviated as H+H). In order to obtain a homogeneous suspension, the cells were gently squeezed through a nylon

gauze, which had been premoistened with medium. Subsequently, they were washed in the same medium, and resuspended in Hanks' balanced salt solution (Gibco, Paisley, Scotland; pH 7.1, 310 mOsm; abbreviated as HBSS). The viability of the nucleated cells was determined either by fluorescence microscopy after differential staining of live and dead cells by, respectively, acridine orange (Sigma, St. Louis, Mo., USA) and ethidium bromide (Sigma) or by light microscopy after applying trypan blue which does not stain viable cells (7). The nucleated cells were counted in a Bürker haemocytometer chamber or with an electronic cytometer (Elzone, Elmhurst, Ill., USA). The desired number of viable cells was then suspended in a volume of 0.5 ml HBSS, and injected into a tail vein of the recipient mouse.

Spleen cells were prepared for transplantation by squeezing small fragments of the spleen through the premoistened nylon gauze. Further preparation of the spleen cell suspension was similar to that described for the bone marrow cells.

During all these manipulations, the cells were kept on melting ice.

4. Subcutaneous and intraperitoneal transplantation of 5T2 MM

The 5T2 MM cells were prepared as described for intravenous transplantation. Subcutaneous transplantation was performed by injection of the cells into the inguinal region of the recipient mice. Thereafter, the presence and growth of a tumour was determined by regular palpation of this region. Development of intraperitoneal transplanted 5T2 MM was ascertained by swelling of the abdomen. The sera of all recipients were also regularly screened for the presence of 5T2 MM protein (see section 5 of this chapter).

5. 5T2 MM immunoglobulin

The "take" and development of 5T2 MM in individual mice were routinely monitored by measuring the 5T2 MM protein in serum samples with the double immunodiffusion test according to Ouchterlony. The antibody activity of this MM protein for DNP conjugates was employed for this assay (4).

When a more sensitive technique was required in order to measure very low serum concentrations of 5T2 MM protein, a competition enzyme-linked immunosorbent assay (ELISA) was used. In this assay, 25 µl of a solution of monoclonal anti-5T2 MM idiotype antibody (145-4.1) in phosphate-buffered saline (PBS) was coated on individual wells of 96-well flexible microtitration plates (Falcon, 3911, Becton Dickinson, Oxnard, Ca., USA) for 1 h at room temperature (RT). Unoccupied adsorption sites on the wells were saturated with a solution of 1% (weight/volume, abbreviated as w/v) bovine serum albumin (BSA) in PBS. Subsequently, the plates were washed, twice with PBS and once with a solution of 0.2% (volume/volume, abbreviated as v/v) Tween-20 in PBS (abbreviated as PBS-T). First, the coated antigen was incubated with 20 µl of serial dilutions of the serum to be tested for 45 min at RT. These dilutions had been prepared in PBS containing 1% (w/v) BSA, 0.02% (w/v) sodium azide, and 10 µg/ml phenol red (abbreviated as PBS/BSA/Azide). Directly thereafter, 20 µl of an appropriate dilution in PBS/BSA/Azide of biotin-conjugated, purified 5T2 MM protein (abbreviated as 5T2-biotin) was added. After 1 h at RT, the plates were washed twice with PBS, once with PBS-T, and, again, twice with PBS. Subsequently, the wells were incubated with 20 µl of an appropriate dilution in PBS/BSA/Azide of avidin conjugated with peroxidase (abbreviated as Av-PO) for 1 h at RT. The plates had been washed again with PBS and PBS-T, before the wells were filled with 50 µl of a 2 mg/ml solution of the staining substrate ortho-phenylenediamine (OPD) (Kodak, Rochester, NY, USA), supplemented with 0.5 mM H_2O_2. After incubation for 15 min at RT in a dark box, the asborbance of individual wells was measured at a wave length of 450 nm using an ELISA reader (Titertek Multiskan MC, Flow Lab., McLean, Va., USA). The concentration of 5T2 MM protein in the different sera was deduced from the degree of inhibition of the binding of 5T2-biotin to the 145-4.1 antibody coated on the plate. Purified 5T2 MM protein in a known concentration was used as the standard in order to calculate the various serum concentrations.

The methods of the various ELISA's in the studies of this thesis were essentially similar. In the following sections, only differences from the procedure here described are mentioned in detail.

5T2 MM immunoglobulin has been isolated from the serum or ascitic fluid of 5T2 MM-bearing mice by affinity chromatography, using protein A and

DNP-BSA immunoadsorbent columns.

A competition radioimmuno assay (RIA) was used for comparison of the binding affinity of 5T2 MM immunoglobulin for different hapten-carrier conjugates with that for DNP-BSA (6). In this assay, the binding of radiolabelled 2,4 DNP-BSA to 5T2 MM immunoglobulin coated on the test plate was determined after addition of serial dilutions of the hapten-carrier conjugates to the individual wells.

Four mouse monoclonal antibodies of BALB/c origin directed to the idiotype of 5T2 MM immunoglobulin were prepared: 145-4.1, 145-8.3, and 145-18.1, all three of IgG1 subclass, and 145-2.1 of IgM class (6). The binding affinities of these four monoclonal antibodies for the 5T2 MM idiotype and their specificity for the binding site of the 5T2 MM immunoglobulin for 2,4 DNP-BSA were investigated in a competition ELISA. The antibodies 145-4.1, 145-8.3, and 145-18.1 had each been labelled with biotin. The binding of each of these biotin conjugates to 5T2 MM immunoglobulin coated on the plate was determined after preincubation of the coated wells with serial dilutions of each of the four (non-conjugated) anti-5T2 MM idiotype antibodies or of DNP-BSA. The relationship between the binding site for 2,4 DNP-BSA on the 5T2 MM immunoglobulin and the 5T2 MM idiotype (as recognized by each of the four monoclonal antibodies) was determined in an ELISA as follows: purified 5T2 MM immunoglobulin was incubated with serial dilutions of each of the monoclonal anti-5T2 MM idiotype antibodies or of 2,4 DNP-BSA. This 5T2 MM immunoglobulin-antibody or -DNP-BSA mixture was subsequently added to individual wells which had previously been coated with 2,4 DNP-BSA. Thereafter, the wells were washed. The binding of 5T2 MM immunoglobulin to DNP-BSA coated on the plate was assessed after addition of, respectively, a PO-conjugated rabbit anti-mouse immunoglobulin antibody (Dakopatts, Glostrup, Denmark) and, after washing, of the substrate solution.

6. Immunofluorescence studies of the 5T2 MM bone marrow and spleen cells

a) Cytoplasmic immunofluorescence

Cytoplasmic immunofluorescence was performed according to Hijmans et al.

(8). Briefly: bone marrow and spleen cell suspensions were spun down on glass slides in a cytospin centrifuge (Construction workshop of the REP-Institutes, TNO). The cell preparations were then fixed for 15 min at -20°C in acid ethanol, and, thereafter, they were washed in PBS. Subsequently, the fixed cells were incubated with a sheep anti-mouse antibody specific for the allotype of the 5T2 MM immunoglobulin (Igh1b) or with a monoclonal anti-5T2 MM idiotype antibody (145-4.1), for 30 min at RT. These antibodies were coupled with FITC or TRITC. After they had been stained, the slides were washed in PBS. The fluorescence pattern of the stained cells was studied with a fluorescence microscope (Zeiss, Ober Kochen, FRG).

b) Surface membrane fluorescence

The monoclonal anti-5T2 MM idiotype antibody 145-4.1 was used to determine the surface membrane 5T2 MM idiotype expression on 5T2 MM bone marrow cells. A 50 µl suspension containing $1x10^6$ cells was incubated with 50 µl of an appropriate dilution of the 145-4.1 antibody for 30 min. This was followed by a second incubation with 50 µl of a FITC-conjugated goat anti-mouse IgG1 antibody (Nordic Immunological Lab., Tilburg, The Netherlands) for, again, 30 min. Before their use, all antibodies were centrifuged at maximum speed for 10 min in a Beckman Airfuge (Beckman Instrumentals b.v., Mijdrecht, The Netherlands) in order to remove aggregated material. The cells were washed in HBSS supplemented with 0.1% (w/v) BSA and 0.1% (w/v) sodium azide (abbreviated as HBSS/BSA/Azide) after each incubation. Finally, the cells were suspended in the desired number in this medium. During the whole staining procedure, the cells were kept on melting ice. The immunofluorescence distribution of the cells was analysed with a fluorescence-activated cell sorter (FACS II, Becton Dickinson, Sunnyvale, Ca., USA) equipped with logarithmic amplifiers. The instrument was used in the configuration as described by Visser et al. (9).

7. Analysis of cellular DNA content

DNA of bone marrow or spleen cells was stained with propidium iodide (PI) (Calbiochem; San Diego, Ca., USA) according to Taylor (10). Briefly: samples of $1x10^6$ cells, which had been suspended in 50 µl of HBSS/BSA/Azide, were each incubated with 750 µl of a HBSS solution containing 50 µg/ml PI and 1% (v/v) Triton X-100 (Sigma Chem. Co., St. Louis, Mo., USA) for 20 min at RT. Analysis of the PI-fluorescence intensity of the stained cells was performed with the FACS II. The signals were linearly amplified.

8. Velocity sedimentation

5T2 MM bone marrow cells were separated on the basis of differences in their sedimentation velocity according to Miller (11). Briefly: 5T2 MM bone marrow cells were suspended in HBSS supplemented with 0.35% (w/v) BSA, and loaded as a thin layer on top of a HBSS column; this column was supplemented with a shallow density gradient ranging from 0.5 to 2% (w/v) BSA, in order to stabilize the fluid column for the prevention of turbulence and convection mixing during loading and sedimentation. The cells were allowed to sediment under the influence of gravity for 3 h, and collected in fractions of equal volume. The number of viable nucleated cells within each fraction was expressed as a function of the sedimentation rate.

9. Radiographic examination of the skeleton

a) Radiographs

Radiographs of individual skeletons were made with a Faxitron X-ray machine (27.5 KW, 0.75 mA, exposure time of 1 min) using Kodak Definix medical radiographic film (no. 502.2157) (4). Before exposure, the skeletons were cleared from surrounding tissues.

b) Microradiographs

In one particular experiment (see chapter IVB), microradiographs were made of the right femur of the mice (4). Previously, the femora were fixed in 70% ethanol, and embedded in methylmethacrylate according to Te Velde et al. (12). Mid-frontal sections of about 115 µm thick had been cut on a Leitz 1,000 Sägemikrotom, before microradiographs of these sections were made with the microradiograph (Philips, PW 1008/80, operated at 20 KV, 30 mA over an exposure time of 15 min). This part of the study was performed at the Clinical Investigation Unit of the Department of Clinical Endocrinology and Metabolism, University Hospital, Leiden, The Netherlands.

10. Histological study

Mice were submitted to a complete necropsy immediately after being sacrificed, and the different representative organs were prepared for histopathological examination following standard procedures (2). The tissue samples were fixed in 10% (v/v) buffered formalin, trimmed, and embedded in paraffin. Bone tissues were decalcified before embedding in paraffin. Histological sections of a thickness of 3 µm were stained with haematoxylin-phloxine-saffron (HPS). Additional stains were applied when indicated.

11. Immunization protocols

a) Subcutaneous immunization

C57BL/KaLwRij mice were immunized subcutaneously with purified 5T2 MM immunoglobulin or with an appropriate (syngeneic) control immunoglobulin. The subcutaneous immunization was performed by injection of the antigen into the inguinal and/or axillary regions, and, when indicated, into the hind footpads. The schedules for immunization are described in the relevant sections. The samples were administered in complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), or HBSS.

Subcutaneous immunization of C57BL/KaLwRij mice with 5T2 MM bone marrow cells or with syngeneic normal bone marrow cells was performed in both inguinal regions according to the specific experimental design. The cells had been obtained and prepared as described for transplantation. The nucleated cells were counted, and suspended in the desired numbers in 200 µl of HBSS for each mouse to be immunized. One hundred µl was injected into each inguinal region.

b) Intravenous immunization

For intravenous immunization of C57BL/KaLwRij mice with 5T2 MM bone marrow cells or with syngeneic normal bone marrow cells, the cells were prepared as for subcutaneous immunization; the desired number of cells was injected in a volume of 0.5 ml of HBSS into a tail vein.

12. Irradiation of 5T2 MM bone marrow cells before immunization

The 5T2 MM cells received 40 Gy gamma-radiation from a ^{137}Cs source in a Gamma cell 20 small animal irradiator (Atomic Energy of Canada, Ltd., Ottawa, Canada) before injection as an immunogen, if it was necessary to abolish their proliferation capacity. During the irradiation, the cells were suspended in H+H medium, and kept on melting ice.

13. Determination of anti-5T2 MM idiotype antibody activity in the sera of C57BL/KaLwRij mice

Sera of C57BL/KaLwRij mice immunized with 5T2 MM immunoglobulin were tested for the presence of anti-5T2 MM idiotype activity by means of a competition ELISA. In this assay, the degree of binding between purified 5T2 MM protein coated on the test plate and a monoclonal anti-5T2 MM idiotype antibody (145-8.3) conjugated with biotin was determined after preincubation of the individual wells with serial dilutions of the sera to be investigated. Further procedures were as described under section 5.

14. Delayed type hypersensitivity experiments

The delayed type hypersensitivity (DTH) reaction was used to monitor the presence of a T-cell-dependent immunity to 5T2 MM immunoglobulin and to 5T2 MM cells in a syngeneic system (13). Male and female C57BL/KaLwRij mice between two and five months of age were used as recipients. 5T2 MM bone marrow cells and normal syngeneic bone marrow cells to be used for immunizations, were obtained from donor mice of the same sex. Recipient mice were immunized subcutaneously with bone marrow cells suspended in HBSS or with MM protein emulsified in CFA. The recipient mice were challenged after seven days (unless otherwise indicated) with either 5T2 MM protein or 5T2 MM cells, injected into their left hind foot in a final volume of 50 µl HBSS. Mice immunized intravenously with bone marrow cells were challenged with 5T2 MM bone marrow cells, either on day 5 or on day 7, according to the particular experiment. The greatest thickness of the left injected foot and the non-injected right foot was measured with a foot thickness-measuring instrument (Construction workshop, REP-Institutes, TNO) 24 h and 48 h after the challenge. DTH reactions were expressed as the relative foot swelling (per cent) according to the following calculation:

[(thickness left foot - thickness right foot)/ thickness right foot] x 100%.

In order to serve as a control for the non-specific swelling caused by challenge with the antigen, a separate group of mice was immunized with a non-relevant (control) antigen, or not immunized at all.

15. Chemotherapy of 5T2 MM

The alkylating agent cyclophosphamide (Endoxan$^{(R)}$, Asta, Bielefeld, FRG) was used for treatment of mice with 5T2 MM. The drug was dissolved in the appropriate solvent shortly before its use. The administration of the solution was performed by intraperitoneal injection.

16. Treatment of 5T2 MM with monoclonal anti-5T2 MM idiotype antibody

Monoclonal anti-5T2 MM idiotype antibody (145-4.1) was isolated from ascitic fluid by protein A affinity chromatography, and subsequently dialyzed against HBSS. The antibody was administered intravenously or intraperitoneally in 250 µl of HBSS. The treatment schedules have been described in the concerning chapter.

17. The bisphosphonate APD

The disodium salt of (3-amino-1-hydroxypropylidene)-1,1-bisphosphonic acid (abbreviated as APD) (4) was kindly provided by Prof. O.L.M. Bijvoet (Clinical Investigation Unit of the Department of Clinical Endocrinology and Metabolism, University Hospital, Leiden, The Netherlands). This compound was mixed in amounts of 500, 1,000, 2,000, or 10,000 ppm (parts per million) in standard food pellets (AM II$^{(R)}$, Hope Farms, Woerden, The Netherlands) for oral administration to the mice in the different experiments.

18. Urine hydroxyproline and creatinine content

Mice were placed in metabolic cages, and their urine was collected daily into flasks. These flasks contained thymol crystals to prevent bacterial growth. The creatinine and hydroxyproline content were measured by Dr. P.H. Reitsma (Clinical Investigation Unit of the Department of Clinical Endocrinology and Metabolism, University Hospital, Leiden, The Netherlands) (4,14). The daily hydroxyproline excretion was expressed as the molar hydroxyproline/creatinine ratio in the 24 h urine. This ratio corrects for errors due to inaccuracies in the daily collection of the urine samples.

19. Scoring of osteolytic lesions on radiographs

For semi-quantitative evaluation, the presence and the extent of the osteolytic lesions in the skeletons as seen on the radiographs were scored as 0 (none), 1 (mild), 2 (moderate), and 3 (severe) for each of the following bones: femur, tibia, humerus, scapula, pelvis, ribs, and vertebrae (4).

20. Quantitative measurement of bone mass on microradiographs

In the microradiographs, metaphyseal trabecular bone mass was assessed by measurement of the surface area of bone in a circular area with a diameter of 1.5 mm, which was located directly proximal to the distal epiphyseal plate of the femur. Epiphyseal trabecular bone mass was determined also in a circular area, but with a diameter of 0.68 mm. This measurement was applied directly distal to the same epiphyseal plate. Cortical bone mass was assessed by measurement of the surface areas of the both cortices over a length of 1.5 mm, starting directly proximal to the same epiphyseal plate. The contours of calcified tissue in the selected areas were drawn on transparant paper using a projection microscope, and the surface areas were subsequently calculated using a MOP-AM02 x-y tablet. The results were expressed in square millimeters of bone (4). This part of the study was performed by Dr. P.H. Reitsma (Clinical Investigation Unit of the Department of Clinical Endocrinology and Metabolism, University Hospital, Leiden, The Netherlands.

21. Scoring of bone and tumour involvement by histology (chapter IVB.1)

A semiquantitative scoring system was used to yield an estimate of the degree of bone destruction in treated and untreated 5T2 MM-bearing mice, and of the involvement of various tissues by the myeloma cells (4). The tumour extent was separately scored in the following compartments: the bone marrow and the extramedullary tissues as described in detail in chapter IVB.1. The bone destruction was evaluated by determination of the number of trabeculae

per arbitrary unit area in the metaphysis of the sternal ends of the ribs and in the distal metaphysis of the femur from mice of the experimental groups. In addition, the degree of cortical bone destruction was assessed in the sternal ends of the ribs and in the distal femoral metaphyses. It was expressed in arbitrary units on a scale ranging from 0 to 2.

22. Scoring of the number of osteoclasts by histology

The effect of transplantation with 5T2 MM and of APD treatment on the number of osteoclasts was examined on histological sections of the ribs. These cells were counted per arbitrary unit of internal bone surface in the rib ends (4).

23. Scoring of bone and tumour involvement by histology (chapter IVB.2)

The extent of growth of 5T2 MM within the bone marrow compartment was scored in a semi-quantitative fashion using a scale ranging from 0 to 2. The tumour growth in extramedullary tissues was scored separately in the lymph nodes, spleen, ovaries, and in the muscle tissue directly adjacent to the bones. The scoring system used in these different organs has been described in detail in chapter IVB.2. The degree of bone destruction was assessed in cortical bone. This examination was performed in the humerus, tibia, femur, vertebrae, pelvis, sternum, ribs, and calvarium. The total score was derived from addition of the scores of the individual bones (grading from 0 to 2 per bone).

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CHAPTER IIIA

THE C57BL/KaLwRij MOUSE 5T2 MULTIPLE MYELOMA

1. The 5T2 MM model

1.1 Introduction

The availability of an appropriate experimental animal model is essential for pre-clinical studies on the biology and therapy of MM. The suitability of such an animal model depends upon specific requirements. Its morphological and clinical characteristics should closely resemble those of MM in man. Moreover, the animal myeloma should be easily transplantable in order to guarantee a continuous supply of MM material for studies in vivo and in vitro.

Several other models of plasma cell malignancy have been reported in the literature. Spontaneous plasma cell neoplasia has been observed in many non-human mammalians, e.g., in dogs, mice, rats, and monkeys (1,2). Some of these plasma cell malignancies, especially those described in dogs, had clinical characteristics which were comparable to those of MM in man (1,3), but others had to be regarded as a local extramedullary plasmacytoma.

Such animal neoplasms are only infrequently detected. When they are grafted, rejection will follow, if the malignant plasma cells are transplanted into allogeneic recipients of the same species. This immunological barrier against engraftment is caused by differences in the histocompatibility complex among non-inbred animals of the same species. Therefore, most spontaneous animal plasma cell neoplasms cannot be used as experimental models for studies on MM.

A few, currently available inbred (genetically identical) strains of mice and rats are particularly prone to the development of a plasma cell tumour. In mice of the C3H strain, typical plasmacytomas were observed to develop in the ileocaecal region (4), which in an advanced stage involved the mesenteric and

mediastinal lymph nodes (5). In spite of the development within members of an inbred mouse strain, only few of these neoplasms could be transplanted successfully. A high incidence of spontaneous ileocaecal plasmacytoma (immunocytoma) has also been observed in the inbred Louvain (Lou) rats (6). These plasma cell tumours are easily transplantable, and are morphologically indistinguishable from the mouse plasmacytomas. However, in contrast with most mouse plasmacytomas, only a minor fraction (3 per cent) of the Lou rat immunocytomas are IgA-producers; most of them (44 per cent) produce and secrete IgE.

The BALB/c mouse strain is sensitive to induction of a plasmacytoma by intraperitoneal implantation of solid plastic foreign bodies (7,8) or intraperitoneal injection of mineral oils (which contain mixtures of many saturated hydrocarbon molecules) (9). The hydrocarbon Pristane, in particular, is a potent inducer of plasmacytoma (10). These plasma cell tumours have developed with an incidence of 40 to 70 per cent. The latent period between the moment of induction and detection of the plasmacytoma ranges from 6 to 20 months.

After induction, the primary BALB/c plasmacytomas have been located in the peritoneal connective tissue. Cells of these plasmacytomas have not been observed to metastasize to the bone marrow. Furthermore, the osteolytic lesions, which are characteristic for MM, are absent (11). Over the past twenty-five years, many different plasmacytomas have been induced in BALB/c mice. Like MM in man, most of these produce and secrete a monoclonal immunoglobulin. They can easily be transplanted into syngeneic recipients. Kobayashi and co-workers have demonstrated that the growth characteristics of the progeny of the induced BALB/c plasmacytomas depend on the route of transplantation (11). Intraperitoneal injection of the plasmacytoma cells into syngeneic recipients resulted in a growth pattern similar to that of the induced primary plasmacytoma, in that the recipient mice developed ascitic fluid and the tumour cells were present in the peritoneal connective tissue (7). The mice died as a consequence of their intra-abdominally growing plasmacytoma. Generally, no metastases occurred in other organs. Bone lesions were only incidentally seen (11). Similar observations were made when the plasmacytomas had been transplanted subcutaneously: a great deal of these mice died without metastases or the presence of bone lesions. However, the mice in which the growth of these plasmacytoma transplants was slow, developed metastases in

their bone marrow. Osteolytic bone lesions also occurred frequently in those animals. For example, they were found in 64 per cent of the animals with a slowly growing MPC-1 plasmacytoma, which allowed the mice to survive more than 40 days after transplantation. In contrast, bone lesions were found in only 22 per cent of the mice with MPC-1 plasmacytoma dying within 30 days (11).

Intravenous transplantation of the BALB/c plasmacytoma cells appeared effective in generating osteolytic lesions in the recipients (11). After intravenous injection of the neoplastic plasma cells in a sufficient number, they were predominantly present in the bone marrow, and more than 70 per cent of the recipients developed bone lesions. These osteolytic lesions were most frequently observed in the proximal part of the tibia, the distal end of the femur, the pelvis, humerus, lumbar and sacral vertebrae, and in the mandibles. Advanced cases, however, with tumour growth into the periostium had osteoplastic changes. The growth of the plasma cell tumour from the vertebral body into the spinal canal often resulted in compression of the spinal cord. The bone destruction was characterized by the absence of trabecular bone in the metaphyseal regions and by severe erosion of the cortex. Of the extra-skeletal tissues, the spleen always contained plasmacytoma cells. Neoplastic cells were also frequently seen in the lungs and liver.

The immunoglobulin-secreting BALB/c plasmacytomas have proven to be very useful tools for many different studies (12). The discovery that these plasmacytomas are monoclonal has consequently resulted in the availability of large amounts of antigen-binding myeloma proteins of identical structure (13, 14). This has enabled to achieve major advances in the knowledge of the molecular structure of immunoglobulins. The BALB/c plasmacytomas have also been employed as models in the study of the processes underlying normal B-cell function, the regulation of the immune response, and of the genetic basis of immunoglobulin production and diversity (15,16,17,18). Hybridisation of normal murine immune B-cells with a malignant fusion partner which has been derived from a BALB/c plasmacytoma, has made it feasible to produce monoclonal antibodies (19).

An essentially different application of these plasmacytomas is their utilization as models of oncogenesis, immunoregulation in, and (immuno)therapy of myeloma (20,21,22,23,24,25). The murine plasmacytoma has appeared useful

for such investigations, although, as a matter of fact, it is not a model for MM, but for a localized plasma cell malignancy.

Aging mice of the inbred C57BL/KaLwRij strain frequently develop disorders in their serum immunoglobulin spectrum (26). When studies were carried out on idiopathic paraproteinaemia (which is also called benign monoclonal gammapathy) (abbreviated as IP), old animals of this strain were occasionally found to have an excessive homogeneous immunoglobulin component in their serum (27,28). Morphological and immunofluorescence examinations of bone marrow and spleen cells of such an animal revealed the presence of MM.

One of these spontaneous C57BL/KaLwRij myelomas, the 5T2 MM, was used as the experimental model for the studies in this thesis. The specific characteristics of the 5T2 MM have, therefore, been described in the section of this chapter that deals with the results of the examinations. In addition, the discussion has been dedicated to the comparison of the 5T2 MM with the frequently used BALB/c plasmacytoma model, and to its resemblance to MM in man.

1.2 Results

a) Origin and intravenous propagation

The 5T2 MM originated spontaneously in an aging male C57BL/KaLwRij mouse in 1976. It has since been propagated for twenty-six successive generations by intravenous injection of bone marrow or spleen cells from 5T2 MM-bearing donor mice into young female and male recipients of the same strain. The injection of a sufficient number of these cells has resulted in the development of 5T2 MM in a large proportion (nearly 100 per cent) of the recipient mice in each successive transplantation generation. The "take" frequency of 5T2 MM has remained stable during the subsequent transplantations. Once a 5T2 MM has developed, it leads to a considerably shortened life span of the recipient, which is only a few months after transplantation. This contrasts with the median survival of approximately twenty-four months in normal C57BL/KaLwRij mice (29).

b) Symptomatology

Mice with advanced 5T2 MM were ill-looking with pilo erection. Often, neurological symptoms were noted. These included paresis or paralysis of the hind limbs, signs of hyperaesthesia, and, incidentally, convulsions.

c) Growth pattern

Histological studies: After intravenous transplantation, the typical 5T2 MM cells were initially found only in the bone marrow compartment of the recipient (Fig.1). The distribution pattern of the 5T2 MM cells within the bone marrow was generally focal.

The myeloma cells were also found in various extramedullary tissues, but only if the 5T2 MM was in an advanced stage of development. The extramedullary organs most often involved were: the spleen (red pulp), the lymph nodes, and the meninges inside the skull and the vertebral column. Extension of the growth of the 5T2 MM into the vertebral canal led to compression of the spinal cord (Fig.2). Less frequently, the 5T2 MM cells were observed in the ovaries, the liver, and the lungs. Within the bone marrow, the myeloma cells largely replaced the normal haematopoietic tissue. In very advanced stages of 5T2 MM, the myeloma cells were present in the muscle tissue surrounding the affected bones.

Immunofluorescence studies: Analysis by immunofluorescence of the cytoplasmic immunoglobulin-positive cells in the bone marrow and spleen of the donor mouse bearing the primary 5T2 MM revealed the presence of numerous cells positive for IgG2a (Fig.3) (27). A similar immunofluorescence pattern of bone marrow and spleen cells was found in transplanted recipient mice with overt 5T2 MM. A large number of cells positive for cytoplasmic 5T2 MM immunoglobulin could be identified within the spleen and, especially, the bone marrow using anti-IgG2a or anti-5T2 MM idiotype antibodies.

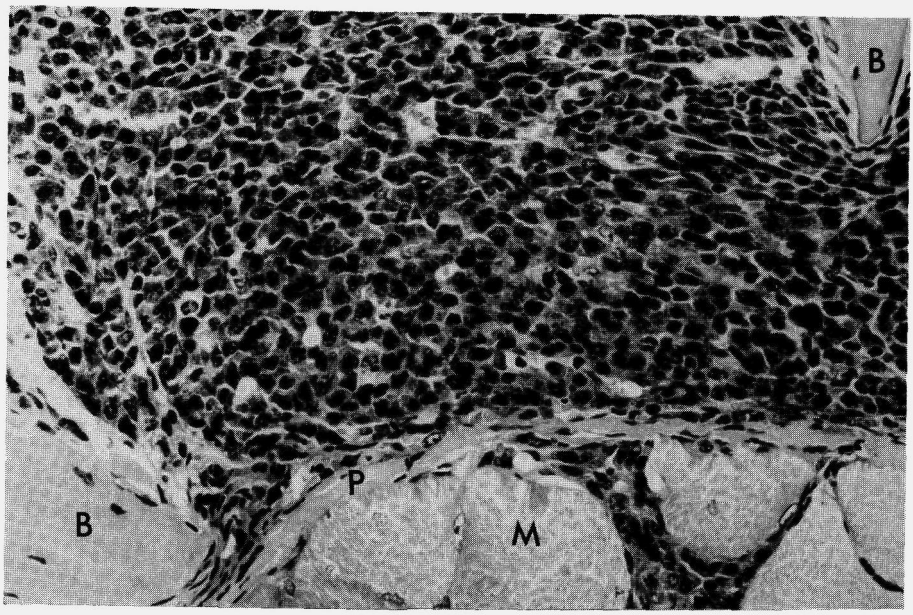


Fig.1: Iliac bone of a 5T2 MM-transplanted mouse. Moderately differentiated *myeloma cells*, proliferating within the bone marrow cavity have destroyed the bone cortex and invaded into the adjacent muscle tissue. HPS x 435.
(M: muscle; B: bone; P: periost).

d) Bone destruction

Radiographic studies: Radiographs of the skeletons of mice with advanced 5T2 MM revealed the presence of diffuse osteoporosis and numerous osteolytic lesions. The most severe osteolytic lesions were observed in the metaphyses of the femora and the tibiae, and in the ribs. Less frequent locations for osteolytic lesions were the humerus, the pelvic bones, the scapula, and the calvarium. The pelvis, sternum, and vertebrae showed mainly a severe osteoporosis (Fig.4).

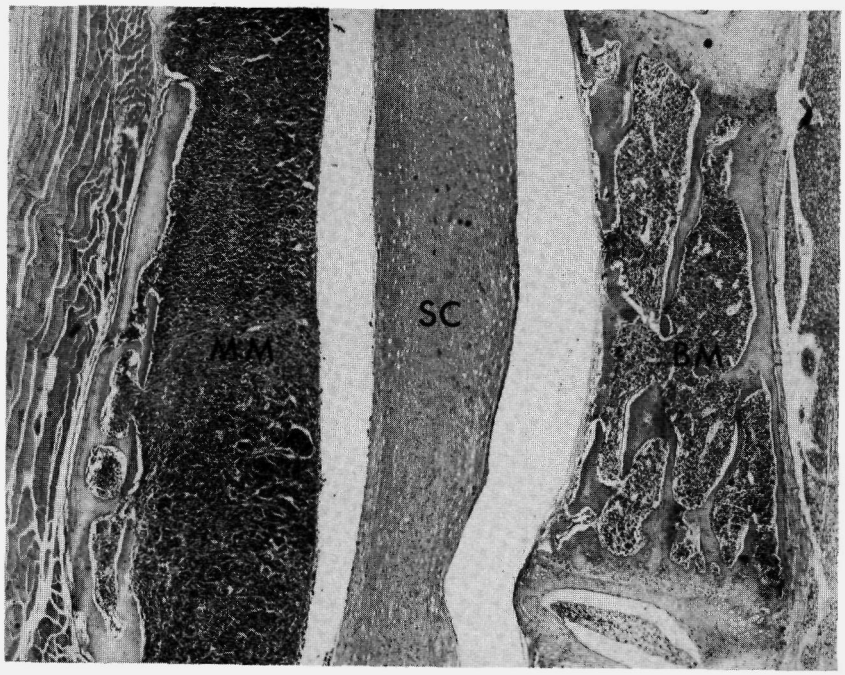


Fig.2: Extension of 5T2 MM cells from the vertebral bone marrow compartment into the vertebral canal with compression of the spinal cord. HPS x 42.
(BM: vertebral bone marrow; SC: spinal cord; MM: myeloma tissue).

Histological studies: At histology, the osteolytic lesions on radiographs appeared to be due to focal destruction of cortical bone, and to a decrease in the amount of trabecular bone. The focal cortical destruction was most extensive where blood vessels passed through the cortex. The decrease in trabecular bone was most striking in the distal metaphyses of the femora and in the proximal metaphyses of the tibiae (Fig.5).

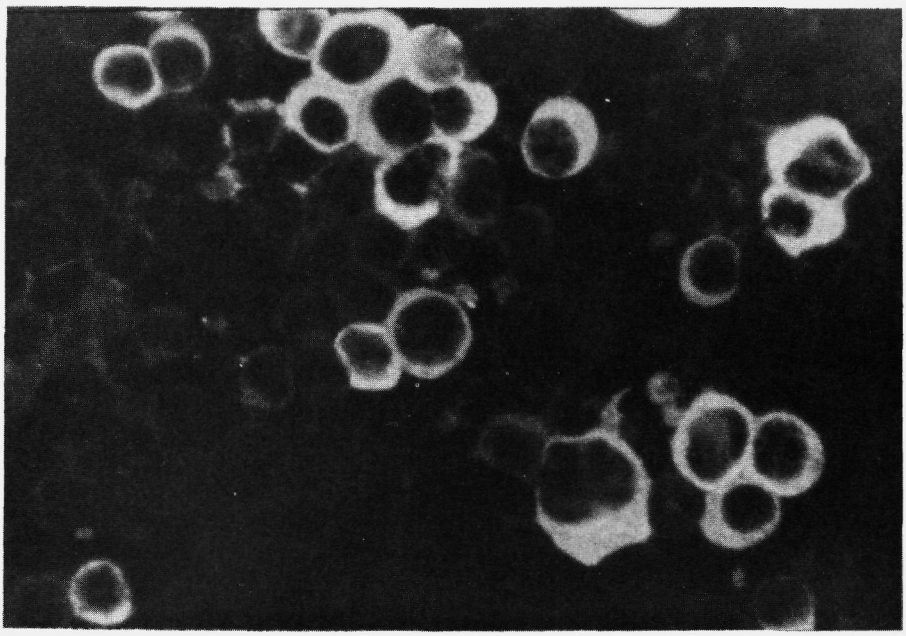
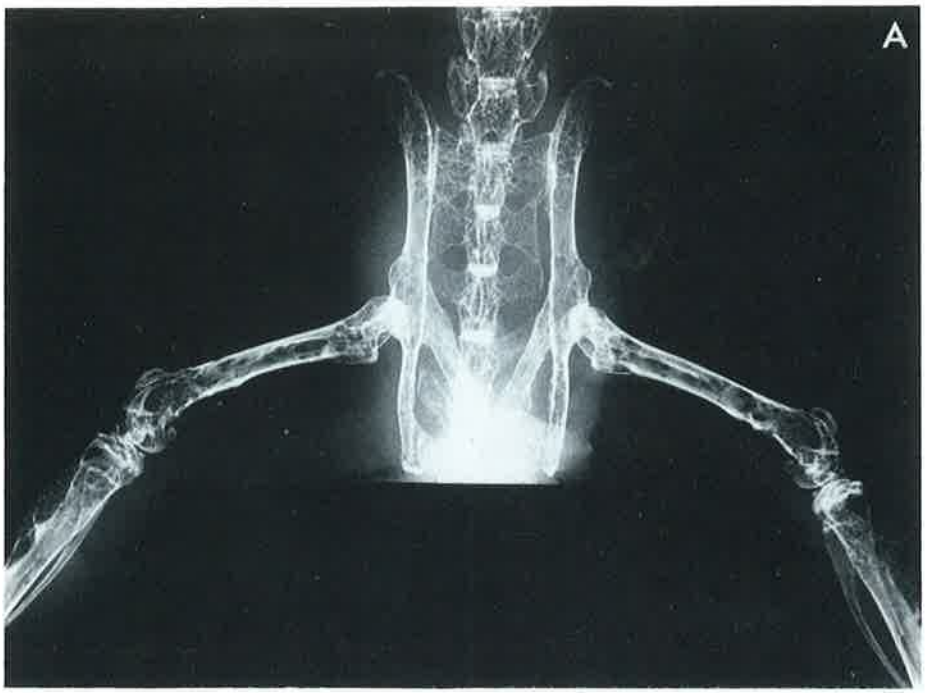


Fig.3: Immunofluorescence in cytoplasmic IgG2a-positive bone marrow cells from the mouse with the primary 5T2 MM.

Fig.4: Radiograph of the lower part of the skeleton of a 5T2 MM mouse (A) and a normal C57BL/KaLwRij mouse (B). Notice the increased radiolucency of the bone tissues of the myeloma mouse, which is characteristic of bone loss.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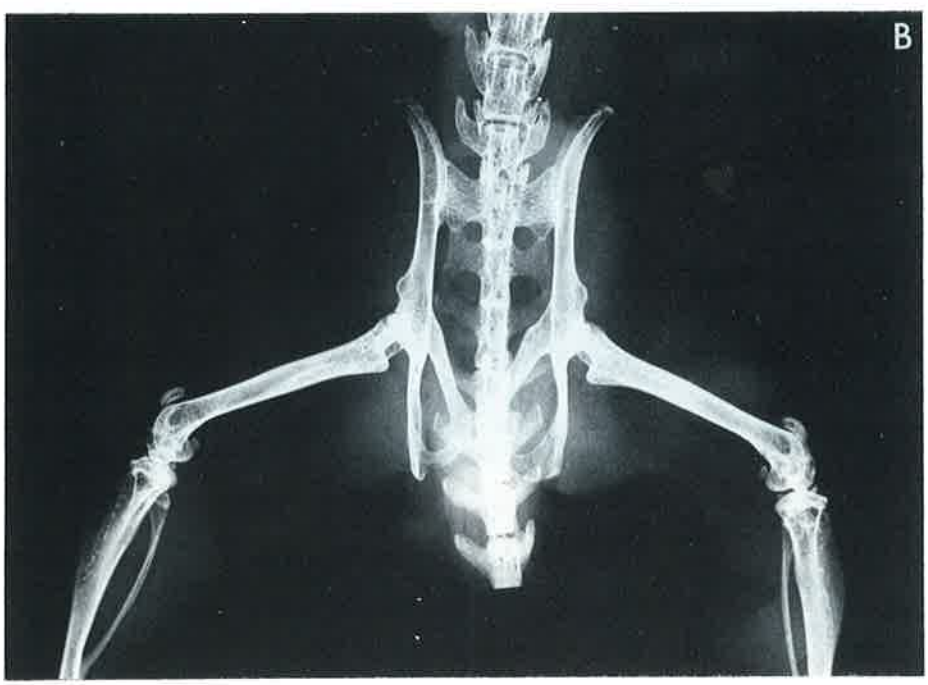
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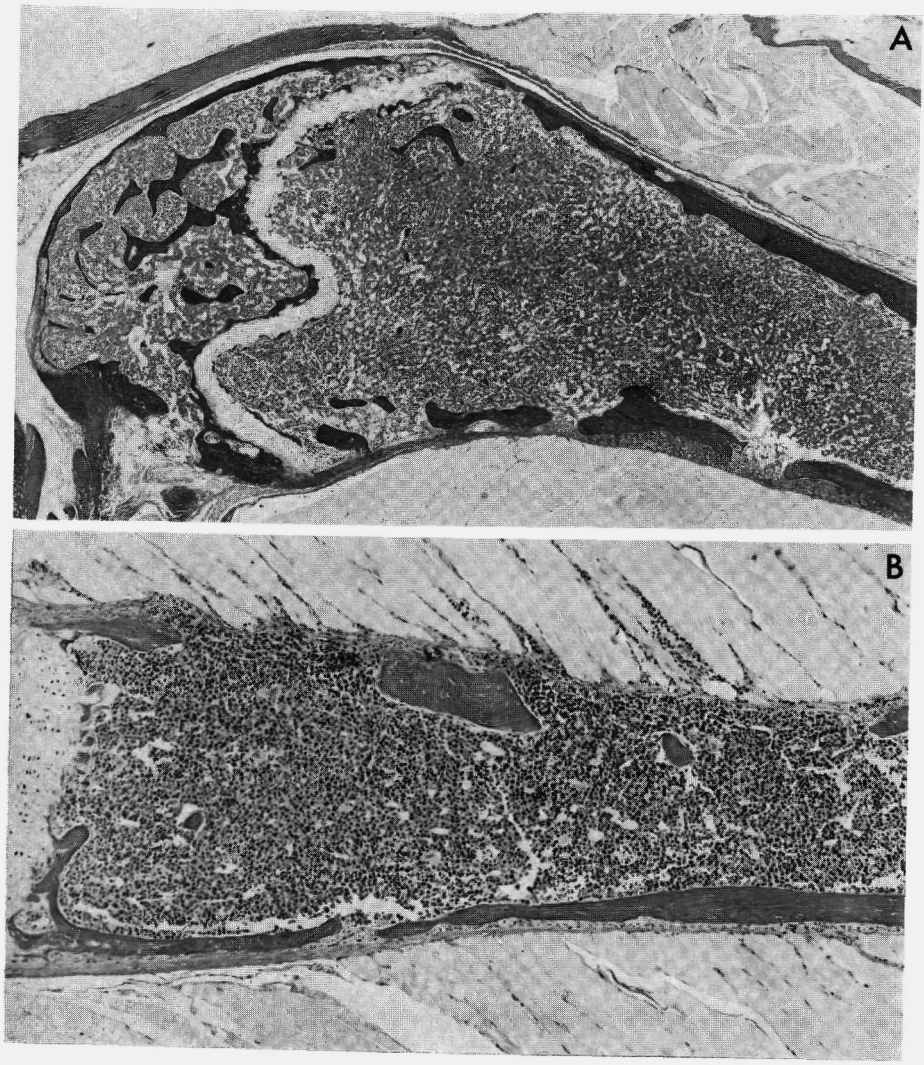

Fig.5: Histological sections of the distal part of a femur (A) and the sternal end of a rib (B) from a 5T2 MM mouse. Notice the severe destruction of the trabecular and cortical bone tissue. Van Gieson x 40 (A) and 120 (B).

e) Further complications caused by 5T2 MM

In very advanced cases of 5T2 MM, the presence of anaemia was indicated by the low haematocrit values in the blood of the animals.

The serum agar electrophoresis (Fig.6) and immunoelectrophoresis pattern of mice with an advanced 5T2 MM showed a strong homogeneous component and only low levels of the normal immunoglobulins (Fig.6).

Occasionally, the urine of 5T2 MM mice contained Bence Jones proteins of the kappa-type.

Fig.7 represents a histological picture of a typical myeloma kidney, as occasionally observed in mice with 5T2 MM. The myeloma kidney is characterized by the presence of proteinaceous casts within the tubules and plasma cell infiltrates in the interstitial tissue.

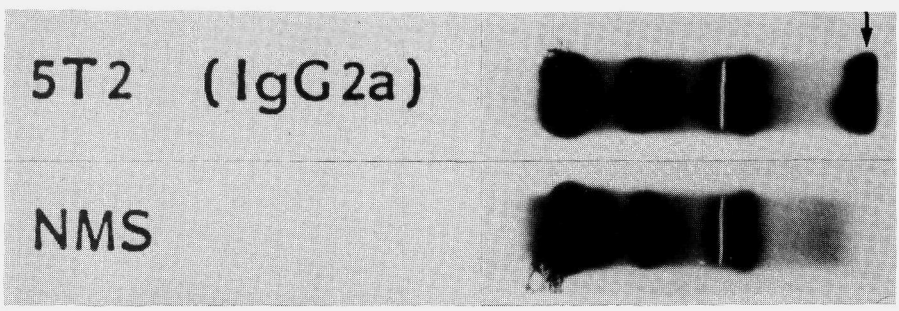

Fig.6: Agar electrophoresis pattern of serum from a 5T2 MM mouse and a normal C57BL/KaLwRij mouse. The homogeneous component in the serum of the myeloma-bearing mouse is indicated (arrow). Notice the decreased amount of normal immunoglobulins in the electrophoresis pattern of the serum from the 5T2 MM mouse.

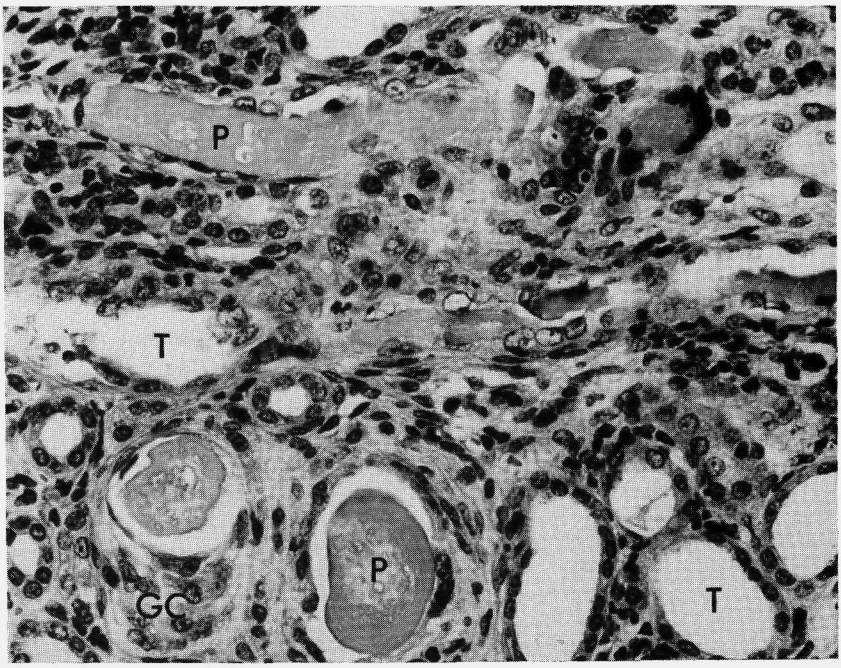

Fig.7: Cortex of a typical myeloma kidney from a 5T2 MM-transplanted mouse showing dense, partly laminated, protein casts within dilated tubules. Tubular epithelium is flattened with focal destruction and multinucleated giant cell formation. In addition, some mononuclear cell infiltration is present in the interstitium. HPS x 435.
(T: tubule; P: protein cast; GC: giant cell).

f) Cytological aspects of the 5T2 MM cell

Examination of 5T2 MM cells by light microscopy revealed a heterogeneous morphology: the cells showed differences in size and maturity. The majority of the 5T2 MM cells had a plasmablastic appearance. These cells were characterized by a high nuclear/cytoplasmic ratio. Occasionally, binucleated cells were visible. Most nuclei were large and contained coarsely dispersed

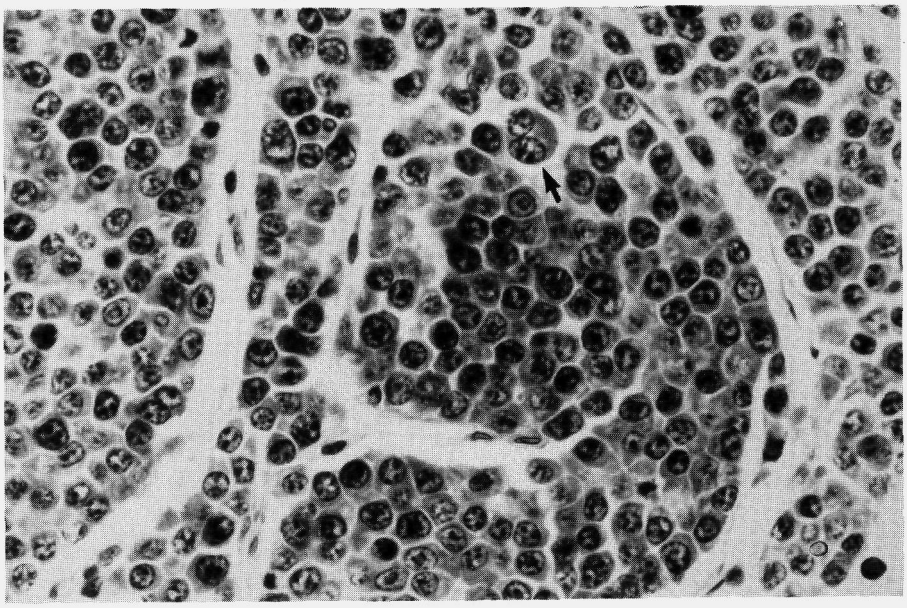

Fig.8: High magnification of a histological section of typical 5T2 MM cells. Noticeable features are the heterogeneity in size of the myeloma cells and the coarsely dispersed nuclear chromatin. Most cells have a large nucleus and only a small amount of cytoplasm. The arrow indicates a binucleated MM cell. HPS x 700.

chromatin. Distinct nucleoli were frequently observed within the nucleus. (Fig. 8).

g) Subcutaneous propagation

Subcutaneous injection of 5T2 MM cells into the inguinal region resulted in the development of a solid subcutaneously located tumour, but only in a low to moderate proportion of the recipient mice. The "take" frequency in different experiments was not constant: no distinct dose-response relationship was observed between the number of injected cells and the percentage of mice showing "take" of 5T2 MM. The majority of the mice with a subcutaneously

located 5T2 myeloma was serologically positive for the 5T2 MM immunoglobulin.

Histological examination revealed the presence of 5T2 MM cells in the bone marrow of more than half of the mice with a large subcutaneous 5T2 tumour. Involvement of other, extramedullary, organs with 5T2 MM cells was only incidentally observed. In contrast, only a minority of subcutaneously transplanted descendants of mineral-oil induced BALB/c plasmacytoma, which were studied for comparison, showed bone marrow involvement, whereas severe infiltration of lymph nodes and peritoneum by the tumour cells was always observed (28) (Table 1).

Table 1 DIFFERENCES BETWEEN SUBCUTANEOUSLY TRANSPLANTED 5T2 MM AND MINERAL OIL-INDUCED BALB/c PLASMACYTOMA

	MM	Plasmacytoma
take frequency	low to moderate	high
bone marrow involvement	high	low
lymph node involvement	low	high (in case of bone marrow involvement)
Cytological pleomorphism	moderate	severe

h) Intraperitoneal propagation

Intraperitoneal injection of the myeloma cells resulted in the development of 5T2 MM in a frequency comparable to that observed after intravenous transplantation. All mice that showed development of the intraperitoneally transplanted MM showed the presence of 5T2 MM immunoglobulin in their serum. Only a few mice developed ascites.

Macroscopic and microscopic examination of the abdomen of the intraperitoneally transplanted mice revealed an extensive infiltration with tumour tissue, predominantly in the mesenteric lymph nodes. In advanced cases, a generalized dissemination of the 5T2 MM was observed in, for example, the bone marrow, spleen, mediastinal lymph nodes, and retroperitoneal space.

Table 2 INHIBITION OF BINDING OF RADIOLABELLED 2,4-DNP-BSA TO 5T2 MM IMMUNOGLOBULIN BY VARIOUS HAPTEN-CARRIER CONJUGATES

hapten-carrier	starting concentration (mg/ml)	dilution: 1/50 (% inhibition)	1/100	1/500
2,4-dinitrophenyl-HSA	1.5	93	89	64
5-acetyluracil-1-BSA	0.5	88	84	54
purine-6-oyl-BSA	0.5	79	68	39
2,4-dinitrophenyl-BSA	0.5	70	55	27
cytidine-BSA	0.5	46	34	14
adenosine-5'-monophosphate-BSA	0.5	37	28	21
2,4-dinitrophenyl-BGG	0.5	11	10	3
uridine-5'-monophosphate-BSA	0.5	7	8	5
guanosine-BSA	0.5	6	3	13
nitroazidephenyl-BSA	0.5	1	--	6
p-azobenzenearsenate-BSA	0.5	--	--	3

HSA: human serum albumin; BGG: bovine gamma globulin;
BSA: bovine serum albumin

i) 5T2 MM immunoglobulin

The 5T2 MM immunoglobulin is an IgG2a-kappa immunoglobulin with a binding specificity for various hapten-carrier conjugates. The relative binding affinities of 5T2 MM protein for various hapten-carrier conjugates as compared with the affinity for 2,4-DNP-BSA is shown in Table 2 (30).

Four monoclonal anti-5T2 MM idiotype antibodies (145-4.1, 145-8.3, 145-18.1, and 145-2.1) were prepared (30), and their binding affinities for this idiotype were compared with each other in a competition ELISA. The

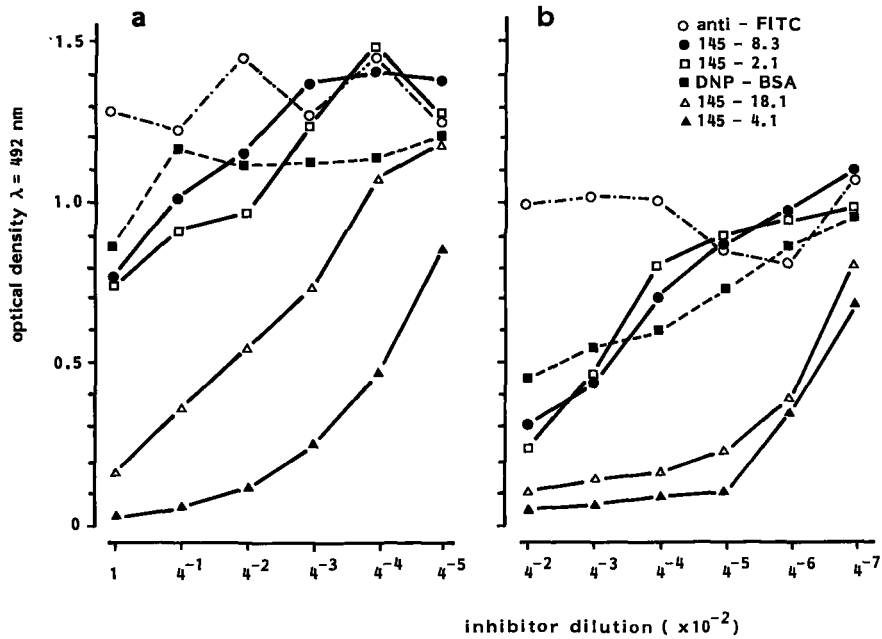

Fig.9, a and b: Binding of the biotin conjugates of the monoclonal anti-5T2 MM idiotype antibodies 145-4.1 (a), 145-8.3 (b), and 145-18.1 (not shown) to 5T2 MM immunoglobulin coated on the plate, in the presence of each of the four anti-5T2 MM idiotype antibodies or of DNP-BSA. The monoclonal anti-FITC antibody 239-3.1 served as a control.

145-18.1 and, especially, the 145-4.1 antibodies showed the highest affinity for the idiotype of the 5T2 MM immunoglobulin. (Fig.9) (30). In addition, the relationship between the antigen-binding site of 5T2 MM immunoglobulin for DNP-BSA and the 5T2 MM idiotype, as recognized by these four monoclonal antibodies, was determined. The binding of the 145-4.1, 145-18.1, and 145-8.3 antibody to 5T2 MM immunoglobulin was only moderately inhibited in the presence of 2,4-DNP-BSA (Fig.9) (30).

In another ELISA, the binding of 5T2 MM immunoglobulin to 2,4-DNP-BSA coated on the plate was not inhibited after preincubation of this immunoglobulin

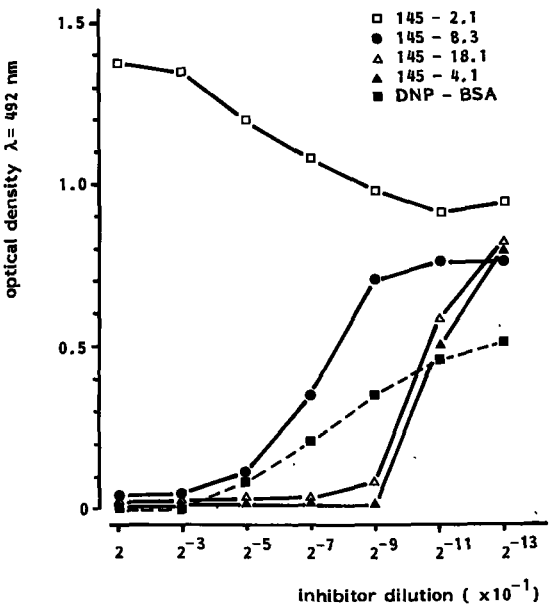

Fig.10: Results of an ELISA to determine the binding of 5T2 MM immunoglobulin to DNP-BSA coated on the plate. Prior to the incubation, the 5T2 MM immunoglobulin was mixed with each of the four monoclonal anti-5T2 MM idiotype antibodies or with DNP-BSA. The degree of binding was measured after a subsequent incubation with a peroxidase-conjugated anti-mouse immunoglobulin antibody, followed by addition of the substrate solution OPD.

with the 145-2.1 antibody. However, inhibition of the binding of 5T2 MM immunoglobulin to DNP-BSA coated on the plate was observed after pre-incubation of the 5T2 MM immunoglobulin with each of the three other anti-5T2 MM idiotype antibodies (Fig.10).

1.3 Discussion

In man, neoplasia is always primary. This is in contrast with many animal tumour models which are maintained in their transplanted form. The transplanted form of a neoplasm can yet be used for studies on many biological characteristics of malignancy. However, only the primary form is appropriate for studies on aetiology, and on the initial growth and distribution pattern.

The 5T2 MM is the animal model for MM used in the different studies described in this thesis. It is one of the myeloma lines (5T MM's) which originated spontaneously in aging C57BL/KaLwRij mice (28). These primary MM's have an unknown aetiology as is the case for MM in man. The genetic background of the C57BL/KaLwRij mouse may play an important role in the origin of their MM. This was demonstrated for the occurrence of benign monoclonal gammapathy in this mouse strain (31). Apart from this, the aetiology may involve disorders in oncogene expression, as has been demonstrated in human Burkitt lymphoma and in BALB/c plasmacytoma (20). Their occurrence may in turn be stimulated by viral factors, or by an increased mutation rate within a given B-cell clone. The increase in number of mutations may be due to repeated monoclonal expansions, which are the result of imbalances between the T- and B-immune system during aging (32).

In the mouse with the primary 5T2 MM (and with the other primary 5T MM's), the myeloma cells were mainly located in the bone marrow and spleen (27, and unpublished results). The malignant character of these atypical bone marrow and spleen cells was expressed by their behaviour after transplantation into young recipients of the same strain. In contrast with the bone marrow and spleen cells from mice with IP, which can only be propagated into young syngeneic recipients to a maximum of four generations (27), transplantation of the MM cells has resulted in "take" of MM in all subsequent generations. The distribution pattern of the primary 5T2 MM has roughly been maintained in the

recipient mice after the many intravenous transplantations. The 5T2 MM cells seemed to have a preference for the bone marrow. This was indicated by their presence in that compartment when 5T2 MM was in an early stage of development, and long before the MM cells were observed in other organs. The typical neurological symptons, i.e., the convulsions and paresis or paralysis of the hind legs of the mice with advanced 5T2 MM, can be explained by the extensive growth of the myeloma from the bone marrow of the skull and the vertebrae into the meninges. This causes compression of the cerebrum and spinal cord.

The primary 5T2 MM and the other 5T MM's are very well comparable to MM in man. Their spontaneous origin has already been mentioned. The typical localization of the 5T2 MM cells in the bone marrow is similar to that of MM cells in human beings. The observation that 5T2 MM cells were also present in the spleen is not in disagreement with the absence of myeloma cells in this organ in human beings: the mouse spleen is a "bone marrow" like, i.e., a haematopoietic organ, whereas the human spleen belongs to the secondary lymphoid tissues, which are of "lymph node" type. Further similarities between MM in man and in the C57BL/KaLwRij mouse are the positive correlation of its incidence with age, the most frequently observed isotype of the myeloma protein, which is IgG, and the presence of bone lesions.

The usefulness of 5T2 MM as an experimental model for the human disease is further determined by its high "take" frequency after intravenous transplantation, and by its rather constant biological behaviour after many subsequent transplantations. The latter property makes 5T2 MM a reliable tool for long-term studies. The results of studies performed in a given transplantation generation remain valid also for subsequent generations.

Not all properties of the transplanted 5T2 MM are, however, comparable to the primary 5T2 MM and to MM in man. Whereas a malignant process is believed to have its origin in one transformed cell, a transplanted tumour starts its growth from many injected cells. Moreover, if injected intravenously, the tumour cells are disseminated immediately. It may be expected that the continuous occurrence of spontaneous mutations will cause variations in the genome of the tumour cells of subsequent generations. This may lead to further deviations of properties from those of the original neoplastic clone. Although such deviations have not yet been demonstrated, further studies,

especially of the primary or early generations of different 5T MM's, are needed to investigate this aspect. However, these limitations of the transplanted form of 5T2 MM do not interfere with its usefulness for the present studies on the immunology and on the bone destruction of MM.

In contrast with MM in C57BL/KaLwRij mice, the plasma cell tumours in BALB/c mice all originated, either after induction or spontaneously, as local plasmacytomas. They were not located in the bone marrow, but most often in the peritoneal connective tissue. Most probably, they originated from the gut-associated lymphoid tissue, as indicated by the observation that most BALB/c plasmacytomas (60 per cent) secrete IgA immunoglobulin (33), which often has antibody activity to antigens present in the digestive tract. This is in contrast with the aetiology of MM in man and in C57BL/KaLwRij mice, which probably originate in B-cells of the systemic immune system.

If propagated intravenously, the BALB/c plasmacytoma resembled the 5T2 MM in that the neoplastic cells were located in the bone marrow, and bone lesions were present. The difference between BALB/c plasmacytoma and 5T2 MM was best illustrated by their behaviour after subcutaneous transplantation. The 5T2 MM cells developed into a subcutaneous tumour only in a low to moderate frequency, whereas the subcutaneously propagated BALB/c plasmacytoma had a high "take" frequency, and grew much more progressively. If a subcutaneously transplanted 5T2 tumour had developed, the 5T2 myeloma cells were also frequently observed in the bone marrow, which was in contrast with the subcutaneous BALB/c plasmacytoma.

Also after intraperitoneal transplantation, 5T2 MM differed significantly from BALB/c plasmacytoma. Ascites development was rarely seen in 5T2 MM, and the 5T2 MM cells disseminated frequently to extraperitoneal organs.

In conclusion, intravenously propagated 5T2 MM resembles MM in man very closely, and is continuously available. It can be used for studies on the immunology and treatment of MM, and on MM-associated bone destruction. However, the aetiology and site of origin of MM can only be studied in its primary form. Considering the differences between 5T2 MM and the plasmacytoma models, 5T2 MM is the most appropriate model for studies on this disease.

In the study described in this chapter, several immunological characteristics of the 5T2 MM immunoglobulin were investigated.

The binding specificity of 5T2 MM immunoglobulin for various hapten-carrier conjugates indicates that its binding site can react with different antigens. Its specificity for DNP conjugates is in part dependent on the structure of the carrier as illustrated by the lack of reactivity of the 5T2 MM immunoglobulin with DNP-BGG (30).

The anti-5T2 MM idiotype antibody 145-4.1 was demonstrated to have the highest binding affinity for the 5T2 MM idiotype (30). Therefore, this antibody was used in different studies for detection of the 5T2 MM cells and their immunoglobulin product, and for attempts to treat this MM by immune regulation.

Three out of the four anti-5T2 MM idiotype antibodies tested recognized an idiotypic determinant that was part of the antigen-binding site of 5T2 MM immunoglobulin for DNP-BSA. Only binding of the 145-2.1 antibody to 5T2 MM protein did not influence its reactivity with DNP-BSA, indicating that this antibody recognized a determinant not directly associated with the binding site for DNP-BSA (30).

CHAPTER IIIA

THE C57BL/KaLwRij MOUSE 5T2 MULTIPLE MYELOMA

2. Identification of 5T2 MM and determination of its extent within the bone marrow by idiotype-specific reagents and by abnormalities in cellular DNA content

2.1 Introduction

Histological examinations of the bone marrow of mice with an advanced 5T2 MM have revealed that a large part of its normal cells has been replaced by myeloma cells. The degree of the bone marrow involvement with myeloma cells depends on the state of development of this neoplasm.

The serum concentration of the myeloma protein reflects to a certain degree the extent of the myeloma in the host. Tumour cell mass in patients with MM was calculated from measurements of rates of synthesis and metabolism of the myeloma immunoglobulin (34,35). The determination of the extent of the myeloma mass on the basis of such calculations is rather complex, and should probably be regarded as an approximation. In man, however, there are no possibilities for making a more exact calculation.

In experimental animal myeloma models such as the 5T2 MM, the proportion of tumour cells within the bone marrow compartment (or within other organs, if involved) can be determined more precisely by the detection of a marker present in or on, and specific for, all myeloma cells. The idiotype of cytoplasmic 5T2 MM immunoglobulin meets this requirement. Since a great deal of the 5T2 MM cells has the morphology of immature plasma cells (see chapter IIIA.1.2.), many of them are also expected to express the 5T2 MM idiotype on their surface membrane.

In search of alternative tumour markers, the distribution pattern of the DNA content of bone marrow cells from mice with 5T2 MM was compared with

that of normal bone marrow cells. The presence of abnormalities in the DNA content of human myeloma cells has been demonstrated by several investigators (36,37,38). Moreover, the percentage of cells with an abnormal DNA content appeared to correlate in an excellent way with the proportion of identifiable plasma cells in the bone marrow of MM patients (36). This indicates that the cellular DNA content is a useful marker in order to estimate the myeloma cell mass.

The aim of the following experiments was to examine and to compare the usefulness of different 5T2 MM-specific markers for identification of 5T2 MM cells, and for determination of the extent of this myeloma within the bone marrow of the mice. Bone marrow from mice which had been transplanted with 5T2 MM was investigated for the presence of 5T2 MM cells by the detection of cytoplasmic 5T2 MM idiotype at different time points after transplantation. In addition, sera from the same mice were investigated for their 5T2 MM immunoglobulin content. In subsequent experiments, the distribution pattern of DNA and the expression of surface 5T2 MM idiotype were studied in bone marrow cells from 5T2 MM mice and from normal C57BL/KaLwRij mice.

2.2 Experimental designs and results

a) Measurement of the serum concentration of 5T2 MM immunoglobulin, and determination of the percentage of cytoplasmic 5T2 MM idiotype-positive cells

Each of 36 female C57BL/KaLwRij mice of two months of age received an intravenous injection of $2x10^5$ bone marrow cells from syngeneic donor mice with a full blown 5T2 MM. The mice were subsequently separated into six experimental groups of equal size. Five weeks later, the first six mice were sacrificed. This procedure was repeated every two weeks until all 36 mice were sacrificed. The only exception was a three weeks interval between the kill of the mice of the third group and those of the fourth group (Fig.1).

The concentration of 5T2 MM immunoglobulin in the serum of each mouse was measured by competition ELISA (see chapter II.5).

A femur was taken from each of three mice randomly selected from each

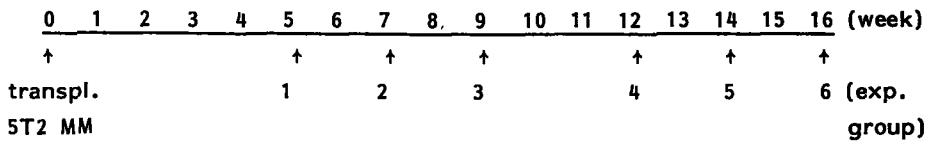

Fig.1: Scheme of time intervals (expressed in weeks) between the day of transplantation of all 36 mice of the six experimental groups with 5T2 MM bone marrow cells and the week that each group of mice was sacrificed.

group of six animals. Cytoplasmic immunofluorescence was performed with the monoclonal anti-5T2 MM idiotype antibody coupled with FITC as described in chapter II.6.a. The number of cells which were positive for cytoplasmic 5T2 MM idiotype was determined in an arbitrarily chosen surface area on each glass slide. One hundred cells were counted on each slide. The results of the determinations of the serum 5T2 MM protein concentration and of the percentage of cytoplasmic 5T2 MM idiotype-positive cells were correlated with each other.

5T2 MM immunoglobulin was clearly detectable in the sera of five out of the six mice of the third group, i.e., nine weeks after the day of transplantation of the 5T2 MM. From that time, the mean serum 5T2 MM protein concentration increased in an exponential fashion. The 5T2 MM protein concentration reached a mean level of 10 mg/ml serum at sixteen weeks after transplantation (Fig. 2).

Occupation of femoral bone marrow by 5T2 MM cells began at about nine weeks after the day of transplantation. The bone marrow from the femora of the six mice that were sacrificed sixteen weeks after transplantation showed about 50 per cent replacement of normal cells by 5T2 MM cells (Fig.3).

A correlation coefficient of 0.81 was found between the serum concentration of 5T2 MM protein and the percentage of cytoplasmic 5T2 MM idiotype-positive plasma cells within the femoral bone marrow. Three mice showed some discrepancies (Fig.4).

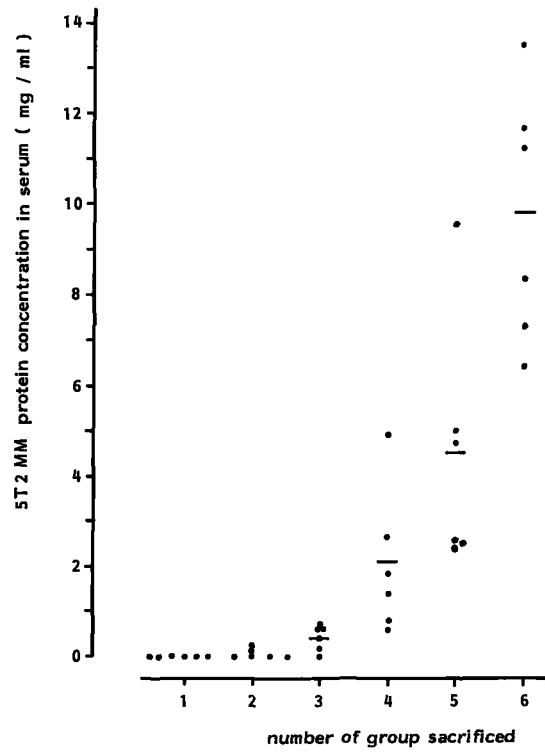

Fig.2: Serum 5T2 MM immunoglobulin concentration (individual and mean values in mg/ml) at different time points after intravenous transplantation of the mice with 5T2 MM bone marrow cells. The measurements were performed by competition ELISA (see chapter II.5). The points of time at which the mice of the different groups were sacrificed are indicated in Fig.1.

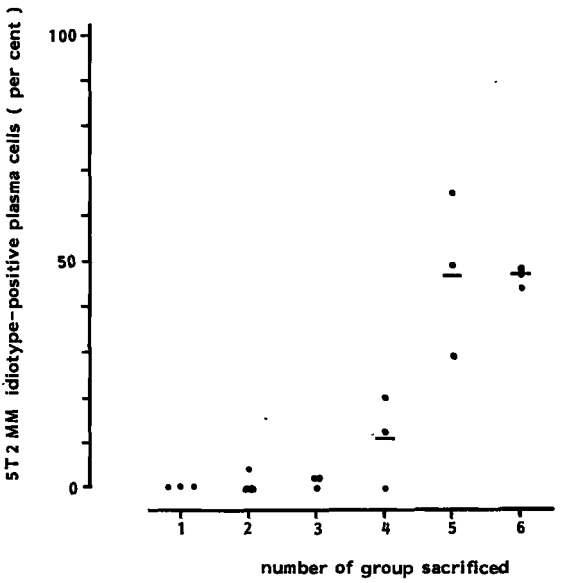

Fig.3: Percentage (individual and mean values) of cytoplasmic 5T2 MM idiotype-positive cells in the femoral bone marrow from mice with 5T2 MM at different periods after transplantation. The presence of the 5T2 MM idiotype within the cells was determined by direct cytoplasmic immunofluorescence as described in chapter II.6.a. In Fig.1 is indicated when each group of mice was sacrificed.

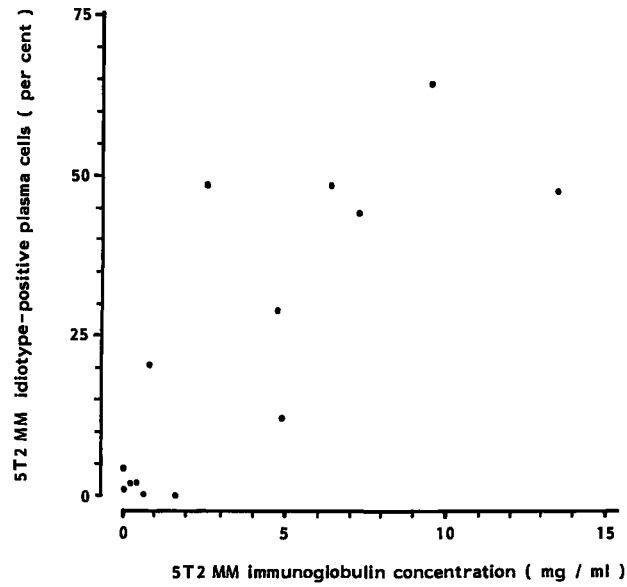

Fig.4: Correlation between the serum concentration of 5T2 MM immunoglobulin and the percentage of femoral bone marrow cells expressing the 5T2 MM idiotype in their cytoplasm. Each point represents the both values of an individual mouse. Linear regression. Correlation coefficient: 0.81 with 13 degrees of freedom; p-value (2-sided test): 0.02.

b) Analysis of nuclear DNA content of 5T2 MM bone marrow cells

Bone marrow cells from three mice with 5T2 MM (mouse 5T2-1, 2, and 3) and from three normal C57BL/KaLwRij mice (mouse NM-1, 2, and 3) were prepared for analysis of their DNA content as described in chapter II.7. The DNA distribution patterns of the bone marrow cells from the three 5T2 MM mice were determined in three separate experiments; therefore, the resulting curve of each individual 5T2 MM mouse was compared with that of a different normal mouse (i.e., 5T2-1 compared with NM-1, etc.). It was not expected that the DNA distribution curves of the three normal mice would differ significantly from each other. In Fig.5a and b, representative examples are shown of DNA

distribution curves of, respectively, 5T2 MM bone marrow cells and normal bone marrow cells.

Three distinct peaks were present in the distribution pattern of the DNA content of bone marrow cells from the mice that had developed 5T2 MM: two large peaks representing the majority of the cells and a small third one. The

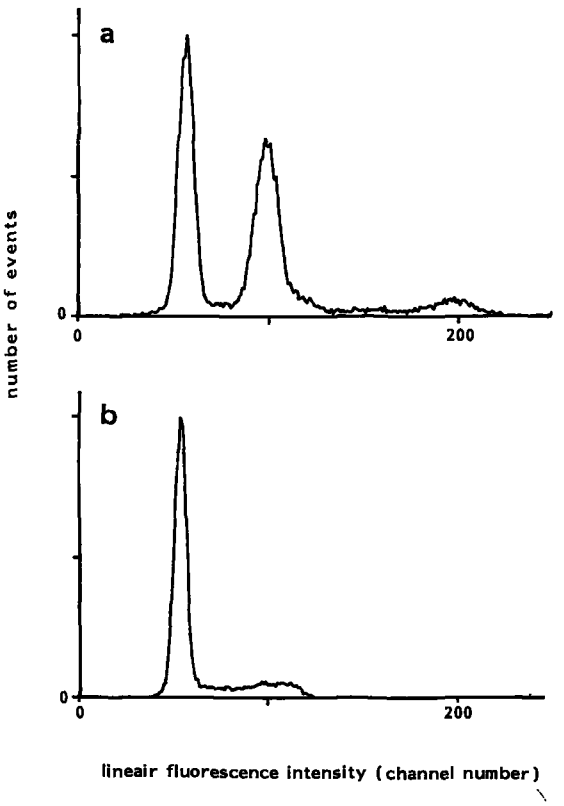

Fig.5a: DNA distribution curve of bone marrow cells from a mouse bearing 5T2 MM.

Fig.5b: Idem of normal C57BL/KaLwRij bone marrow cells. The cells were stained with propidium iodide before the analysis of their fluorescence intensity (see chapter II.7).

cells within the first peak expressed the same fluorescence intensity as the non-cycling cells (in Go/G1 phase) of normal bone marrow (=2n). The second peak was located at about 1.75 times the fluorescence intensity of the first one (=3.5n). The position of this peak in the DNA distribution curve corresponded with that of the cycling cells in the late S phase in the DNA distribution curve of the normal bone marrow. The third peak was located at two times the fluorescence intensity of the second peak (=7n). The size of the fraction of cells with an aneuploid content of DNA was determined by comparison of the DNA distribution curve of 5T2 MM bone marrow cells with that of bone marrow cells from a normal mouse. The cells with a DNA content of 2n and 4n within the 5T2 MM bone marrow population were assumed to be mainly normal diploid and tetraploid cells in, respectively, the Go/G1 and G2/M phase of the cell cycle. The cells containing 3.5n and 7n DNA were considered as, respectively, the non-cycling and cycling (G2/M phase) aneuploid cells (with the exception of the cycling cells with a normal content of DNA in the S phase, which also contain about 3.5n DNA). The aneuploid cells with a DNA content between 3.5n and 7n were regarded as being in the S phase of the cell cycle. The ratio between cycling and non-cycling cells with a normal DNA content from the 5T2 MM bone marrow was tentatively assumed to correspond with the ratio between cycling and non-cycling cells from normal bone marrow. This assumption enabled the estimate of the percentage of cycling cells with a normal DNA content within the 5T2 MM bone marrow. The percentage of cells with an aneuploid content of DNA was calculated by subtracting the percentage of cycling cells with a normal content of DNA from the percentage of cells with a DNA content exceeding 2n.

The calculated data of the various mice which were analysed are given below:

Mouse 5T2-1: The fraction of cells with a diploid content of DNA had a size of 63 per cent. The remaining 37 per cent of all cells comprised the fraction of the aneuploid cells together with that of the cycling normal cells.

Mouse NM-1: Not in cycle (Go/G1 phase) was 80 per cent of all bone marrow cells. All remaining cells (20 per cent) were dividing, i.e., in S, G2, or M phase. The cycling cell fraction had a size of (20:80)x the percentage of the Go/G1 fraction. From this ratio, it was assumed that the proportion of cycling bone marrow cells with a normal DNA content had a value of 15.8 per

cent [calculation: (20:80)x63% (=Go/G1 fraction 5T2-1)]. It was concluded from these calculation that 21.2 per cent (calculation: 37%-15.8%) of all cells in the bone marrow of mouse 5T2-1 were aneuploid.

Mouse 5T2-2: Forty-two per cent of all bone marrow cells had a diploid content of DNA. The aneuploid cells and the cycling normal cells formed a fraction of 58 per cent of the whole marrow population.

Mouse NM-2: The fraction of non-dividing cells was composed of 78 per cent of the bone marrow cells. The cycling cells (22 per cent) formed a fraction with a size of (22:78)x the percentage of the Go/G1 fraction. The percentage of cycling cells with a normal content of DNA from the bone marrow of mouse 5T2-2 was assumed to be 11.6 [(22:78)x42%]. The fraction of aneuploid cells contained 46.4 per cent (58%-11.6%) of all bone marrow cells from mouse 5T2-2.

Mouse 5T2-3: The fraction of diploid cells had a percentage of 68. Therefore, all aneuploid and cycling normal cells together formed a fraction of 32 per cent.

Mouse NM-3: The non-cyling fraction had a size of 82 per cent (Go/G1); eighteen per cent of the cells belonged to the S/G2/M fraction, i.e., (18:82)x the percentage of Go/G1 fraction. In a similar way as was calculated for the above-mentioned mice, the proportion of aneuploid cells in the bone marrow from mouse 5T2-3 was determined to be 17 per cent.

c) Proportion of surface membrane 5T2 MM idtiotype-positive cells within the bone marrow of 5T2 MM-transplanted mice.

The same three 5T2 MM mice (5T2-1, 2, and 3) and normal C57BL/KaLwRij mice (NM-1, 2, and 3) were investigated in order to determine the expression of the 5T2 MM idiotype on the surface membrane of 5T2 MM bone marrow cells. Staining of the bone marrow cells and FACS analysis was performed as described in chapter II.6.b.

The fluorescence distribution of bone marrow cells from mouse 5T2-1 and from mouse NM-1 is illustrated in Fig.6A. No distinct separate population of 5T2 MM idiotype-positive cells could be discerned. When compared with the fluorescence pattern of 5T2 MM bone marrow cells only incubated with FITC-conjugated goat anti-mouse IgG1 antibody (second step control, see chapter

II.6.b; distribution pattern not shown), the bone marrow cell compartment of mouse 5T2-1 contained a fraction of surface membrane idiotype-positive cells of 10 per cent.

The fluorescence of the bone marrow cells from mouse 5T2-2 was, in general, of a higher intensity than that of the bone marrow cells from mouse NM-2 (Fig.6B). Compared to the second step control (not shown), a fraction of 20 per cent of the 5T2 MM bone marrow cells was regarded as surface membrane idiotype-positive.

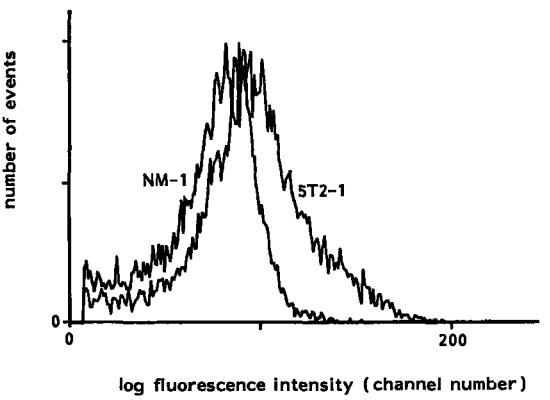

Fig.6A: Distribution of bone marrow cells from mouse 5T2-1 and from mouse NM-1 according to their surface membrane immunofluorescence intensity after incubation of the cells with monoclonal anti-5T2 MM idiotype antibody 145-4.1, followed by a second step incubation with a FITC-conjugated goat anti-mouse IgG1 antibody.

In Fig.6C, the fluorescence distributions of bone marrow cells from the mice 5T2-3 and NM-3 are illustrated. After subtracting of the fluorescence intensity of the 5T2 MM cells only incubated with the second step (not shown), 7 per cent of the bone marrow cells from mouse 5T2-3 were considered to express the 5T2 MM idiotype on their surface membrane.

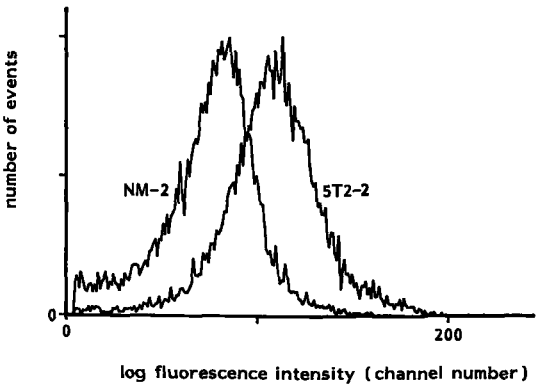

Fig.6B: Distribution of bone marrow cells from mouse 5T2-2 and from mouse NM-2 according to their surface membrane immunofluorescence intensity. The cells were incubated with anti-5T2 MM idiotype antibody 145-4.1 and FITC-conjugated goat anti-mouse IgG1 antibody (see chapter II.6.b).

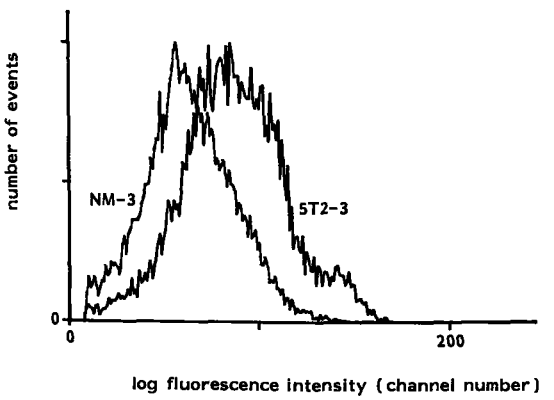

Fig.6C: Distribution of bone marrow cells from mouse 5T2-3 according to their surface membrane 5T2 MM idiotype immunofluorescence. The immunofluorescence intensity distribution of the bone marrow cells from mouse NM-3 is shown for comparison (see legends Fig.6A and B).

2.3 Discussion

For a good characterization of the biological behaviour of the 5T2 MM, data with respect to its extent and growth rate are needed. Such data can only be obtained by detection of features specifically related to the 5T2 MM cell. The 5T2 MM idiotype is the clone-specific marker of this myeloma. The 5T2 MM idiotype-positive immunoglobulin is present at three different locations: in the cytoplasm and on the surface membrane of 5T2 MM cells, and in the circulation and the other extracellular fluid compartments of the mice. Detection of the 5T2 MM idiotype in serum samples is relatively easy (see chapter II.5). It can be performed repeatedly while keeping the mice alive, which is a prerequisite for longitudinal studies. In contrast, determination of the number of positive cells within the bone marrow or other organs by immunofluorescence microscopy is time-consuming, and only possible after the animals have been sacrificed. At about nine weeks after transplantation, when the serum 5T2 MM immunoglobulin became clearly detectable, the first 5T2 MM cells were visible in the bone marrow. This observation indicates that monitoring of the serum 5T2 MM protein concentration after intravenous transplantation of the mice with 5T2 MM bone marrow cells is a reliable method to determine the moment that the myeloma cells begin to expand within the bone marrow. Where the 5T2 MM cells locate in the mouse directly after transplantation has not yet been established. The rapid increase in involvement of the bone marrow with 5T2 MM cells suggests that the bone marrow offers the favourable biological conditions for "homing" of the 5T2 MM cells. The increase of the mean percentage of cytoplasmic 5T2 MM idiotype-positive cells within the bone marrow as a function of time paralleled that of the mean serum concentration of 5T2 MM immunoglobulin to a reasonable degree. Therefore, serial determinations of the serum 5T2 MM immunoglobulin concentration are useful to estimate the size and the growth rate of the neoplastic process. A limitation of the cytoplasmic immunofluorescence technique is the necessity of fixation of the cells to enable binding of the reagents to the immunoglobulins within the cell. This technique can, therefore, not be used for purification of a population of viable 5T2 MM cells as, for example, in cell sorter experiments. The proportion of cytoplasmic 5T2 MM idiotype-positive cells within the heterogeneous bone marrow population to be transferred into syngeneic recipients can be assessed by fluorescence

microscopy of parallel samples (for example, in order to determine the minimal number of cells that will result in "take" of the 5T2 MM), but this takes a lot of time. The possibility to detect cytoplasmic immunoglobulin by flow cytometry offers new perspectives in this respect (39,40). This technique enables a rapid analysis of the proportion of myeloma cells within the heterogeneous bone marrow. Flow cytometry is an objective method, leading to reproducible data. The applicability of flow cytometry for analysis of the cytoplasmic fluorescence distribution of 5T2 MM cells requires further study.

The fraction of aneuploid cells within the 5T2 MM bone marrow population can be regarded as the neoplastic cells. This implies that a rather accurate assessment of the proportion of tumour cells within the 5T2 MM bone marrow is possible by flow cytometric analysis of the cellular DNA content. The procedure is easy and rapid (41). However, just as for cytoplasmic immunofluorescence, the cells have also to be subjected to fixation for propidium iodide staining. Besides the calculation of the size of the whole myeloma fraction, the DNA distribution pattern permits the assessment of the proportion of cycling 5T2 MM cells as well. The question whether 5T2 MM cells with a diploid DNA content are also present within the bone marrow can only be answered with certainty by correlation of the proportion of cells with an aneuploid DNA content with that of cells with another 5T2 MM-specific marker such as cytoplasmic idiotype. Barlogie et al. (40) reported the identification of a diploid myeloma cell population next to an aneuploid population in 6 out of 48 (i.e., 12.5 per cent) patients by combined flow cytometric analysis of DNA ploidy and cytoplasmic myeloma immunoglobulin. In the human situation, the focal distribution of myeloma cells within the bone marrow compartment complicates a reliable assessment of the percentage of tumour cells by DNA distribution or cytoplasmic immunoglobulin analysis of the cells from bone marrow aspirates or biopsies. A high proportion of the total bone marrow compartment of 5T2 MM-bearing mice is, however, available for processing, thereby increasing the chance for an accurate determination of the percentage of 5T2 MM cells.

Unlike cytoplasmic immunofluorescence, labelling of surface membrane determinants is possible on viable cells. With the proper reagents, one can purify the desired cell populations from heterogeneous suspensions, and subsequently study them in vitro or in vivo. The usefulness of monoclonal

surface membrane-bound immunoglobulin to serve as a marker of neoplastic B-cells has been established in several studies (42,43,44,45,46,47,48). For 5T2 MM, this property of surface membrane-bound immunoglobulin was tested in three representative 5T2 MM-bearing mice. In these experiments, the indirect (two-step) immunofluorescence technique was used. Theoretically, direct (one-step) fluorescence is more optimal, since it leads to less background staining than the indirect technique. However, if the specific fluorescence signals which are yielded by the direct method are too weak, the indirect technique must be applied in order to amplify the signals. The resulting fluorescence patterns of bone marrow cells from 5T2 MM-bearing mice showed the presence of cells that were idiotype-positive (Fig.6A-C). However, the distribution of these idiotype-positive cells according to their fluorescence intensity did not result in a curve with a distinct separate peak. This observation indicates that the 5T2 MM population within the bone marrow consists of different subpopulations of cells expressing the surface membrane 5T2 MM idiotype in different densities. This was supported by histology, that revealed differences in size of the 5T2 MM cells, which might in part reflect the presence of differences in degree of differentiation.

When correlated with the proportion of 5T2 MM cells within the bone marrow of the same mouse on basis of DNA distribution analysis, only 40 to 50 per cent of the 5T2 MM cells were detected by their idiotype on the surface membrane: mouse 5T2-1 showed 21 per cent cells with aneuploid DNA and only 10 per cent idiotype-bearing cells; in mice 5T2-2 and 5T2-3 these proportions were, respectively, 46 per cent versus 20 per cent, and 17 per cent versus 7 per cent. The idiotype on the surface membrane of 5T2 MM cells is apparently not a suitable marker for determination of the size of the myeloma mass, or for purification of all 5T2 MM cells from the heterogeneous bone marrow population. However, it deserves further study to establish whether this marker can be used for identification of subsets of precursor cells of the 5T2 MM clone. In addition, the presence of the tumour-specific antigen, i.e., the idiotype on at least a fraction of the myeloma cells is a promising condition for initiation of studies on immunological treatment of this malignancy by anti-idiotype antibodies. If the proliferating compartment of the 5T2 MM consists of surface membrane idiotype-positive cells, it may be expected that it is possible to retard, or even terminate its growth by such immunological tools.

CHAPTER IIIA

THE C57BL/KaLwRij MOUSE 5T2 MULTIPLE MYELOMA

3. Separation and characterization of subsets of bone marrow cells from mice bearing the 5T2 MM

3.1 Introduction

Multiple myeloma has long been regarded as a neoplasm of monoclonal plasma cells. In the last decade, however, results from a number of studies have indicated that less differentiated B-cells may also be part of the myeloma cell clone. Neoplastic cells of transplanted murine plasmacytomas have been demonstrated to differentiate from small non-secreting clonogenic cells to large plasmacytoid cells secreting the monoclonal immunoglobulin (49,50). In man, precursor cells in various stages of differentiation, which express the myeloma idiotype, have been observed in the peripheral blood and in the bone marrow (reviewed in 51 and 52,53). These findings suggest that the malignant transformation leading to the development of MM has already occured in a precursor of the plasma cell. Further support for this hypothesis was offered by the observation of chromosomal abnormalities in plasma cells as well as in cells with a B-lymphocyte phenotype of a patient with plasma cell leukaemia (54). These chromosomal abnormalities showed a similarity with those reported in cases of MM. However, functional proof of the participation of the idiotype-bearing B-cells in the myeloma clone has not yet been delivered. Stimulation in vitro of such B-cells in the presence of mitogens such as Pokeweed mitogen or Staphylococcus Aureus did not lead to a subsequent differentiation of these cells into more mature cells of the immunoglobulin-secreting phenotype (55,56). Moreover, it was suggested by other investigators that the idiotype-bearing lymphocytes in MM are in fact T-lymphocytes binding the myeloma immunoglobulin by Fc-receptors with specificity for its isotype (57). Recently,

myeloma precursors of lymphoid morphology which lacked surface and cytoplasmic immunoglobulin, but expressed the acute lymphoblastic leukaemia antigen (CALLA) were identified in the bone marrow of patients with MM. Stimulation in vitro of these cells with the phorbol ester 12-0-tetradecanoylphorbol-13 acetate (TPA) resulted in their transformation into plasma cells that synthesized the myeloma-specific immunoglobulin (58). This observation indicates the existence of a functional relationship between more differentiated MM cells and their precursors. However, the exact role of the idiotype-bearing B-lymphocyte has not become clear in that study.

Studies in a suitable experimental animal MM model may be valuable for obtaining more insight into the precursors of the neoplastic clone. The following study represents an attempt to characterize the individual subpopulations of the 5T2 MM clone. Bone marrow cell suspensions from 5T2 MM-bearing mice were fractionated according to their sedimentation velocity. The phenotype of the cells within the different fractions was studied by analysis of their surface membrane 5T2 MM idiotype expression and of their nuclear DNA content. In addition, the potential of these cells to develop into a typical 5T2 MM was determined after their transplantation into syngeneic recipients. The results of this study indicate that the 5T2 MM clone involves cells in various stages of differentiation.

3.2 Experimental design and results

Bone marrow cells from male and female C57BL/KaLwRij mice with an advanced 5T2 MM were harvested (see chapter II.3), and separated on the basis of differences in their sedimentation velocity (see chapter II.8). The number of nucleated cells within the 5T2 MM bone marrow cell suspension and within each fraction was determined with an electronic cytometer (see chapter II.3). Aliquots of cells were taken from the unseparated 5T2 MM bone marrow cell suspension and from each fraction. These cells were prepared for analysis of their surface membrane 5T2 MM idiotype expression (see chapter II.6.b) and nuclear DNA content (see chapter II.7).

Cells from the unfractionated 5T2 MM bone marrow and from selected fractions were resuspended in HBSS, and transferred intravenously into young

syngeneic recipient mice (see chapter II.3). Each experimental group comprised four recipients. Each mouse received 0.5×10^6 cells. The development of 5T2 MM was monitored weekly in serum samples from all recipients as described in chapter II.5.

For morphological examination, glass slides with cells from the different fractions were prepared using a cytocentrifuge (see chapter II.6.a). The cell preparations were stained with May-Grünwald-Giemsa, and examined by light microscopy.

Two peaks were observed in the distribution curve of the 5T2 MM bone marrow cells according to their sedimentation velocity (Fig.1). The first peak contained cells with a sedimentation rate of 3 to 5 mm/h; the second peak

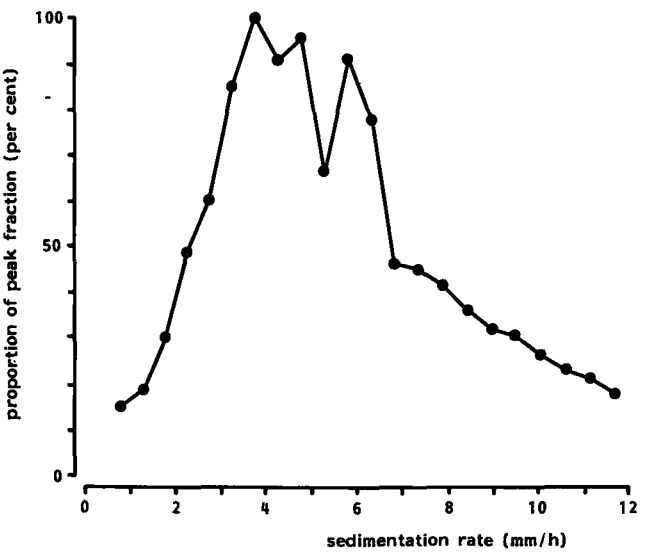

Fig.1: Distribution profile of 5T2 MM bone marrow cells according to their sedimentation velocity. The cells were sedimented by 1 g for 3 h at 4°C (see chapter II.8). The proportion of cells in each fraction is expressed as the percentage of the number of cells in the peak fraction. The largest fractions were found at sedimentation rates of 3 to 5 and 6 mm/h.

consisted of larger cells with a sedimentation rate of 6 mm/h. A relatively high proportion of all cells sedimented with a velocity of more than 6 mm/h. The typical character of this distribution curve was observed in separate experiments. Fig.1 serves as the standard distribution curve and has been incorporated in Figs.2, 3, and 5.

All mice injected intravenously with unfractionated 5T2 MM bone marrow cells showed development of 5T2 MM after twelve weeks, as demonstrated by the presence of 5T2 MM protein in their sera. A more rapid development of 5T2 MM was observed in mice which received cells from fractions with a sedimentation rate of about 8 mm/h. 5T2 MM protein was detected in the sera of these recipients as early as six weeks after transplantation. In contrast, 5T2 MM protein was detectable in the sera from mice transplanted with the smaller cells (sedimentation rate less than 5 mm/h) only after 18 weeks (Fig.2).

Twenty-three per cent of the cells within the unfractioned 5T2 MM bone marrow expressed the 5T2 MM idiotype on their surface membrane. After separation of the 5T2 MM bone marrow cell suspension, the highest proportion (16-39 per cent) of surface membrane 5T2 MM idiotype-positive cells was present within the fractions containing the larger cells, i.e., the cells with a sedimentation rate exceeding 6.5 mm/h. The fractions with the smallest cells (sedimentation rate less than 3 mm/h) contained a higher precentage (16-19 per cent) of 5T2 MM idiotype-positive cells than the fractions with cells of intermediate size (sedimentation rate between 3 and 6 mm/h) (6-12 per cent) (Fig.3).

The distribution of the DNA content of unfractionated 5T2 MM bone marrow cells and of bone marrow cells from a normal C57BL/KaLwRij mouse is illustrated in Fig.4. About 40 per cent of all cells within the unfractionated 5T2 MM bone marrow suspension belonged to the neoplastic clone on the basis of the aneuploid content of DNA (see chapter IIIA.2.2.b).

Individual cell fractions obtained by the velocity sedimentation procedure contained different numbers of 5T2 MM cells in, respectively, the Go/G1 (3.5n) and G2/M (7n) phase of the cell cycle. Within the fractions with larger cells (sedimentation rate exceeding 6 mm/h), an increase in the proportion of non-cycling 5T2 MM cells (3.5n) was observed as compared with the unseparated 5T2 MM bone marrow population and with the fractions containing cells with

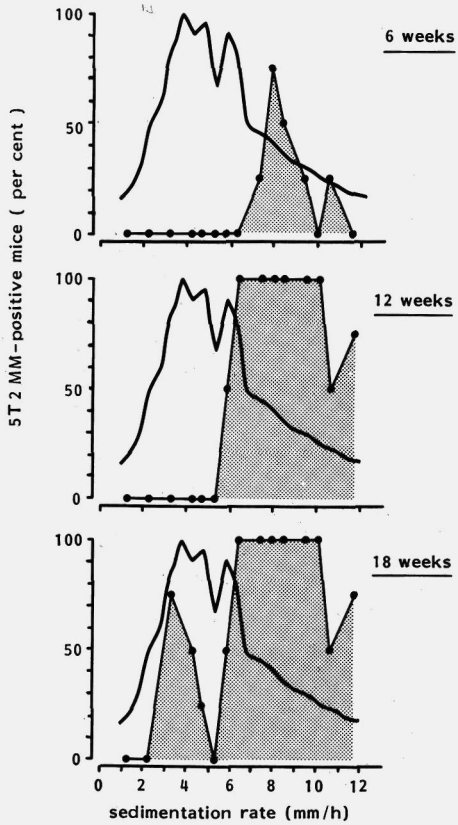

Fig.2: Distribution of cells which caused the development of 5T2 MM according to their sedimentation rate. Mice of the experimental groups (n=4) were each injected intravenously with 0.5×10^6 cells from one of the different fractions of 5T2 MM bone marrow cells obtained by velocity sedimentation. The percentage of recipients developing 5T2 MM is shown at, respectively, 6, 12, and 18 weeks from the day of transplantation. In each figure, the total 5T2 MM bone marrow sedimentation profile is indicated (full continuous line). Mice which received cells with a sedimentation rate of about 8 mm/h had a detectable MM already at 6 weeks after transplantation. In contrast, transplantation of cells with a sedimentation rate less than 5 mm/h resulted in the development of 5T2 MM only after 18 weeks.

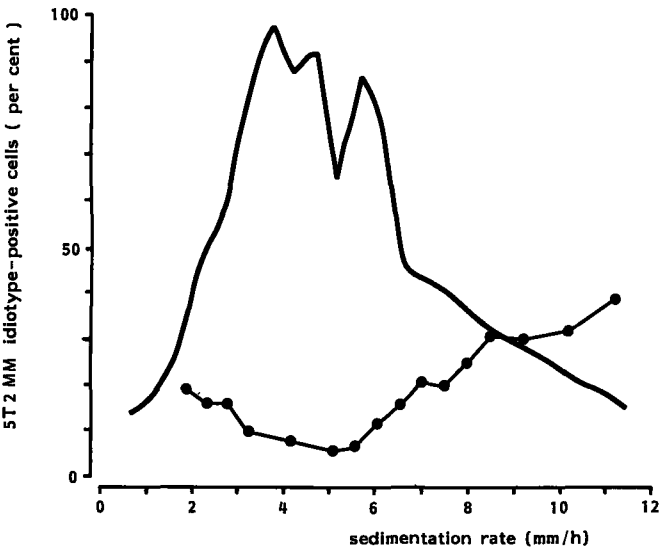

Fig.3: Distribution of surface membrane 5T2 MM idiotype-positive cells in individual fractions according to their sedimentation rate. The sedimentation velocity profile of unfractionated 5T2 MM bone marrow cells is indicated (full continuous line). The cells were stained according to the procedure described in chapter II.6.b. The highest percentages of 5T2 MM idiotype-positive cells were found in the fractions of cells with a sedimentation rate exceeding 6.5 mm/h. Non-fractionated 5T2 MM bone marrow contained 23 per cent of 5T2 MM idiotype-positive cells.

sedimentation rates less than 6 mm/h (Fig.5). An increase in the proportion of non-cycling 5T2 MM cells (3.5n) was also observed in the fractions with a sedimentation rate of about 3 mm/h (Fig.5). A distinct third peak, representing the 5T2 MM cells with 7n DNA (G2/M phase), was only observed in the DNA distribution curves of the fractions containing cells with sedimentation rates exceeding 9 mm/h, i.e., the largest cells (Fig.5).

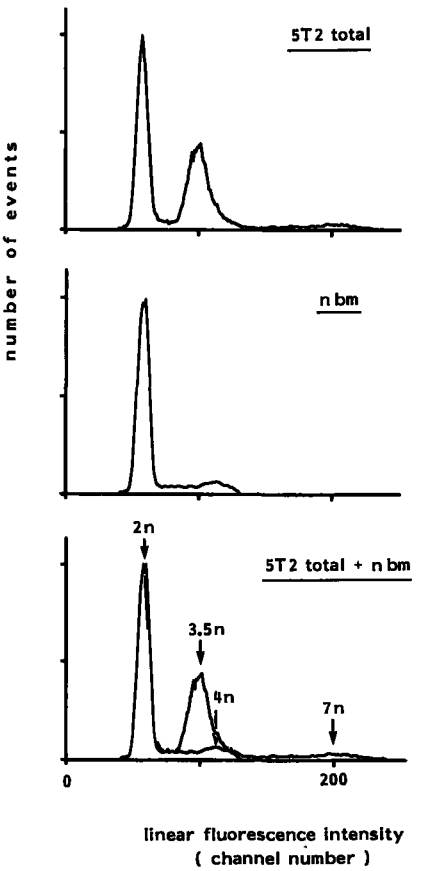

Fig.4: DNA histogram of 5T2 MM bone marrow cells. The nuclear DNA was stained with propidium iodide (see chapter II.7), and the fluorescence intensity was subsequently determined by analysis in a fluorescence-activated cell sorter (FACS II) using linear amplification of the signals. The DNA distribution of normal mouse bone marrow cells is shown for comparison.
The first peak in the 5T2 MM bone marrow DNA distribution curve corresponds with non-cycling normal bone marrow cells; the second peak is located at about 1.75 times the fluorescence intensity of the first one, and the third peak represents cells with twice the fluorescence intensity of the second peak. A number of at least 10^4 events (cells) was analysed for the determination of each DNA histogram.
(nbm: normal C57BL/KaLwRij mouse bone marrow).

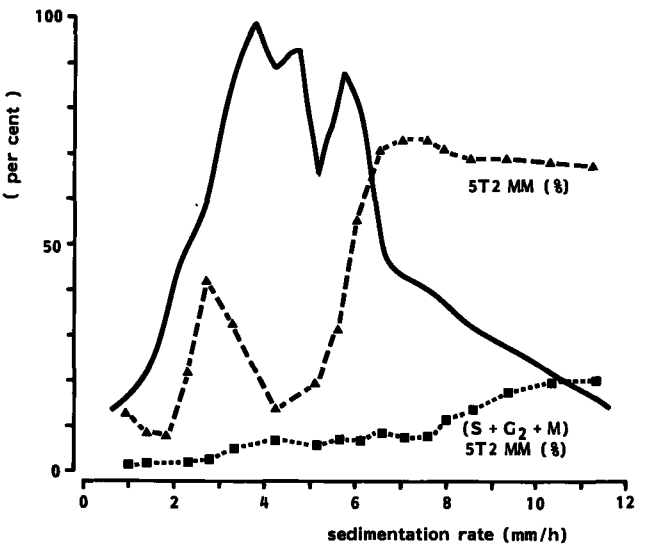

Fig.5: Distribution of all 5T2 MM cells (-▲-) and of cycling 5T2 MM cells (-■-) according to their DNA content within fractions obtained by velocity sedimentation. All 5T2 MM cells were calculated as the proportion of cells with a DNA content exceeding 2n minus the proportion of cycling non-myeloma bone marrow cells. The latter proportion was calculated by assuming that the proportion of cycling normal bone marrow cells in 5T2 MM bone marrow and in normal mouse bone marrow was identical. The 5T2 MM cells with a DNA content exceeding 3.5n were assumed to reflect the cycling 5T2 MM cells in S/G2/M phase. The sedimentation velocity profile of total 5T2 MM bone marrow cells is indicated (full continuous line).

3.3 Discussion

In the present study, the question was addressed whether the observed morphological heterogeneity of the 5T2 MM cells reflects the presence of

different MM subpopulations. Therefore, 5T2 MM bone marrow cells were separated according to their size, and subsequently tested for their DNA content, surface membrane 5T2 MM idiotype expression, and their capacity to develop into a typical 5T2 MM.

A large number of cells had to be processed because the potential of cells from individual fractions to develop into a tumour was tested by transplantation in vivo. For this reason, the velocity sedimentation procedure was chosen for fractionation of the cell suspensions. Cells in normal bone marrow with sedimentation rates of 3 mm/h and 5 mm/h have been reported to be lymphoid cells and myeloid cells, respectively (59,60). The relatively high proportion of cells with a sedimentation rate exceeding 6 mm/h reflected large abnormal myeloma cells.

Cells of fractions with sedimentation rates of a very broad range (from 3 to about 11 mm/h) caused the development of 5T2 MM after their intravenous transfer into syngeneic recipient mice. However, 5T2 MM bone marrow cells with a sedimentation rate of 8 mm/h required a shorter period of time to develop into an overt myeloma as compared with the unfractionated 5T2 MM bone marrow cells or cells from the other fractions. This indicated that the fractions containing cells with a sedimentation rate of 8 mm/h included more of the cycling tumour cells than the other fractions. The increase in time interval between the transplantation of the smaller cells (sedimentation rate between 3 and 5 mm/h) and the development of 5T2 MM might be explained by the assumption that these cells were 5T2 MM cells in an earlier stage of differentiation than the larger cells, i.e., the smaller cells had first to differentiate into a more mature stage before they were equally able to proliferate. This assumption was supported by light microscopy, which revealed a plasmacytoid appearance of the cells from the fractions with a moderate to high sedimentation rate, whereas the slower sedimenting cells had a lymphoid cell morphology. Contamination of the fractions containing the smaller cells with large cells cannot be totally excluded. However, this is unlikely to be the major cause of the observed pattern because corresponding results were obtained in three different experiments.

Based on the DNA distribution analysis, a substantial increase in the percentage of myeloma cells was observed in the fractions with sedimentation rates between 6 and 11 mm/h when compared with the proportion of 5T2 MM

cells in unfractionated 5T2 MM bone marrow. This supports the hypothesis that the high proportion of large cells in the 5T2 MM bone marrow reflects mainly the presence of the myeloma cells. In addition, the observation that intravenous transplantation of cells from the large cell fractions into syngeneic recipient mice resulted in the development of a myeloma after a relatively short time interval is in agreement with the finding that a high percentage of proliferating 5T2 MM cells (7n DNA) was present within these cell fractions. However, the first 5T2 MM's were detected in recipient mice which were transplanted with cells from fractions with sedimentation rates between 8 and 9 mm/h. These fractions contained a lower percentage of cells with 7n DNA than the fractions with sedimentation rates exceeding 9 mm/h. This lack of correlation between the maximum percentage of 7n DNA-containing cells and the maximum rate of development of 5T2 MM after transplantation might be explained by the presence of binucleated 5T2 MM cells, which were actually not in cycle, within the fractions with sedimentation rates of more than 9 mm/h (see chapter IIIA.1.2.f).

The unfractionated 5T2 MM bone marrow cell population contained about 20 per cent of surface membrane-bound 5T2 MM idiotype-positive cells. Nearly 40 per cent of these cells were demonstrated to belong to the 5T2 MM clone on the basis of their aberrant content of DNA. This implies that about 50 per cent of these cells expressed the 5T2 MM idiotype on their surface membrane. Corresponding percentages of surface membrane 5T2 MM idiotype-positive cells were found in the bone marrow from the 5T2 MM mice which have been described in the previous chapter (chapter IIIA.2). The hypothesis that the heterogeneity of the 5T2 MM cell population is at least in part a reflection of differences in maturity of the myeloma cells is supported by the heterogeneity in the expression of the surface idiotype as demonstrated by idiotypic analysis of the different cell fractions. The idiotype-positive cells within the large cell fractions might be of the plasmablastic phenotype.

Non-cycling myeloma cells, which were demonstrated by the DNA distribution analysis to be present mainly within the fractions with sedimentation rates between 2.5 and 3.5 mm/h, might represent small precursor cells. Apparently, these small cells had to differentiate into a more mature myeloma cell before they could proliferate. The following observations support this assumption: a) typical myeloma cells are in general of larger size than pre-

cursor cells of the B-cell lineage, i.e., the immature and mature B-lymphocytes (49,50); b) the increase in proportion of tumour cells within the small cell fractions occurred simultaneously to the increase in proportion of surface membrane 5T2 MM idiotype-positive cells in these fractions, suggesting an increase in the percentage of 5T2 MM idiotype-bearing B-lymphocytes; c) within the small cell fractions, no substantial number of cycling cells containing 7n DNA could be demonstrated. Therefore, one might conclude that small, non-proliferating 5T2 MM cells are less mature myeloma cells, presumably of the B-lymphocyte phenotype.

In conclusion, the morphological heterogeneity of the 5T2 MM bone marrow cell population is also reflected in the different neoplastic proliferation capacities of subsets of this clone. The clear-cut differences in time interval between transplantation of the different cell fractions into syngeneic mice and the development of 5T2 MM may reflect the differences in maturity of the 5T2 MM cells: small non-dividing cells showed a delayed development of 5T2 MM probably due to their relatively early stage of differentiation; in contrast, the majority of the proliferating MM cells (7n DNA) was found within the large cell fractions. Transplantation of cells of these fractions resulted in a relatively rapid onset and development of 5T2 MM.

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CHAPTER IIIB

IMMUNE REGULATION OF 5T2 MULTIPLE MYELOMA

1. Immune response to 5T2 MM idiotype

1.1 Introduction

Malignancies of B-cells such as MM are of monoclonal origin. The idiotype of their immunoglobulin product is a unique clonal marker as is the case for individual clones of normal B-cells, and can be regarded as a tumour-specific antigen (1, reviewed in 2).

According to the network hypothesis of Jerne (3), idiotypes of normal immunoglobulins are targets involved in the immunological regulation of the normal immune response. It has been demonstrated in several studies that malignant immunoglobulin-secreting cells of the B-cell lineage are susceptible to idiotype, isotype, and antigen-specific immune regulation. This indicates that such neoplastic cells have retained some of their physiologic properties (reviewed in 4 and 5). The majority of those studies utilized mineral oil-induced murine plasmacytomas of BALB/c origin (MOPC-plasmacytomas). Subcutaneous immunization of BALB/c mice with the immunoglobulin secreted by the MOPC-315 plasmacytoma (an IgA-lambda2 immunoglobulin, abbreviated as M-315) resulted in the induction of a humoral idiotype-specific immune response (6). In addition, the immunized mice appeared to be relatively resistant to a subsequent subcutaneous or intravenous challenge with numbers of MOPC-315 cells that proved to be lethal in naive or control-immunized mice (7,8). The induction of isologous anti-idiotype antibodies has also been reported for other induced BALB/c plasmacytomas (9,10). The mechanism of the idiotype-specific resistance to transplantation of plasmacytoma cells is not completely understood. It is not clear whether serum anti-idiotype antibodies actually participate in the establishment of this resistance. In immunized BALB/c mice,

no positive correlation could be demonstrated between the serum level of antibodies to the M-315 idiotype and the degree of resistance against transplantation of MOPC-315 cells (11). Moreover, it was demonstrated in the same study that passive administration of serum containing anti-MOPC-315 idiotype antibodies to BALB/c mice simultaneously with and after implantation of MOPC-315 cells did not inhibit the development of this plasmacytoma. However, inhibition of tumour growth was achieved after passive transfer of anti-MOPC-315 idiotype antibodies throughout a period ranging from three weeks before to more than one week after intravenous inoculation of the mice with the corresponding plasmacytoma cells (12). BALB/c mice injected with antibodies to the idiotype of the syngeneic TEPC-15 plasmacytoma showed a delayed development of this tumour, when they had been transplanted with TEPC-15 cells one day later (13). The results of those studies show clear discrepancies. The anti-idiotype antibodies are probably not the only effectors of the idiotype-specific tumour immunity. Evidence for the participation of other elements of the immune system has been obtained in a number of other studies. Addition of complete Freund's adjuvant, for example, is required for immunization in order to obtain full expression of idiotype-specific resistance against MOPC-315 plasmacytoma (14). This indicates that specific T-cells and/or non-specific effectors, like macrophages, are involved in this resistance. Further studies have extended this observation. Intraperitoneal immunization of BALB/c mice with free light chains of M-315 gave rise to antibodies with specificity for these light chains, but not for the intact M-315. However, the mice were rendered resistant to "take" of the MOPC-315 plasmacytoma, which expressed only the intact M-315 (15). Moreover, when BALB/c mice had been immunized with free variable domains of M-315 light chains (Vl-315), they also showed resistance against subsequent challenge with MOPC-315 cells in spite of the absence of detectable antibodies directed to those variable domains (15). In contrast, no resistance was induced by immunization with the free variable domain of the heavy chain of M-315 (Vh-315). These findings corresponded with the observation that M-315-specific T-helper cells were detected after immunization with free light chains of M-315, or with Vl-315, but that they were not found after immunization with Vh-315 (15). Thymectomy which had been performed in mice shortly after immunization with M-315 led to the abolition of the established resistance

against engraftment of this plasmacytoma. This observation suggests that a thymus-dependent mechanism is involved in the maintenance of this resistance. (16).

More information about T-cell involvement has been provided in two other reports (17,18). Those investigators made use of a delayed type hypersensitivity (DTH) reaction in order to measure effector T-cell function. A DTH reaction was elicited to M-315 or to M-315-coupled splenocytes if BALB/c mice had been immunized subcutaneously with one of these antigens. Intravenous immunization caused suppression of the DTH reaction.

The findings that the growth of malignancies of immunoglobulin-secreting cells can be inhibited by immunological manipulations, offer prospects for immunotherapeutic strategies. It was therefore decided to determine whether the 5T2 MM exhibits a behaviour comparable to the induced BALB/c plasmacytomas with respect to idiotype-specific immune regulation. Attempts were made to induce an idiotype-specific immunity to 5T2 MM immunoglobulin and 5T2 MM bone marrow cells in C57BL/KaLwRij mice. The effect of subcutaneous immunization of mice with 5T2 MM immunoglobulin was determined with respect to the induction of a humoral anti-5T2 MM idiotype activity, and to the survival of the animals after subsequent intravenous transfer of 5T2 MM cells. In addition, the participation of T-cells in the 5T2 MM idiotype-specific immunity was examined by induction and suppression of the DTH reaction to 5T2 MM immunoglobulin and to 5T2 MM bone marrow cells.

1.2 Experimental designs and results

a) Humoral anti-5T2 MM idiotype response and resistance against "take" of 5T2 MM.

Four groups of female C57BL/KaLwRij mice of 3.5 months of age were included. The mice of three groups were repeatedly immunized subcutaneously with 50 µg of 5T2 MM immunoglobulin, 200 µg of 5T2 MM immunoglobulin, or 200 µg of 5T14 MM immunoglobulin, another MM protein of C57BL/KaLwRij origin (IgG1-kappa). Mice of the fourth group were not immunized. Each group consisted of six mice, with the exception of the 200 µg of 5T2 MM immuno-

globulin group that consisted of eleven mice. All immunogens were injected into the inguinal and axillary regions. Besides, the first immunization dose was also injected into the hind foot pads (see chapter II.11.a). The immunization protocol is shown in Table 1.

Small blood samples were drawn from each mouse at one week after each immunization, and the sera were tested for the presence of anti-5T2 MM idiotype antibodies by means of a competition ELISA (see chapter II.13).

The results of the determination of the anti-5T2 MM idiotype activity in the sera after nine immunizations, i.e., after 6.5 months, are shown in Fig.1.

Table 1 IMMUNIZATION PROTOCOL

Mice group	n	Immunization(s.c.)* protein	dose	Injection** in	Immunization*** period
1	6	5T2	50 µg	CFA,IFA,PBS(3x),CFA(5x)	8 months
2	11	5T2	200 µg	CFA,IFA,PBS(3x),CFA(5x)	8 months
3	6	5T14	200 µg	CFA,IFA,PBS(3x),CFA(5x)	8 months
4	6	-	-	-	-

* Each mouse of Group 1, 2, and 3 received 10 immunizations, which were performed by subcutaneous (s.c.) injection of the antigen into the inguinal and axillary regions; during the first immunization, the antigen was injected also into the hind foot pads. The first five immunizations were performed once a week; the sixth, seventh, and eighth immunizations were monthly; the time interval between the eighth and ninth immunization was three months; the final immunization was performed six weeks after the previous one.

** The injections for the first immunization were given in CFA, those for the second in IFA, the injections for the next three immunizations were given in PBS, and all subsequent immunizations were performed by injection in, again, CFA.

*** The period between the initial and the final (tenth) immunization was nearly eight months.

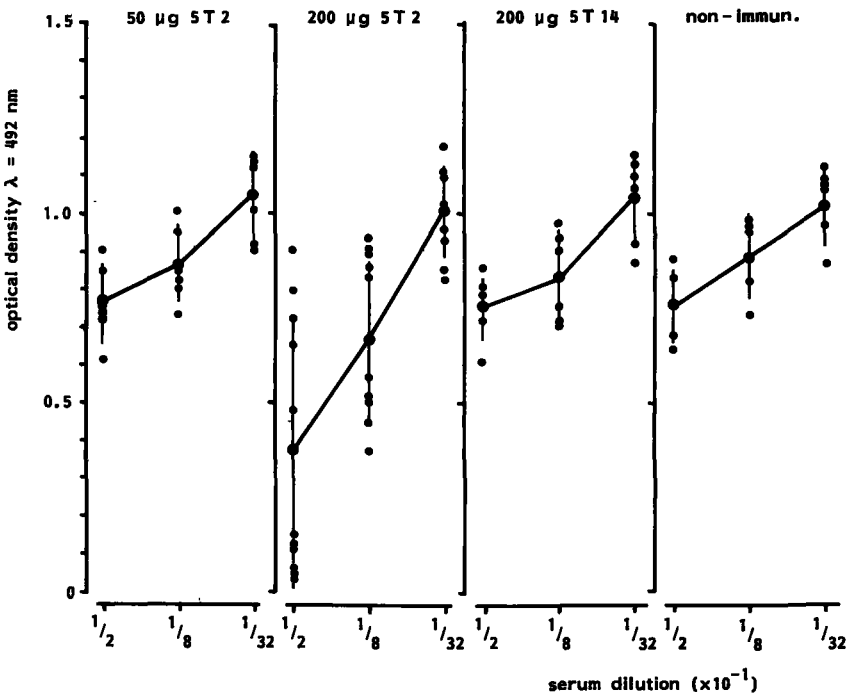


Fig.1: Determination of the anti-5T2 MM idiotype antibody activity in the sera of C57BL/KaLwRij mice immunized with 5T2 MM immunoglobulin. The degree of binding (as tested by solid phase competition ELISA) between the 5T2 MM immunoglobulin coated on the plate and a biotin-conjugated monoclonal anti-5T2 MM idiotype antibody (145-8.3) is expressed in optical density values after a second incubation with avidin-labelled peroxidase. The presence of anti-5T2 MM idiotype antibodies in the sera from the immunized mice was tested by inhibition of the binding between the solid phase 5T2 MM immunoglobulin and the 145-8.3 antibody. Various dilutions of the sera from the individual mice of this experiment were added to the coated wells, one hour before the addition of the 145-8.3 antibody.

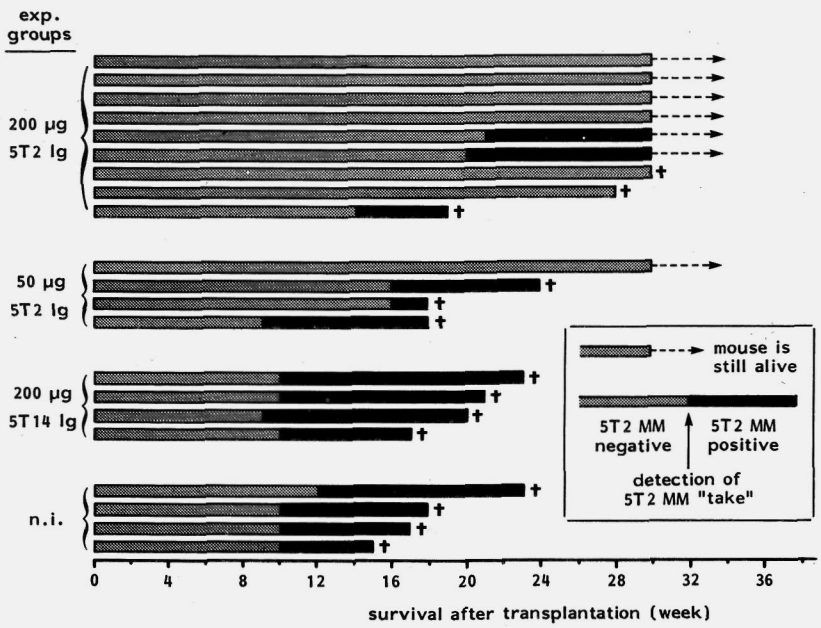


Fig.2: Survival of mice repeatedly immunized with 50 or 200 µg of 5T2 MM immunoglobulin, or with 200 µg of 5T14 MM immunoglobulin, and of non-immunized controls after intravenous transplantation of $1x10^5$ 5T2 MM bone marrow cells. When indicated, the "take" of 5T2 MM, as determined by the presence of serum 5T2 MM immunoglobulin, has been illustrated in the bars representing the individual mice. The life span of the animals immunized with 200 µg of 5T2 MM immunoglobulin was significantly increased ($p<0.05$, Mann-Whitney U-test). (Ig: immunoglobulin; n.i.: not immunized).

In the group repeatedly immunized with 200 µg of 5T2 MM immunoglobulin, six out of eleven mice showed antibody activity to the 5T2 MM idiotype. Animals that had repeatedly received the injections of 50 µg of 5T2 MM immunoglobulin, and both control groups did not respond.

After completion of the immunization procedure (i.e., after eight months) the mice of all four groups were transplanted intravenously with $1x10^5$ 5T2 MM bone marrow cells. This number of cells had previously proven to be lethal for

naive C57BL/KaLwRij mice. "Take" of 5T2 MM was monitored by detection of the 5T2 MM immunoglobulin in the individual sera by the double immunodiffusion technique according to Ouchterlony (see chapter II.5). Six out of nine mice (*) immunized with 200 µg of 5T2 MM immunoglobulin did not show "take" of 5T2 MM after transplantation. The same observation was made in only one out of four mice (*) immunized with 50 µg of 5T2 MM immunoglobulin. All control mice showed "take" of 5T2 MM within twelve weeks after transplantation (Fig.2).

Eight of the nine mice immunized with 200 µg of 5T2 MM immunoglobulin survived significantly longer than the control animals. Two out of the eight mice with a prolonged life span showed serologically the development of 5T2 MM. In the group immunized with 50 µg of 5T2 MM immunoglobulin, only one mouse had a prolonged survival, and did not show the presence of serum 5T2 MM immunoglobulin (Fig.2).

The relationship between the serum anti-5T2 MM idiotype activity in the mice immunized with 200 µg of 5T2 MM immunoglobulin, the "take" of 5T2 MM, and the survival of the animals is shown in Table 2.

b) DTH response to 5T2 MM immunoglobulin and 5T2 MM bone marrow cells

The occurrence of a specific DTH response to 5T2 MM immunoglobulin and 5T2 MM bone marrow cells was investigated in C57BL/KaLwRij mice (see chapter II.14).

Mice were immunized subcutaneously with 40 µg of 5T2 MM immunoglobulin, and subsequently challenged with 30 µg of 5T2 MM immunoglobulin five, seven, nine, or eleven days later. A control group of mice was immunized with 40 µg of 5T14 MM immunoglobulin seven days prior to the challenge with 30 µg of 5T2 MM immunoglobulin. Forty-eight hours after the challenge, a specific DTH response was observed in the animals challenged five or seven days after immunization with 5T2 MM immunoglobulin. The highest mean DTH response to 5T2 MM immunoglobulin (14.4 per cent, after substraction of the mean DTH response in the 5T14 MM immunoglobulin-immunized mice) was measured in the

(*) Two out of the six or eleven mice of the original groups were used in another experiment.

Table 2 RELATIONSCHIP BETWEEN THE SERUM ANTI-5T2 MM IDIOTYPE ACTIVITY, THE "TAKE" OF 5T2 MM, AND THE SURVIVAL OF THE MICE REPEATEDLY IMMUNIZED WITH 200 MICROGRAMS OF THE 5T2 MM IMMUNOGLOBULIN.

Mouse (no.)	anti-5T2 MM idiotype antibody in serum	5T2 MM Ig in serum	Survival* (relative)
1	+	-	prolonged
2	+	-	prolonged
3	+	-	prolonged
4	+	-	prolonged
5	+	-	prolonged
6	-	+	prolonged
7	-	-	prolonged
8	-	+	not prolonged
9	-	+	prolonged

* In comparison with the non-immunized control mice.
(Ig: immunoglobulin).

mice challenged after seven days (Fig.3). Therefore, hereafter only data of DTH reactivity in mice challenged seven days after immunization will be shown (with the exception of the experiment illustrated in Fig.6).

In dose-response experiments, it was demonstrated that a clear-cut specific DTH reaction (9.4 per cent) was elicited, if mice had been immunized subcutaneously with 25 µg of 5T2 MM immunoglobulin, and subsequently challenged with 20 µg of 5T2 MM immunoglobulin. Increase of the immunization dose of 5T2 MM immunoglobulin did not result in a higher DTH response (Fig.4).

The mice of the different groups, which were immunized subcutaneously with 5T2 MM bone marrow cells in numbers ranging from $8x10^4$ to $5x10^6$, were

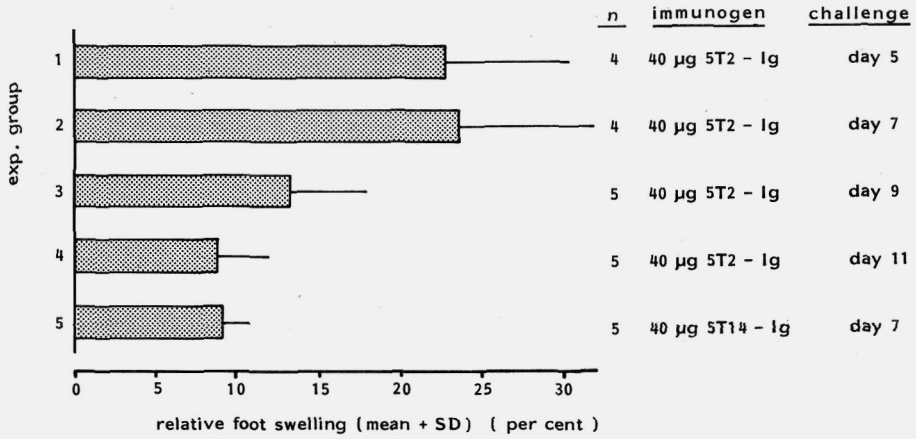


Fig.3: Kinetics of the DTH responsiveness to 5T2 MM immunoglobulin. Mice of Groups 1-4 were immunized subcutaneously with 40 µg 5T2 MM immunoglobulin in CFA, and challenged with 30 µg 5T2 MM immunoglobulin in HBSS after five, seven, nine, and eleven days, respectively. Mice of Group 5 were immunized with 40 µg 5T14 MM immunoglobulin, and challenged with 5T2 MM immunoglobulin seven days later. Forty-eight hours after the challenge, the foot swelling was measured.

all challenged with $5x10^6$ 5T2 MM bone marrow cells. The foot swelling was measured after 48 hours. The maximum specific DTH reaction (9-11 per cent) was reached after immunization with $1.25x10^6$ or $5x10^6$ 5T2 MM bone marrow cells (Fig.5).

Intravenous immunization of mice with 5T2 MM bone marrow cells did not result in a specific DTH reaction after challenge with 5T2 MM bone marrow cells (Fig.6). The mice of Groups 1-5 received an intravenous injection with different numbers of viable 5T2 MM bone marrow cells. Challenge with $3x10^6$ 5T2 MM bone marrow cells was performed after five days.

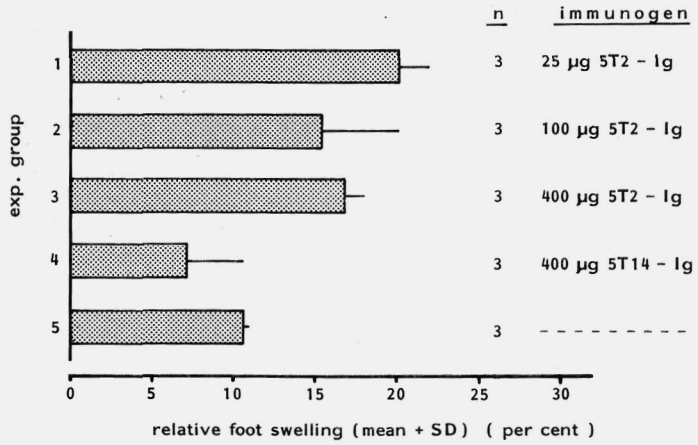
When mice were immunized subcutaneously with 200 µg of 5T2 MM immunoglobulin, and challenged with $5x10^6$ 5T2 MM bone marrow cells seven days later, a specific DTH reaction was observed 48 hours after the challenge. A

Fig.4: Relationship between the immunization dose of 5T2 MM immunoglobulin and the size of the DTH reaction to this antigen. The mice of Groups 1-3 were immunized subcutaneously with 25, 100, and 400 µg 5T2 MM immunoglobulin, respectively, and the mice of Group 4 with 400 µg 5T14 MM immunoglobulin. Group 5 consisted of mice which were not immunized at all. Seven days later, all mice were challenged with 20 µg 5T2 MM immunoglobulin. The relative foot swelling was measured 48 hours after the challenge. The mean relative foot swelling of mice of Group 5 was subtracted for determination of the specific DTH response.

similar observation was made when the mice had been immunized subcutaneously with $5x10^6$ 5T2 MM bone marrow cells. After intravenous immunization with the same number of 5T2 MM bone marrow cells, no DTH reaction was elicited (Fig. 7).

Besides a primary DTH response, a secondary response could be elicited in mice immunized subcutaneously with 40 µg of 5T2 MM immunoglobulin or $10x10^6$ 5T2 MM bone marrow cells, and challenged twice with, respectively, 30 µg of 5T2 MM immunoglobulin or $10x10^6$ 5T2 MM bone marrow cells. The second challenge was given six weeks after the first one. A specific DTH reaction of 19 per cent and 22 per cent was observed after a second challenge with, respectively, 5T2 MM immunoglobulin and 5T2 MM bone marrow cells. The secondary type of DTH reaction was not elicited if the mice had been immunized with 5T14 MM immunoglobulin or normal mouse bone marrow cells (Fig.8). Proliferation of the 5T2 MM bone marrow cells used for immunization and for the first challenge was prevented by prior irradiation (40 Gy from a ^{137}Cs source, see chapter 11.12).

Fig.5: DTH reaction in mice immunized subcutaneously with different numbers of 5T2 MM bone marrow cells. Seven days later, the mice were each challenged with $5x10^6$ 5T2 MM bone marrow cells. The mice of Group 5 were not immunized and served as controls. The relative foot swelling was measured 48 hours after the challenge. (bm: bone marrow).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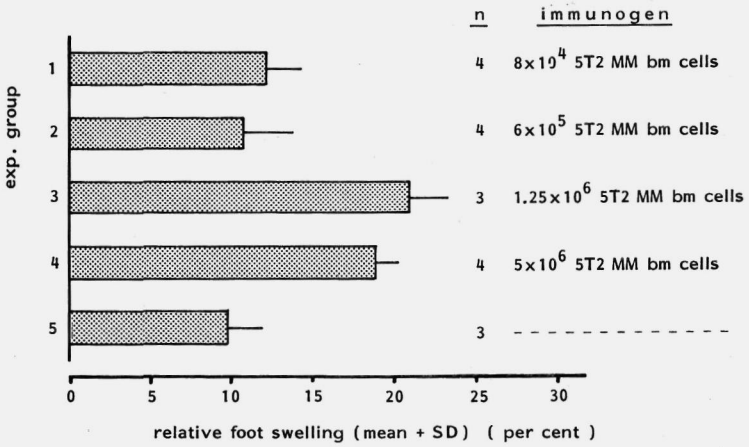
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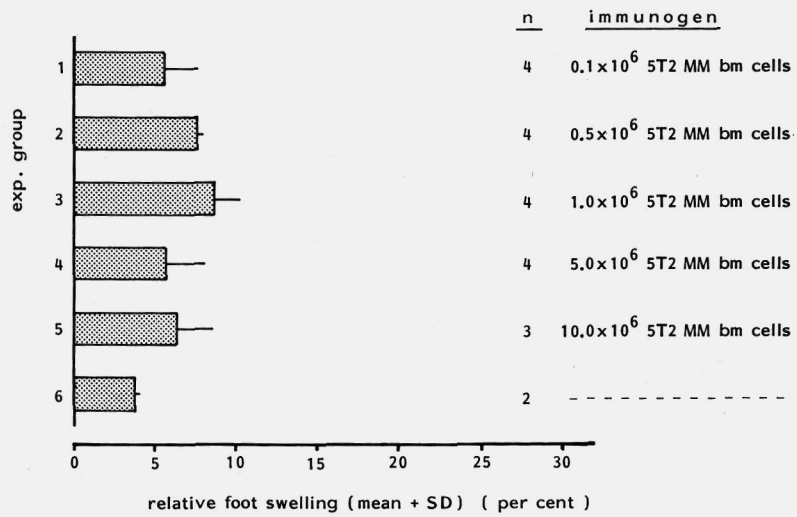
relative foot swelling (mean + SD) (per cent)

Fig.6: Relative foot swelling of the mice immunized intravenously with different numbers of 5T2 MM bone marrow cells. The mice were challenged with 5T2 MM bone marrow cells after five days. Forty-eight hours later, the DTH reaction was measured. The mice of Group 6 received only a HBSS injection for immunization and served as controls.
(bm: bone marrow).

Fig.7: Relative foot swelling of the mice immunized with different immunogens and via different routes: Group 1) 200 µg 5T2 MM immunoglobulin subcutaneously; Group 2) 200 µg 5T14 MM immunoglobulin subcutaneously; Group 3) $5x10^6$ 5T2 MM bone marrow cells subcutaneously; Group 4) $5x10^6$ C57BL/KaLwRij bone marrow cells subcutaneously; Group 5) $5x10^6$ 5T2 MM bone marrow cells intravenously; Group 6) $5x10^6$ C57BL/KaLwRij bone marrow cells intravenously. DTH reactions were measured 48 hours after challenge with $5x10^6$ 5T2 MM bone marrow cells. A specific DTH reaction was observed only in the experimental groups of mice which had been immunized subcutaneously with 5T2 MM immunoglobulin or with 5T2 MM bone marrow cells. Group 1 vs Group 2: $p<0.01$; Group 3 vs Group 4: $p<0.001$; Group 3 vs Group 5: $p<0.001$ (two-tailed Mann-Whitney U-test).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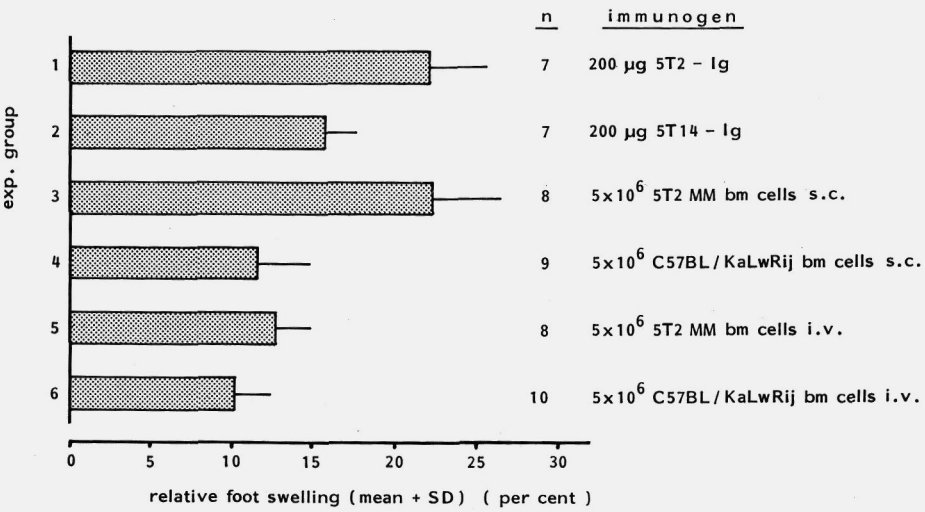
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9
5x10^6 C57BL/KaLwRij bm cells s.c.
8
5x10^6 5T2 MM bm cells i.v.
10
5x10^6 C57BL/KaLwRij bm cells i.v.
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relative foot swelling (mean + SD) (per cent)

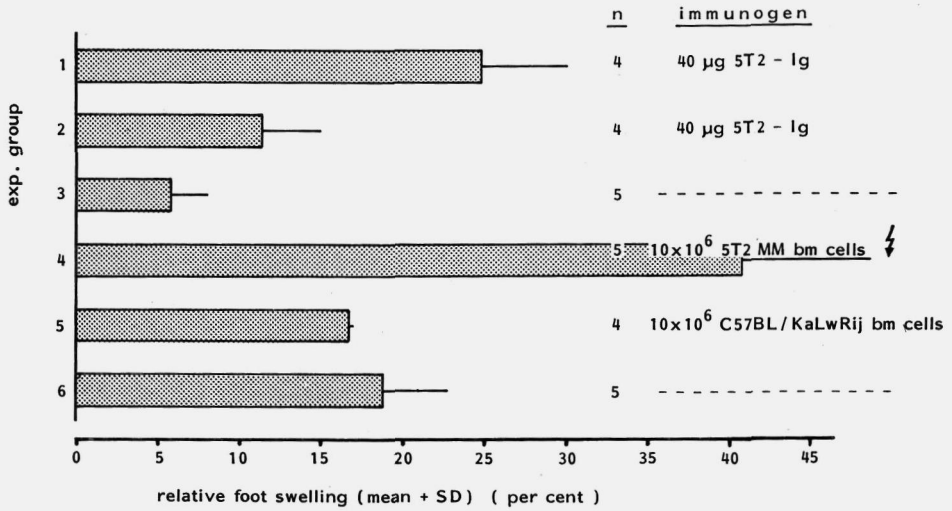

Fig.8: Secondary DTH reaction to 5T2 MM immunoglobulin and 5T2 MM bone marrow cells. The relative foot swelling was determined 48 hours after challenge with 30 µg 5T2 MM immunoglobulin (Groups 1-3), or with $10x10^6$ 5T2 MM bone marrow cells (Groups 4-6). The mice of Groups 1, 2, 4, and 5 received a second challenge six weeks after the first one. Immunization: Group 1) 40 µg 5T2 MM immunoglobulin subcutaneously; Group 2) 40 µg 5T14 MM immunoglobulin subcutaneously; Group 4) 5T2 MM bone marrow cells subcutaneously; Group 5) C57BL/KaLwRij bone marrow cells subcutaneously. The mice of Groups 3 and 6 were never immunized and served as controls for background DTH response values.

1.3 Discussion

An intensive immunization with 5T2 MM immunoglobulin was required to induce a humoral response to the idiotype of this protein in C57BL/KaLwRij mice. The anti-5T2 MM idiotype antibodies were detected after an immunization period of more than six months. Moreover, the induction of the anti-5T2 MM idiotype activity depended on the dose of the immunogen. The antibodies were

demonstrated only in the mice which had been repeatedly immunized with 200 µg of 5T2 MM immunoglobulin. However, the duration of the immunization period appeared to be of more importance than the dose of the 5T2 MM immunoglobulin. Mice repeatedly immunized subcutaneously with 200, 400, or 600 µg of 5T2 MM immunoglobulin, but for a shorter period of time, did not develop a humoral anti-5T2 MM idiotype response (results not shown). These observations are not in agreement with those in most MOPC plasmacytomas. Sakato and co-workers (9) reported the induction of isologous serum anti-idiotype antibodies to the immunoglobulin of the MOPC-167 plasmacytoma (an IgA-kappa immunoglobulin) after only two injections with as little as 10 µg of this immunoglobulin. Other investigators demonstrated that three weekly subcutaneous injections of 200 µg of MOPC-315 immunoglobulin (M-315) were sufficient to induce anti-idiotype antibodies in BALB/c mice (7,11). The MOPC proteins had been subjected to mild reduction and alkylation before immunization (7). This procedure might have influenced the antigenicity of these proteins, but this has not been assessed. The reason for the low immunogenicity of the idiotype of 5T2 MM immunoglobulin in syngeneic mice is not clear. Sakato and Eisen (10) reported that only one out of five different BALB/c plasmacytoma immunoglobulins did not result in a humoral anti-idiotype response in syngeneic mice after an immunization period which exceeded five weeks. They explained the unresponsiveness to that particular idiotype (T15) by the presence of tolerance due to the natural abundance of this idiotype in BALB/c mice. Whether a relatively high frequency of the 5T2 MM idiotype is present in normal C57BL/KaLwRij mice has not yet been established.

The survival of the immunized C57BL/KaLwRij mice after transplantation with 5T2 MM cells was also shown to depend on the immunization dose. None of the mice repeatedly immunized with 200 µg of 5T2 MM immunoglobulin and showing serum anti-idiotype activity had detectable 5T2 MM protein in their sera; three out of four mice not showing serum anti-5T2 MM idiotype antibodies were serum 5T2 MM immunoglobulin-positive. These findings suggest that anti-5T2 MM idiotype antibodies play an active role in the idiotype-specific 5T2 MM resistance.

The observation of a specific DTH reaction after subcutaneous immunization of C57BL/KaLwRij mice with 5T2 MM immunoglobulin or 5T2 MM bone marrow cells indicates that T-cells are involved in the immune response to these

isologous antigens. Mice immunized subcutaneously with 5T2 MM immunoglobulin demonstrated specific foot swelling after challenge with either 5T2 MM immunoglobulin or 5T2 MM bone marrow cells. This implies that this T-cell-dependent immune response was directed to identical determinants on both antigens. Moreover, the DTH reaction was elicited in a syngeneic system, which makes it likely that the DTH responses to 5T2 MM immunoglobulin and 5T2 MM bone marrow cells were directed to the 5T2 MM idiotype. The specificity of the reactions was confirmed by the absence of a specific DTH response after subcutanous immunization with 5T14 MM immunoglobulin or with normal syngeneic bone marrow cells. The specific DTH responses were dose-dependent. A subcutaneous immunization dose of 25 to 40 µg 5T2 MM immunoglobulin resulted in the maximum DTH reaction; higher doses of this protein did not result in an increased swelling. Subcutaneous immunization with more than 1.5×10^6 5T2 MM bone marrow cells resulted in the maximum observed DTH response. However, the exact number of myeloma cells in the bone marrow of a mouse bearing 5T2 MM is variable and depends on the stage of development of the MM. In this study, only donor mice with a high serum level (i.e., around 10 mg/ml or higher) of 5T2 MM immunoglobulin were used. Based on this parameter, the percentage of 5T2 MM cells in the bone marrow was estimated to be 30 to 40 per cent. The time interval between immunization and challenge was also relevant: challenge of the mice seven days after immunization with 5T2 MM immunoglobulin resulted in the highest responses.

The observed primary DTH reactions to 5T2 MM immunoglobulin and 5T2 MM bone marrow cells in C57BL/KaLwRij mice were rather weak as compared with DTH reactions in an allogeneic system. This finding is consistent with that of Abbas and co-workers (18), who also observed a weak specific DTH reaction to M-315 coupled to syngeneic splenocytes in syngeneic BALB/c mice. The weak specific DTH response may be a reflection of the low antigenicity of the 5T2 MM idiotype as has already been observed for the induction of humoral anti-5T2 MM idiotype immunity.

The induction of a DTH response to 5T2 MM bone marrow cells is dependent on the route of immunization. Unlike subcutaneous immunization, intravenous immunization with 5T2 MM bone marrow cells did not result in a specific response after challenge with 5T2 MM bone marrow cells.

In conclusion, both a humoral and a T-cell-dependent immune response

were induced to the 5T2 MM idiotype in a syngeneic system. However, an intense immunization protocol was needed for the induction of the anti-5T2 MM idiotype antibodies. Both, the humoral and the cellular immune mechanisms seem to play a role in the idiotype-specific resistance against the development of 5T2 MM.

CHAPTER IIIB

IMMUNE REGULATION OF 5T2 MULTIPLE MYELOMA

2. Immunological treatment of 5T2 MM

2.1 Introduction

Immunity directed to the idiotype of 5T2 MM was demonstrated to result in inhibition or even prevention of the growth of this malignancy in C57BL/KaLwRij mice (see chapter IIIB.1). However, this immunity had to be induced in the animals before the 5T2 MM cells were injected. Such manipulations are clearly irrelevant for clinical practice. Perhaps of more practical application would be the treatment of B-cell malignancies by passive immunization with anti-idiotype antibodies. Experiments on treatment of malignant B-cell lymphoma and plasmacytoma in animals by passive administration of polyclonal or monoclonal anti-idiotype antibodies have already been described in several reports (19,20,21), and attempts have been made to treat malignant B-cell lymphoma in man by infusion of murine monoclonal anti-idiotype antibodies (22,23). With the currently available hybridoma technology, monoclonal antibodies can be prepared in sufficient quantity in individual cases. In case of MM, however, anti-idiotype antibody treatment is very complex. The high serum content of idiotype-bearing myeloma immunoglobulin would bind the administered anti-idiotype antibodies, and thus prevent them reaching the target cells. Moreover, the formation of immune complexes in the circulation by the interaction between idiotype and anti-idiotype could cause serious damage in several organs, especially in the lungs and kidneys. Treatment of MM with anti-idiotype antibodies could only be considered if the serum concentration of circulating idiotype were very low. This means that the patient should first be treated with cytotoxic drugs to such an extent that only a small number of myeloma cells persists. The extent of this "minimal

residual disease" can be monitored by sensitive immunological techniques such as immunoblotting, ELISA, or RIA, which enable the detection of microgram quantities of myeloma immunoglobulin. A concentration of idiotype-bearing immunoglobulin of only 200 µg per ml of serum has already been suggested to be a contraindication for antibody treatment of B-cell malignancies in man (23).

The 5T2 MM was used as a model to investigate whether treatment of MM with anti-idiotype antibodies is feasible. The aim of the first experiment was to determine the effect of repeated administration of allogeneic monoclonal anti-5T2 MM idiotype antibodies shortly after intravenous injection of the 5T2 MM cells on the development of this neoplasm in the mice. Free serum 5T2 MM idiotype was not detectable at the time of infusion of the antibodies. The second study represents a more practical approach of immunological treatment of MM: the condition of the "minimal residual disease" was mimicked by treatment of the 5T2 MM-bearing mice with an alkylating agent prior to the infusion of the anti-5T2 MM idiotype antibodies.

2.2 Experimental designs

The monoclonal anti-5T2 MM idiotype antibody of BALB/c origin 145-4.1 was employed in both studies (24).

In the first experiment, there were five groups each comprising six three-month-old female C57BL/KaLwRij mice. Each mouse was injected intravenously with a suspension of $1x10^6$ bone marrow cells from two donors bearing an advanced 5T2 MM. Group 1 consisted of mice which were not further treated. Three days after transplantation of the 5T2 MM cells, treatment of the mice of the other four groups with the 145-4.1 antibody was initiated. The antibody was prepared for treatment in vivo as described in chapter II.16. The mice of Group 2 received three intravenous injections with the 145-4.1 antibody at intervals of 48 h. The first injection consisted of 200 µg of the 145-4.1 antibody; at each of the two subsequent injections, 500 µg of the antibody was administered. Group 3 consisted of mice that were treated in the same way as those of Group 2, except that the 145-4.1 antibody was injected intraperitoneally. The mice of Group 4 received the 145-4.1 antibody intravenously according to a different schedule. The first three injections were

given as described for the mice of Group 2 but the mice then received six additional injections, each with 500 µg of the 145-4.1 antibody at varying intervals. Group 5 consisted of mice that were injected intraperitoneally with the anti-5T2 MM idiotype antibody in a similar way as the animals of Group 4. The experimental design is shown in Fig.1.

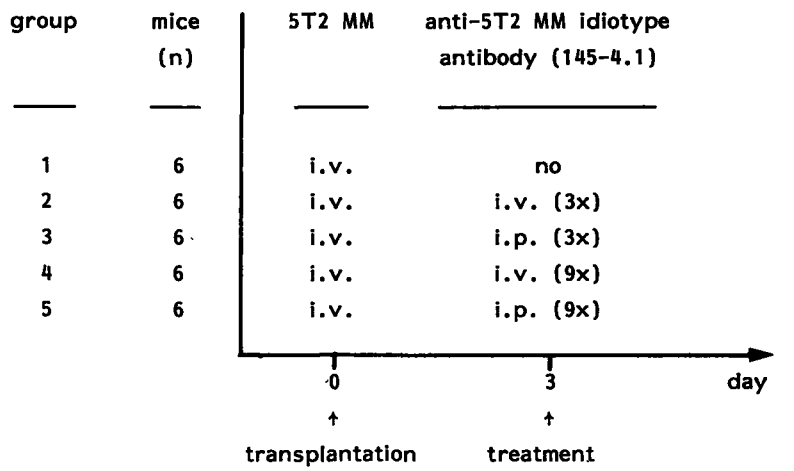

Fig.1: Experimental design of the study on the effect of treatment with the anti-5T2 MM idiotype antibody 145-4.1 on "take" and the development of 5T2 MM. All mice were injected intravenously with $1x10^6$ 5T2 MM bone marrow cells. Three days later, treatment of the mice of Groups 2 to 5 with the 145-4.1 antibody was initiated. The first injection which was given to the mice of Groups 2 and 3 contained 200 µg of the antibody; at the second and third injection, 500 µg of the antibody was administered. The three injections were given intravenously (Group 2) or intraperitoneally (Group 3) at intervals of 48 hours. Next to the first three injections of the 145-4.1 antibody, the mice of Groups 4 (intravenously) and 5 (intraperitoneally) received six additional injections of 500 µg of the antibody. The mice of Group 1 remained untreated. Mice: female C57BL/KaLwRij.
(i.v.: intravenously; i.p.: intraperitoneally).

In the second experiment, three-month-old female C57BL/KaLwRij recipient mice were each injected with $5x10^5$ bone marrow cells from two 5T2 MM-bearing

donor mice. After three months, when all mice had a well developed 5T2 MM, they received an intraperitoneal injection of 500 µg cyclophosphamide (chapter II.15). The sera of all mice were tested for their 5T2 MM immunoglobulin content by competition ELISA (chapter II.5). A serum content of the 5T2 MM protein of less than 0.2 mg/ml was arbitrarily chosen as low enough to initiate anti-idiotype treatment. The anti-idiotype antibodies were prepared for treatment in a way as described for the first experiment. The mice were then separated into five experimental groups. The nine mice of Group 1 did not receive any further treatment. Group 2 comprised seven mice, each of which

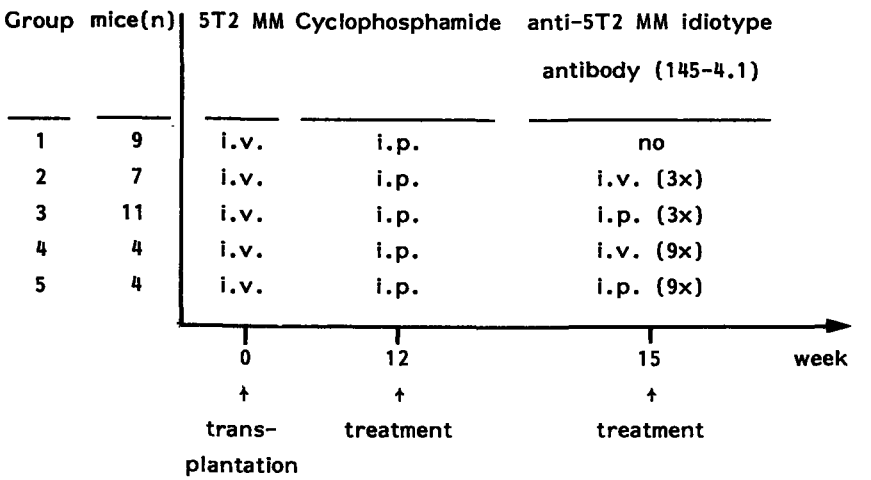

Fig.2: Experimental design of the study on the effect of anti-5T2 MM idiotype antibody (145-4.1) treatment on the growth of 5T2 MM after its reduction by treatment with cyclophosphamide. All mice were injected intravenously with $5x10^5$ 5T2 MM bone marrow cells. Twelve weeks later, when all mice were serologically 5T2 MM idiotype-positive, they received an intraperitoneal injection of 500 µg cyclophosphamide. The mice of the Groups 2 to 5 were treated with the 145-4.1 antibody in the same way as described for the first experiment (see legend Fig.1). This treatment was initiated three weeks after the cyclophosphamide injection, i.e., when the serum concentration of 5T2 MM immunoglobulin was < 200 µg/ml. Mice: female C57BL/KaLwRij.
(i.v.: intravenously; i.p.: intraperitoneally).

received three intravenous injections of the anti-5T2 MM idiotype antibody 145-4.1 at intervals of 48 hours. The eleven mice of Group 3 were treated in the same way, except that the antibody was administered intraperitoneally. Again, the first injection consisted of 200 µg and the other two injections of 500 µg of the 145-4.1 antibody. Group 4 consisted of three mice. After the initial three intravenous injections, they received six additional intravenous injections of 500 µg of the antibody each. The four mice of Group 5 were treated in a similar way, except that the antibodies were administered intraperitoneally (Fig.2). Thereafter, the presence of 5T2 MM paraprotein was regularly tested in the serum of all mice by the aforementioned competition ELISA.

2.3 Results

a) First experiment

Treatment of the mice of Groups 2, 3, 4, and 5 was initiated only three days after transplantation of the 5T2 MM cells, i.e., before serum 5T2 MM immunoglobulin was detectable by a sensitive technique such as competition ELISA (described in chapter II.5). The sensitivity limit of this assay was less then 50 µg/ml. Six weeks after transplantation, all mice of Group 1 showed a clearly detectable amount of 5T2 MM immunoglobulin in their sera. This indicated a growth rate of the transplanted myeloma in these animals which corresponded to the already known growth rate of 5T2 MM. All mice of Group 1 died within twenty weeks of transplantation. In contrast, 5T2 MM was not detectable in the animals of the other four groups at six weeks. At twenty weeks after transplantation, only two out of the six mice of Group 3 (those injected intraperitoneally with 145-4.1 three times) had a detectable amount of 5T2 MM immunoglobulin in their sera. One of the mice of Group 4 (treated intravenously with 145-4.1 for more than three times) died within this period without becoming seropositive for 5T2 MM immunoglobulin. All other mice remained free of a detectable serum 5T2 MM idiotype. These mice showed a prolonged survival without symptoms of a serious disease. Hence, the development of a non-secreting variant of 5T2 MM in these animals could be excluded (Fig.3).

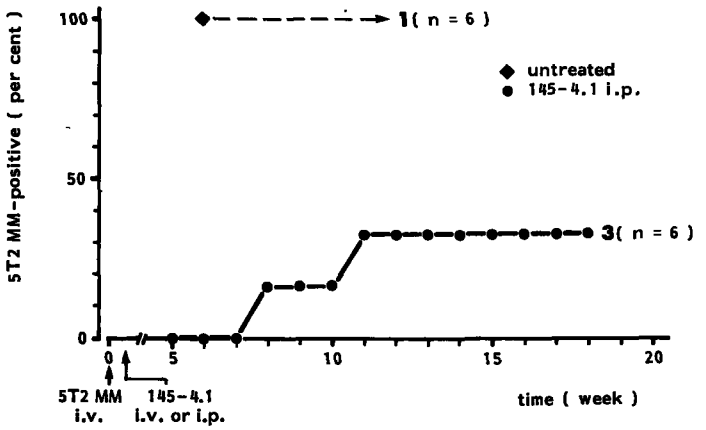

Fig.3: Percentage of mice transplanted with 5T2 MM bone marrow cells in which 5T2 MM was detected after treatment with anti-5T2 MM idiotype antibody. The horizontal axis indicates the time after transplantation of 5T2 MM. Group 1 comprised control mice which were not treated after transplantation. The mice of Groups 2, 4, and 5 did not show "take" of 5T2 MM (not illustrated).
(i.v.: intravenously; i.p.: intraperitoneally).

b) Second experiment

All recipient mice developed 5T2 MM within two to three months of receiving an intravenous injection of 5T2 MM bone marrow cells. Cyclophosphamide treatment resulted in reduction of the growth of the myeloma as was reflected by the decrease in the serum content of 5T2 MM protein. Even at two weeks after administration of this alkylating agent, no paraprotein was detectable in the serum of any of the mice by the double immunodiffusion technique according to Ouchterlony (chapter II.5). However, with the more sensitive competition ELISA, a quantitative determination of the residual serum 5T2 MM protein content could be made. Three weeks after the cyclophosphamide injection, the content of 5T2 MM immunoglobulin in the serum of nearly all mice was below 0.2 mg/ml. Treatment with the 145-4.1 antibody was initiated ten days later. Directly after the initial intravenous injection of the

145-4.1 antibodies, most animals of Groups 2 and 4 looked ill and appeared to suffer respiratory distress. These symptoms were absent after the subsequent injections. The mice of Groups 3 and 5 did not show any symptoms of illness after they had received their first intraperitoneal injection of the antibody. The results of this experiment are shown in Fig.4. All mice that were not

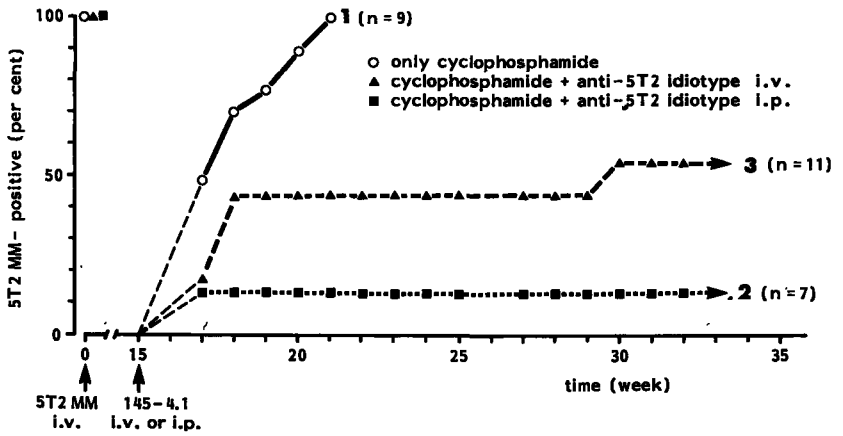

Fig.4: *Percentage of mice transplanted with 5T2 MM bone marrow cells in* which 5T2 MM was detected after treatment with cyclophosphamide (Groups 1 to 3) and anti-5T2 MM idiotype antibody 145-4.1 (Groups 2 and 3). On the horizontal axis, the time after transplantation is indicated. The results of Groups 4 and 5 are not shown. (i.v.: intravenously; i.p.: intraperitoneally).

treated with the 145-4.1 antibody (Group 1) had a clearly detectable amount of serum 5T2 MM paraprotein six weeks after the day the animals of the other groups had received their first anti-idiotype injection. Only one out of the seven mice of Group 2 had a progressive myeloma five weeks after the initiation of the antibody treatment. Prolonged intravenous administration of the 145-4.1 antibodies to the mice of Group 4 did not improve the results. However, this group was very small and, therefore, not suitable to evaluate whether the difference between Groups 2 and 4 was significant. Intraperitoneal

administration of the anti-5T2 MM idiotype antibodies was less effective. Fifteen weeks after the first antibody injection, 5T2 MM was detected in six out of the eleven animals of Group 3. Treatment with more than three intraperitoneal injections was even less successful: three out of the four mice of Group 5 developed 5T2 MM within five weeks of the first injection.

2.4 Discussion

Treatment of C57BL/KaLwRij mice with the monoclonal anti-5T2 MM idiotype antibody 145-4.1 three days after intravenous injection of 5T2 MM cells prevented the development of an overt myeloma in most cases. All mice that had been injected with the same number of 5T2 MM cells, but not treated with the antibodies, showed a typical progressive development of 5T2 MM. It is not known where the 5T2 MM cells were located in the mice at three days after their intravenous injection. The preferential location of 5T2 MM is the bone marrow, but whether this organ is reached selectively after transplantation of the cells remains to be determined. Therefore, the site of interaction between the 5T2 MM cells and the injected 145-4.1 antibodies in the mice of this experiment is not known. The anti-idiotype antibodies can only bind to target cells that express the idiotype on their surface membrane. The predominant cell type in MM has a plasmablastic or plasmacellular phenotype (25). Mature plasma cells contain large amounts of cytoplasmic immunoglobulin, but no surface membrane-bound immunoglobulins. Immature plasma cells or plasma-(immuno-)blasts express both cytoplasmic and surface immunoglobulin (23). The morphology of most of the 5T2 MM cells corresponds to that of plasmablast. The results of this study suggest that the 5T2 MM cells expressing the idiotype on their surface membrane play an essential role in the development of this myeloma after transplantation. The sequence of events which was apparently decisive for the prevention of the development of the myeloma was most likely induced by the binding of the 145-4.1 antibodies to the surface membrane 5T2 MM idiotype-positive cells. Normal regulation of the immune response is mediated by multiple interactions among idiotypes and corresponding anti-idiotype structures (3,26,27). In experimental animal models for plasma cell malignancy, it has been shown that neoplastic B-cells are also

influenced by auto-immune, idiotype-specific signals (4,28,29). The mechanism of idiotype-specific suppression of myeloma growth has not yet been elucidated. Anti-idiotype antibodies elicited by active immunization with a syngeneic plasmacytoma paraprotein were probably not the only mediators of the suppression of the transplanted corresponding plasmacytoma (5,11,16). Idiotype-specific suppressor T-cells are possible candidates for causing the inhibition of plasmacytoma growth. In normal mice, it has been shown that passive administration of anti-idiotype antiserum leads to the induction of idiotype-specific suppressor T-cells (30,31,32). In such idiotypically-suppressed mice, the particular idiotype of an antibody directed to a defined antigen was no longer detectable, indicating that this clone might have been heavily suppressed or even eliminated. By analogy, the 5T2 MM clone might become suppressed by stimulation of pre-existing 5T2 MM idiotype-specific suppressor T-cells after the introduction or generation of sufficient anti-5T2 MM idiotype antibodies. This assumption is in agreement with the observation of other investigators that idiotype-specific T-cells of BALB/c mice which had been immunized with a plasmacytoma idiotype could inhibit the growth of this plasmacytoma in vitro (33). Idiotype-specific suppressor T-cells have been demonstrated to be naturally present in the mouse prior to passive immunization with anti-idiotype antiserum (34). Other possible mechanisms responsible for the suppression of the development of the transplanted 5T2 MM in this study are complement-dependent cytotoxicity and antibody-dependent cytotoxicity by recruitment of killer cells or macrophages. However, the 145-4.1 anti-5T2 MM idiotype antibody belongs to the IgG1-subclass. Murine IgG1 antibodies do not activate complement and are not very efficient in the binding of such effector cells.

The experimental conditions of the first study differed greatly from the situation with which myeloma patients are confronted. Besides the fact that 5T2 MM is a transplanted myeloma and no longer a primary one, the mice were treated shortly after transplantation of the 5T2 MM cells, i.e., at the beginning of its development, whereas in humans whose disease has been diagnosed, the myeloma is in a more or less advanced stage. The aim of this experiment was only to examine whether the growth characteristics of 5T2 MM could be manipulated by passive idiotype-specific immunity. In the second study, experimental conditions were created which resembled the clinical

situation more closely. The mice were allowed to develop an overt myeloma before any treatment was initiated. Subsequently, the circulating 5T2 MM immunoglobulin idiotype was reduced to a very low level in order to make anti-idiotype antibody treatment possible. In the clinic, attempts to reduce the tumour mass in MM patients are frequently made by treatment with alkylating agents, especially with melphalan and cyclophosphamide often in combination with prednisone (35,36,37,38,39). B-cell neoplasms in mice are also sensitive to alkylating agents (19,40). In contrast to man, treatment with these agents may even lead to complete cure. The 5T2 MM was demonstrated to be sensitive to melphalan in combination with prednisone, or to cyclophosphamide. Cyclophosphamide was arbitrarily chosen for treatment of the mice in this study. A single intraperitoneally injected dose of 500 µg (i.e., 25 mg/kg) of this agent appeared to result in marked tumour reduction without achieving a permanent cure of the animals. Although the serum 5T2 MM paraprotein was barely detectable at three weeks following cyclophosphamide treatment, its concentration was nevertheless sufficiently high to cause serious side effects shortly after intravenous administration of the anti-idiotype antibodies, probably as a consequence of immune complex formation. The subsequent anti-idiotype antibody injections did not cause any harm to the mice, indicating that no or very little free 5T2 MM protein persisted in the serum, and that the antibodies could reach the target cells. The results of the second study confirm those of the first one in that passive anti-idiotype treatment of 5T2 MM with the 145-4.1 antibody is possible, if the myeloma mass is very small. The effector mechanism of this mode of treatment has to be established in further studies.

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CHAPTER IVA

BONE DESTRUCTION IN 5T2 MULTIPLE MYELOMA

1. APD treatment: dose determination and toxicity:

1.1 Introduction

Even in those myeloma patients who respond well to chemotherapy, bone destruction remains a major problem (1). In the case of a good response to treatment, the tumour load of a myeloma patient will be reduced to between 1 and 10 per cent of its pretreatment mass (2). Patients with advanced lytic bone lesions (i.e., with stage III MM according to Durie and Salmon) have a measured myeloma cell number of approximately $1\text{-}2\text{x}10^{12}$ before the initiation of treatment (3). Consequently, more than $10\text{x}10^{9}$ myeloma cells still persist in the patient after successful induction of remission. Although tumour reduction of this magnitude is associated with a significant decrease in the serum and/or urine level of myeloma protein and with relief of subjective symptoms, the bone lesions frequently fail to heal.

The skeletal destruction in MM is the result of a pathologically increased osteoclastic bone resorption. Apart from osteolysis and diffuse osteoporosis, this may cause hypercalcaemia in approximately one third of the patients (4).

The bone resorption-inhibiting potency of the bisphosphonates (previously called diphosphonates) may offer new possibilities for treatment of this bone disease. The bisphosphonates are synthetic compounds structurally related to inorganic pyrophosphate, which is characterized by the linkage of two phosphate groups by an oxygen atom (P-O-P). In bisphosphonates, the phosphate groups are linked by a carbon atom. The resulting P-C-P bond is resistant to enzymatic hydrolysis and, therefore, stable in biological systems (reviewed in 5). The dual phosphonate groups have a high affinity for the mineral component of bone, which consists almost entirely of hydroxyapatite

crystals. Bisphosphonates inhibit bone resorption in vivo. This property of the bisphosphonates, in combination with their relatively low toxicity, have led to their clinical application for treatment of diseases with pathological bone resorption such as Paget's disease of bone (6,7,8,9,10,11,12) and cancer-associated hypercalcaemia (13,14,15,16,17). The three most widely studied agents are (1-hydroxyethylidene)-1,1-bisphosphonate (EHDP), (dichloromethylidene)bisphosphonate (Cl_2MDP), and (3-amino-1-hydroxypropylidene)-1,1-bisphosphonate (APD). The modes of action of the bisphosphonates on the inhibition of bone resorption in vivo are not precisely known. The physicochemical stabilization of bone minerals after adsorption of bisphosphonate has been proposed as a possible working mechanism. However, differences have been observed between the potency of the bisphosphonates to inhibit crystal dissolution in vitro and their suppressive activity on bone resorption in vivo (reviewed in 5). Apparently, another mode of action, for example on bone resorbing cells, is responsible in vivo. There is evidence that Cl_2MDP inhibits bone resorption through a cytotoxic effect (18,19,20). The inhibition of osteoclastic bone resorption by APD has recently been explained by interference of adsorbed bisphosphonate with the recognition of bone mineral by osteoclasts (21). EHDP causes mineralization defects in addition to its effect on resorption. This impairment of mineralization causes osteomalacia at doses which are needed for effective inhibition of bone resorption (6,10,22,23). This limits its use in treatment of disorders characterized by increased bone resorption. Cl_2MDP is slightly more potent than EHDP with respect to inhibition of bone resorption, and its application in therapeutic doses is not associated with osteomalacia (7,10). APD is a much more potent inhibitor of bone resorption than the other two bisphosphonates (24). It does not impair mineralization nor is it toxic for bone cells at doses effective for resorption. The inhibitory effect of APD on bone resorption has a very rapid onset, and can be monitored by the urinary hydroxyproline excretion. A significant reduction in the excretion of urinary hydroxyproline in rats was observed within a week of the initiation of treatment (24). In half of the patients suffering from Paget's disease who were treated with APD, the urinary hydroxyproline values also became normal within a week (10). In contrast, similar effects were observed only after three to six months during treatment with EHDP or Cl_2MDP, which could be explained by their lower potency (10). APD has proved to be the

most suitable bisphosphonate for the treatment of Paget's disease (9,10,11,12). APD also inhibits tumour-induced osteolysis in man (25), and is very potent in the treatment of severe tumour hypercalcaemia (14,15,16,17).

Since APD showed a very active inhibition of osteoclastic bone resorption, it was expected that this compound could be used for the prevention and/or treatment of the skeletal destruction in MM. Such an application of APD would require a long period of treatment, often in combination with cytotoxic chemotherapy. Prolonged treatment with APD is generally well tolerated, but whether this will also be the case in patients with advanced MM has not yet been established. Side effects of APD exist. These appeared not to be harmful for patients with a non-malignant disorder, but could conceivably be toxic for severely weakened myeloma patients. For example, in patients with Paget's disease of bone or with tumour-induced hypercalcaemia who have been treated with APD, a transient decrease of approximately 50 per cent has been observed in the peripheral lymphocyte count. This decrease begins between 12 and 24 hours after initiation of the treatment, and lasts for about five days (10,17). The aetiology of the transient lymphocytopenia is unknown. Anti-cancer drugs for the treatment of MM such as the alkylating agents suppress haematopoiesis in the bone marrow. This might be not without risk in combination with the lymphocytopenic or other cellular effects of APD. Therefore, a study on the efficacy of APD to prevent, retard, or even repair the bone destruction in MM should be preceeded by a study in an animal model of MM. Such a study could preclude the possibility of specific toxic side effects of this agent in this disease.

1.2 Experimental design and results

The mouse 5T2 MM was chosen as the preclinical model. Previous to the beginning of the treatment of the myeloma-bearing mice, a dose-finding study was performed with three different quantities of APD in order to test their effect and toxicity. Three groups were formed, each consisting of four three-month-old male C57BL/KaLwRij mice. The animals were placed in metabolic cages, and received standard food pellets containing APD in amounts of 500, 2,000 or 10,000 ppm during their entire life span. The mice were

regularly examined on potential side effects. The three groups were small, because the only aim was to determine the suitable dose. This study, therefore, pertains to tolerability; the toxicity of APD has been further investigated in the treatment studies reported in chapter IVB.1 and 2.

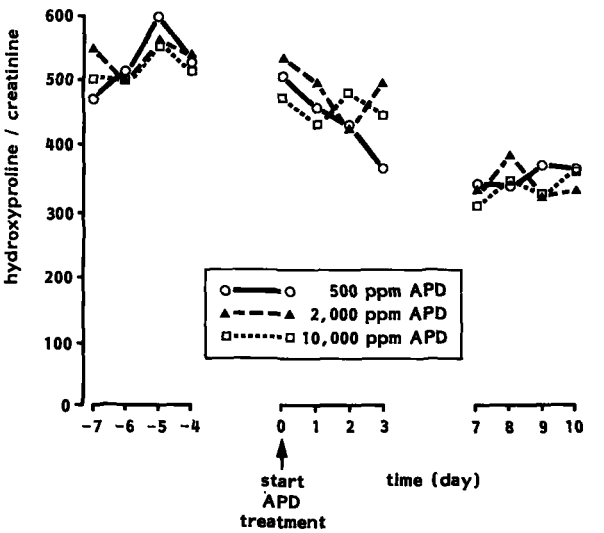

Fig.1: Hydroxyproline/creatinine ratio in the urine of the mice of the three groups treated with 500, 2,000, or 10,000 ppm of APD. The days of collection of the urine are indicated on the abcissa; the mean values of the hydroxyproline/creatinine ratio of the four mice of each group are shown on the ordinate.

a) Effect of APD on the urine hydroxyproline excretion

Inhibition of bone resorption by APD was assessed from the urine hydroxyproline excretion. The urine hydroxyproline excretion was expressed as the hydroxyproline/creatinine ratio (see chapter II.18) (Fig.1). For this purpose, urine samples of the mice of the three groups were collected each 24 h one week before, and two weeks after the initiation of the treatment with 500, 2,000, or 10,000 ppm of APD.

The treatment with the three different doses of APD resulted in a decrease in the mean hydroxyproline/creatinine ratio in the 24 h urine of the four mice of each group. This effect was observed already in the first week of administration of APD. In the second week of APD treatment, the mean hydroxyproline/creatinine ratio did not change further. Furthermore, the three doses of APD used were all equally effective in the inhibition of bone resorption.

b) Effect of APD on the blood cell count

The possible toxicity of APD for the haematopoietic tissue was assessed by determination of the peripheral blood cell count in three out of the four mice of each group during treatment with the different doses of APD. The haematocrit values determined in small blood samples of the individual mice of each group are shown in Table 1.

Mice which received 500 or 2,000 ppm of APD had normal haematocrit values at 8.5 and 18.0 months from the beginning of treatment. After 21.5 months of treatment, a low haematocrit was measured in the blood of one mouse receiving 2,000 ppm of APD. Only one out of the three mice which received 10,000 ppm of APD was still alive after a treatment period of 8.5 months. By that time, its blood had a low haematocrit. The other two mice died six and three weeks earlier, respectively. The mouse that died three weeks before had then a haematocrit of only 9 per cent.

The number of erythrocytes in the blood of the individual mice corresponded with the haematocrit values. This is illustrated in Table 2.

The number of white cells in the blood of the mice which received 500 or 2,000 ppm of APD remained within the normal range after 8.5 months of treatment. A low leucocyte count was observed in the blood of one out of the three mice which had been treated with 500 ppm of APD during a period of 18 months. This animal and one mouse which had received 2,000 ppm of APD showed low leucocyte values after 21.5 months of treatment. One mouse treated with 500 ppm of APD died five weeks previously. The only one of the mice receiving 10,000 ppm of APD that was still alive after nine months of treatment had a blood-leucocyte number comparable to those of the mice treated with 500 or 2,000 ppm of APD (Table 3).

Table 1 HAEMATOCRIT IN THE BLOOD OF THE MICE TREATED WITH 500, 2,000, OR 10,000 PPM OF APD.

Mouse (no.)	APD (ppm)	Haematocrit (%)				
		8.5	9	18	21.5	(mo)*
2	500	47	n.d.	41	44	
3		47	n.d.	42	†	
4		47	n.d.	42	46	
6	2,000	47	n.d.	44	27	
7		40	n.d.	43	39	
8		48	n.d.	35	46	
10	10,000	26	15	†		
11		†***	†	†		
12		9**	†	†		

* months of APD treatment.

** mouse no. 12 died already at 8 months after the initiation of the APD treatment.

*** mouse no. 11 was found dead after 7 months; no blood was available for the different tests.

(n.d.: not done; † : dead).

Table 2 NUMBER OF ERYTHROCYTES IN THE BLOOD OF THE MICE TREATED WITH 500, 2,000 OR 10,000 PPM OF APD.

Mouse (no.)	APD (ppm)	Number of erythrocytes ($\times 10^4/mm^3$ blood)				
		8.5	9	18	21.5	(mo)*
2	500	942	n.d.	799	907	
3		902	n.d.	858	†	
4		938	n.d.	862	902	
6	2,000	932	n.d.	877	486	
7		961	n.d.	869	797	
8		1081	n.d.	732	901	
10	10,000	n.d.	455	†		
11		†***	†	†		
12		†**	†	†		
controls (n=3, mean)	-	931	n.d.	832	897	

* months of APD treatment.

** mouse no. 12 died at 8 months after the initiation of the APD treatment.

*** mouse no. 11 was found dead after 7 months; no blood was available for the different tests.

The mean number of erythrocytes of three untreated age-matched mice is indicated at 8.5, 18, and 21.5 months.

(n.d.: not done; † : dead).

Table 3 NUMBER OF LEUCOCYTES IN THE BLOOD OF THE MICE TREATED WITH 500, 2,000 OR 10,000 PPM OF APD.

Mouse (no.)	APD (ppm)	Number of leucocytes ($\times 10^3$/mm^3 blood)				
		8.5	9	18	21.5	(mo)*
2	500	8.7	n.d.	6.9	7.12	
3		8.5	n.d.	9.2	†	
4		7.0	n.d.	4.3	5.16	
6	2.000	8.4	n.d.	6.6	8.46	
7		8.5	n.d.	12.2	7.22	
8		8.9	n.d.	8.4	5.56	
10	10.000	n.d.	6.5	†		
11		†***	†	†		
12		1.3**	†	†		
controls (mean)	-	n.d.	n.d.	10.1 (n=3)	12.0 (n=2) (9.7-14.3)	

* months of APD treatment.

** mouse no. 12 died already at 8 months after the initiation of APD treatment.

*** mouse no. 11 was found dead after 7 months; no blood was available for the different tests.

The mean number of leucocytes of three and two untreated age-matched mice is indicated at 18 and 21.5 months, respectively.

(n.d.: not done; † : dead).

Table 4 NUMBER OF PLATELETS IN THE BLOOD OF THE MICE TREATED WITH 500, 2,000 OR 10,000 PPM OF APD

Mouse (no.)	APD (ppm)	Number of thrombocytes ($\times 10^3/mm^3$ blood)				
		8.5	9	18	21.5	(mo)*
2	500	372	n.d.	848	n.d.	
3		382	n.d.	892	†	
4		565	n.d.	478	n.d.	
6	2,000	418	n.d.	716	n.d.	
7		498	n.d.	1080	n.d.	
8		510	n.d.	948	n.d.	
10	10,000	n.d.	4	†		
11		†***	†	†		
12		†**	†	†		
controls (n=3; mean)	-	154	n.d.	806	n.d.	

* months of APD treatment.

** mouse no. 12 died at 8 months after the initiation of APD treatment.

*** mouse no. 11 was found dead after 7 months; no blood was available for the different tests.

The mean number of platelets of three untreated age-matched mice is indicated at 8.5 and 18 months. (n.d.: not done; † : dead)

The platelet counts determined in the blood of the mice which received 500 and 2,000 ppm of APD showed some variation. However, the mean values of these two groups were in the same range after 8.5 months of treatment. Eighteen months from the beginning of treatment, a somewhat lower platelet count was measured in the blood of one of the mice which had received 500 ppm of APD. The large difference between the values determined after 8.5 months and 18 months is not clear. The only mouse treated with 10,000 ppm of APD which was available for determination of its platelet count showed a very low value after nine months of treatment, shortly before its death (Table 4).

c) Effect of APD treatment on survival

Three out of the four mice of each group were allowed to complete their life span. Their survival time values are shown in Table 5. The mean survival time

Table 5 SURVIVAL DATA OF C57BL/KaLwRij MICE TREATED WITH 500, 2,000, OR 10,000 PPM OF APD.

APD		APD		APD	
Mouse (no.)	500 ppm	Mouse (no.)	2,000 ppm	Mouse (no.)	10,000 ppm
2	30.5 months	6	25.5 months	10	11 months
3	22.7	7	26	11	8.5
4	35	8	25.7	12	9.3
Mean	29.4		25.7		9.6

of the mice treated with 500 or 2,000 ppm of APD was near to or exceeded the median survival time of this strain of mice, which had been determined to be 24 months (26). Mice treated with 10,000 ppm of APD all died within one year.

d) Histological findings

The mice treated with 10,000 ppm of APD were killed when they were moribund. They were prepared for histological examination as described in chapter II.10. Striking abnormalities were observerd in the haematopoietic tissues and the liver. The bone marrow showed signs of focal hypoplasia and necrosis; the lymphoid tissues were atrophic, and showed some areas with lysis of nuclei. A very low degree of erythropoiesis was observed in the spleen of one mouse. Extensive multifunctional and pericentral necrosis was present in the liver.

1.3 Discussion

The aim of this study was to establish which dose of orally administered APD inhibited bone resorption effectively, while being well tolerated by normal C57BL/KaLwRij mice.

The quantity of hydroxyproline excreted in the urine reflects the rate of bone collagen metabolism (27). The hydroxyproline levels are elevated in the urine of patients with disorders characterized by an increased bone resorption such as Paget's disease of bone and MM (28,29). Inhibition of bone resorption leads to a decrease in the urinary excretion of hydroxyproline, as already observed in experimental animals and in patients treated with bisphosphonates. The efficacy of the three different doses of APD used in this study was judged from changes in the rate of the excretion of hydroxyproline in the urine of the treated mice. Each 24 hours, the urine of the mice was collected. However, it was hardly possible to collect all the urine without losing some of it. For that reason, the 24 h urinary hydroxyproline content was expressed as the hydroxyproline/creatinine ratio (see chapter II.18). A decrease of the mean urinary hydroxyproline excretion of about 30 per cent was observed in each group of mice during the first week of APD treatment. No further

changes in the mean excretion levels were measured in the second week. This observation is in agreement with the studies of Reitsma and co-workers on the effect of APD on bone and mineral metabolism in rats (24). The three doses of APD used in this study did not show clear-cut differences in their effect on the urinary hydroxyproline excretion; they were apparently equally active in inhibition of bone resorption. However, the highest dose of APD seemed to be very toxic. All mice that received 10,000 ppm of APD died within nine months, and in those animals available for determination of the blood cell count, a serious anaemia and thrombocytopenia had developed. One mouse also had a very low leucocyte count. Moreover, histopathological examination revealed very serious abnormalities, which in part corresponded with the presence of a serious anaemia. No abnormalities were found in the blood of the mice of the other two groups up to eighteen months from the beginning of the treatment. Those animals had a life span within the normal range as previously estimated for this mouse strain (26).

The exact amount of APD consumed by the animals can only be estimated. The mean daily food intake of the mice was determined to be approximately 5 grams. This means that the mice which received 500, 2,000, and 10,000 ppm of APD had a daily intake of, respectively, 2.5, 10, and 50 mg of APD. A normal C57BL/KaLwRij mouse weighs about 20 g. The daily consumption of APD per kg by the individual mice of each group would then be 125, 500, and 2,500 mg, respectively. APD has a molecular weight of 279 (10). This indicates that the mice consumed 0.45, 1.8, or 9.0 mmol APD per kg each day. The resorption in the gut of APD is low. In studies in the rat, it has been determined to be about 0.2 per cent (30). Starting from the assumption that mice have an equal resorption capacity, it can be calculated that their effective daily APD intake has been, respectively, 0.9, 3.6, and 18 µmol/kg. In rats, inhibition of osteoclastic bone resorption starts with a dose of APD of 0.016 µmol/kg/day, and is maximal with 16 µmol APD per kg/day. Disturbances of mineralization and of longitudinal growth of bone occurred if the rats received an effective daily dose exceeding 40 µmol/kg/day (24). This means that the doses of APD used in this study were in the range that appeared to be effective on bone resorption in rats without causing osteomalacia or growth reduction. However, a dose of 10,000 ppm of APD appeared to be too toxic for long-term use, and is not of practical value for studies on the influence of

APD on the bone destruction in MM. Long-term use of doses of 500 or 2,000 ppm of APD was well tolerated by the mice. Of these, an oral dose of 2,000 ppm was tentatively chosen in the first study on the treatment of the mouse 5T2 MM with APD.

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CHAPTER IVB

TREATMENT OF THE BONE LESIONS IN 5T2 MULTIPLE MYELOMA WITH THE BISPHOSPHONATE APD

1. Effects of short-term treatment with the bisphosphanate APD on 5T2 MM

1.1 Introduction and experimental design

The potentially beneficial effect of APD treatment on bone destruction in mice with 5T2 MM and the possible direct toxicity of APD for the myeloma cells were investigated in a short-term study. In the previous study (see chapter IVA), the dose of 10,000 ppm of APD was found to be toxic for haematopoietic and lymphoid tissues. APD in a dose of 2,000 ppm was not toxic for normal lymphoid tissues; however, its toxicity for malignant lymphoid cells, for example, the 5T2 MM cells was not excluded. Therefore, the growth and dissemination pattern of 5T2 MM in non-treated mice was compared with that in APD-treated mice.

Five groups of 3-month-old male C57BL/KaLwRij mice were used in the present study. Group 1 consisted of sixteen normal untreated control mice. All mice of Groups 2 and 3, each consisting of sixteen animals, and of Group 5, which consisted of five animals, received an intravenous injection with $1x10^6$ bone marrow cells from a pool of four 5T2 MM-bearing donor mice. The mice of Group 2 were not treated with APD. Treatment of the mice of Group 3 with APD was initiated when the 5T2 MM was in an advanced stage, i.e., two months after transplantation, while the five mice of Group 5 were treated with APD already from the day of transplantation of 5T2 MM. Group 4 comprised sixteen normal control mice that were treated with APD in parallel with those of Group 3. APD was admixed in the standard food pellets in an amount of 2,000 ppm.

Sixteen weeks after transplantation of the 5T2 MM bone marrow cells, all

mice of the five groups were sacrificed, and subjected to necropsy and complete histological examination (see chapter II.10).

All mice of Groups 2, 3, and 5 were examined for "take" and development of the 5T2 MM by determining the 5T2 MM protein in serum samples obtained at 8, 15, and 16 weeks after transplantation (see chapter II.5). The 5T2 MM protein concentration in these samples was measured in a solid-phase competition RIA using a monoclonal antibody to the 5T2 MM idiotype (145-2.1) coated on the individual wells of 96-well flexible microtitration plates (Falcon, 3911) and purified 5T2 MM protein labelled with iodine-125 (1,2). The first incubation was done with serial dilutions of the different sera, the second with the iodinated 5T2 MM protein. The results were calculated from calibration curves obtained with a purified 5T2 MM protein standard.

The bone destruction in the treated and untreated 5T2 MM-bearing mice was investigated by radiography, microradiography, and histology.

The extent of growth of 5T2 MM in the individual mice was examined histologically in the bone marrow space of the skull, humerus, ribs, thoracic and lumbar vertebral columns, pelvic bones, femur, and tibia, and in extramedullary tissues.

1.2 Results

a) "Take" and development of 5T2 MM in the transplanted mice

The 5T2 MM protein appeared shortly after transplantation in all mice of Groups 2, 3, and 5, indicating a 100 per cent "take" of 5T2 MM. The typical pattern of evolution of this MM was demonstrated by the progressive increase of the serum content of the 5T2 MM protein. In all sixteen mice transplanted with 5T2 MM bone marrow cells and not treated with APD (Group 2), the mean serum content of 5T2 MM protein was 6.3, 19.9, and 22.8 mg/ml after, respectively, 8, 15, and 16 weeks. The mean concentration of 5T2 MM protein in the sera of the APD-treated 5T2 MM-bearing mice (Groups 3 and 5) did not differ significantly from that of the animals of Group 2 (Table 1).

Table 1 CONCENTRATION OF 5T2 MM IMMUNOGLOBULIN IN THE SERUM FROM UNTREATED AND APD-TREATED 5T2 MM-TRANSPLANTED MICE

	Group 2 (5T2 MM)			Group 3 (5T2 MM, APD after "take")			Group 5 (5T2 MM, APD directly)		
weeks after transplantation	8	15	16	8	15	16	8	15	16
number of animals	16	16	15	15	15	16	4	5	5
mean (mg/ml)	6.3	19.9	22.8	6.6	25.1	21.9	7.5	15.5	21.8
range	2.4-9.2	13-35.3	16.9-40.3	2.2-9.4	10.3-53.9	11.3-34.3	5.6-10	10-24.3	10.8-37.7
SEM	0.4	1.4	1.6	0.6	3.2	1.6	0.9	2.5	4.8

Statistical evaluation (Student-T test):
Groups 2 vs 3: $p>0.05$, 2 vs 5: $p>0.05$ (8 weeks); Groups 2 vs 3: $p>0.05$, 2 vs 5: $p>0.05$ (15 weeks).
Groups 2 vs 3: $p>0.05$, 2 vs 5: $p>0.05$ (16 weeks).
SEM: standard error of the mean.

b) Skeletal radiography

The radiographs of the skeleton (see chapter II.9.a) of the untreated MM mice of Group 2 demonstrated typical lesions. The rounded or oval defects with sharply defined margins, which were well-separated or merging together and without reactive marginal sclerosis were most prominent in the metaphyseal parts of the femora and the tibiae, and also in the ribs, scapulae, and mandibles. The pelvis, sternum, and vertebrae showed mainly diffuse osteoporosis. The bodies of the vertebrae were often increased in volume, and the intervertebral space was diminished. In two cases, spiculae were observed on the femora (in the distal metaphyseal part) and tibiae (in the proximal metaphyseal part). Effects on the skull could not be evaluated for technical reasons.

In the two groups of APD-treated mice, i.e., Groups 3 and 5, the changes, as described for the mice of Group 2, were much less pronounced, and severe destruction of the bones or even a periosteal reaction was absent. The differences between the treated (Groups 3 and 5) and untreated mice with 5T2 MM (Group 2), as expressed in arbitrary units (see chapter II.19), were significant ($p<0.001$ using the two-tailed Mann-Whitney U-test) (Fig.1).

c) Microradiography of the femur

Examples of microradiographs of the right femurs from the mice of Groups 1, 2, 3, and 4 are illustrated in Figs.2a-2d. The data from the microradiographic studies are presented in Fig.3 (see chapter II.9.b and II.20).

The effect of the treatment of normal mice with APD (Group 4) on the general morphological features of the distal part of the femur was minor. The only noticeable difference from the untreated control mice (Group 1) was an increase in the surface area of the lateral side of the cortex, whereas the medial side remained unchanged.

The distal part of the femur in the untreated 5T2 MM mice of Group 2 was severely affected. Compared with the normal mice, the amount of trabecular bone in the metaphyseal region appeared to be greatly reduced (Figs.2a and 2b). In addition, the surface area of the cortical bone of the femora from the untreated 5T2 MM mice was smaller, and the endosteal surface showed an

undulated pattern. The presence of changes in the metaphyseal region contrasted with an absence of distinct effects caused by the neoplastic process on the surface area of the calcified structures in the epiphysis (Fig.3).

The loss of bone in the femora from 5T2 MM-bearing mice treated with APD (Group 3) was less than the loss in the untreated mice. This protection by APD was most evident in the trabecular bone of the metaphysis, but did not alter the effect of 5T2 MM on cortical bone. (Figs.2a-2d and 3).

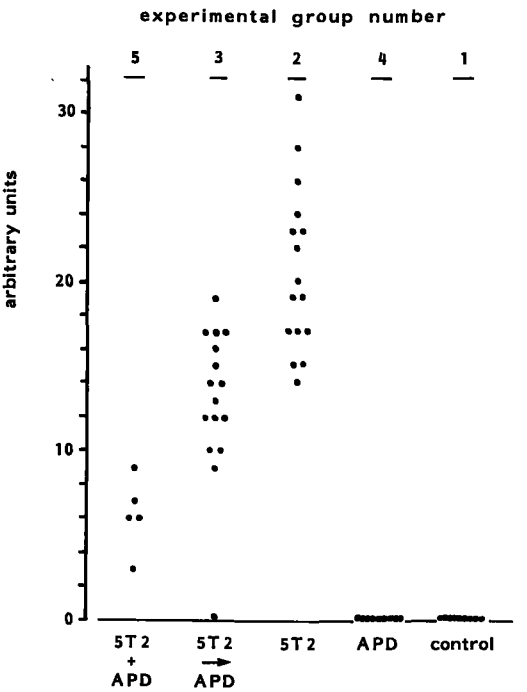
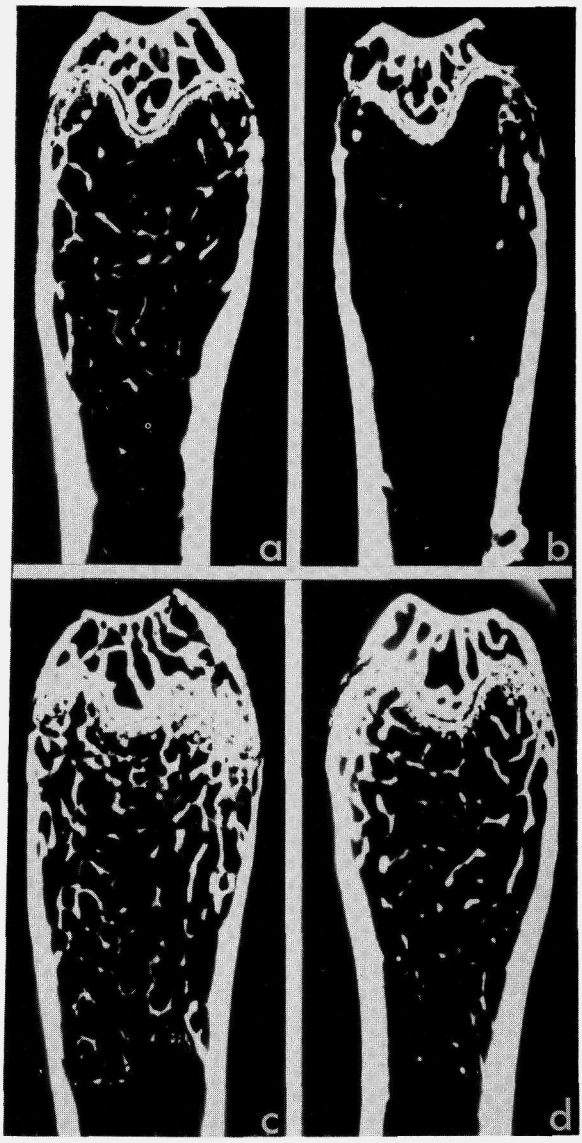


Fig.1: Score of the bone lesions in the radiographs of the skeletons of the untreated (Group 2) and the treated (Groups 3 and 5) mice with 5T2 MM. The presence and extent of the lesions is indicated in arbitrary units on the Y-axis. Differences between Groups 2 and 3: $p<0.001$, Groups 2 and 5: $p<0.001$, Groups 3 and 5: $p<0.005$ (two-tailed Mann-Whitney U-test).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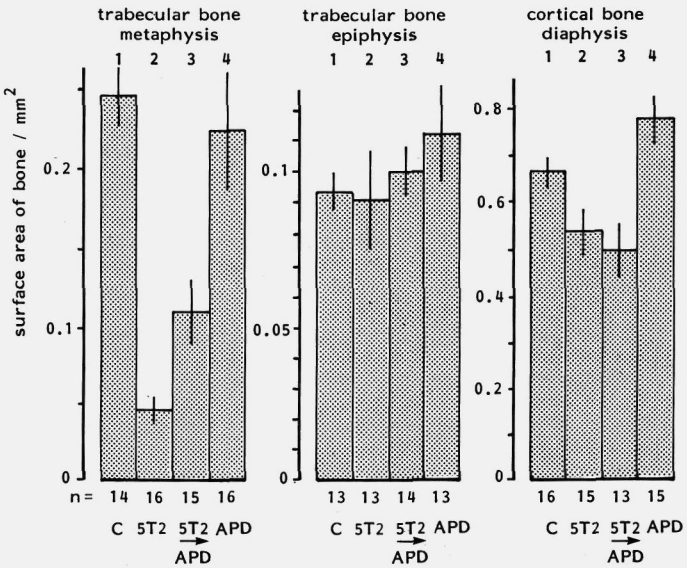


Fig.3: Relation of the surface area of trabecular bone in the metaphysis and the epiphysis, and of cortical bone in the metaphysis in microradiographs of the right femora from the mice of the different experimental groups (1-4). The surface area of trabecular bone in the metaphysis of APD-treated 5T2 MM mice (Group 3) was increased compared with that of untreated 5T2 MM mice (Group 2) ($p<0.01$, two-tailed Mann-Whitney U-test).

Figs.2a-2d: Microradiographs of a frontal section of the right femur characteristic for each of the following experimental groups: (a) untreated normal control mice (Group 1), (b) untreated 5T2 MM mice (Group 2), (c) 5T2 MM mice treated with APD after "take" (Group 3), and (d) normal control mice treated with APD (Group 4). Notice the striking decrease in number and thickness of trabeculae, and the focal cortical damage in the femur of Group 2, and the increase in number of trabeculae in the femora of the APD-treated mice of Groups 3 and 4 (x16).

d) Histology

Tumour extent (Table 2): In Table 2, a combined score is shown for the involvement of various extraskeletal tissues with 5T2 MM cells. The tumour involvement was scored semi-quantitatively on an arbitrary scale running from 0 to 3 (0: no, 1: slight, 2: moderate, and 3: severe involvement) in the spleen, lymph nodes, and other, non-lymphoid organs. The tumour extent in the bone marrow space of various bones, and of the epiphysis and metaphysis of the femora was also scored in a semi-quantitative way in arbitrary units (0: no or dubious, 1: moderate, and 2: severe involvement) (Table 2). The results were again evaluated with the two-tailed Mann-Whitney U-test. The three myeloma groups did not differ significantly in the degree of intra- and extramedullary growth of 5T2 MM cells. The spleen, lymph nodes, and meninges were the most frequently involved extraskeletal tissues. Microscopic examination of the bone marrow of 5T2 MM-bearing mice revealed that normal bone marrow constituents were replaced by myeloma cells. Admixture with remaining normal haematopoietic cells was variable for the different bones, but this was difficult to quantify. The fraction of normal bone marrow cells seemed larger in the epiphysis of the femora and tibiae than in the metaphysis. A similar difference was observed between the thoracic and the lumbar vertrebral column.

Focal bone marrow necrosis with or without myelofibrosis was observed in the ribs, the proximal epiphysis of the tibia, and in the distal epiphysis of the femur (in that order of frequency of involvement) in a minority of the 5T2 MM mice of all the three groups (Group 2: 5/16, Group 3: 6/16, and Group 5: 2/5).

Bone destruction (Table 3): Radiographs indicated that the lesions in the mice with 5T2 MM were most pronounced in the ribs, the distal part of the femur, and the proximal part of the tibia. Two out of these three bone areas, i.e., the sternal end of the ribs and the distal metaphysis of the left femur were selected for semi-quantitative assessment of the degree of bone destruction (see chapter II.21). Statistics were based on the two-tailed Mann-Whitney U-test. The bone lesions appeared to be due to focal destruction of cortical and trabecular bone (see also Figs. 4a-4d and 5a-5d). The focal

Table 2 EFFECT OF APD TREATMENT ON TUMOUR EXTENT IN MICE WITH 5T2 MM

Tissues involved	Experimental group	Mean	SEM	Range	n	Statistical evaluation
Extramedullary:	1	0	0	0	16	
spleen	2	7.00	0.37	5-9	16	2-3: p>0.05
lymph nodes	3	6.38	0.46	3-9	16	2-5: p>0.05
other organs	4	0	0	0	16	3-5: p>0.05
	5	7.20	0.73	5-9	5	
Intramedullary:	1	0	0	0	16	
femur epiphysis	2	1.94	0.06	1-2	16	2-3: p>0.05
	3	2.0	0	2-2	16	2-5: p>0.05
	4	0	0	0	16	3-5: p>0.05
	5	2.0	0	2-2	5	
femur metaphysis	1	0	0	0	16	
	2	2	0	2-2	16	2-3: p>0.05
	3	1.94	0.06	1-2	16	2-5: p>0.05
	4	0	0	0	16	3-5: p>0.05
	5	2	0	2-2	5	
Various bones:						
skull	1	0	0	0	16	
humerus	2	2	0	2-2	16	2-3: p>0.05
ribs	3	1.94	0.06	1-2	16	2-5: p>0.05
vertebral column	4	0	0	0	16	3-5: p>0.05
pelvic bones	5	2	0	0	5	
femur						
tibia						

Statistical evaluation: two-tailed Mann-Whitney U-test.

SEM: standard error of the mean.

Table 3 EFFECTS OF APD TREATMENT ON BONE MORPHOLOGY IN MICE WITH 5T2 MM AND CONTROL MICE

Cortical destruction	Experimental group	Mean	SEM	Range	n	Statistical evaluation
Ribs	1	0	0	0	16	
sternal ends	2	1.6	0.16	0-2	16	1-2: $p<0.001$
	3	1.23	0.17	0-2	13	2-3: $p>0.05$
	4	0	0	0	15	2-5: $p<0.05$
	5	0.8	0.20	0-1	5	3-5: $p>0.05$
Femur	1	0	0	0	15	
distal metaphysis	2	1.6	0.16	0-2	16	1-2: $p<0.001$
	3	1.56	0.16	0-2	16	2-3: $p>0.05$
	4	0	0	0	16	2-5: $p>0.05$
	5	1.2	0.2	1-2	5	3-5: $p>0.05$
Number of trabeculae per arbitrary unit area						
Ribs	1	1.43	0.17	1-3	14	1-2: $p>0.05$
sternal ends	2	1.25	0.20	0-3	16	1-4: $p<0.001$
	3	4.27	0.92	1-15	15	2-3: $p<0.001$
	4	3.3	0.40	1-7	15	2-5: $p<0.001$
	5	6.4	1.12	4-9	5	3-5: $p<0.05$
Femur	1	16.6	0.87	13-25	14	1-2: $p<0.001$
distal metaphysis	2	6.3	1.15	1-20	16	1-4: $p<0.05$
	3	12.7	2.15	2-27	15	2-3: $p<0.05$
	4	18.8	1.02	9-25	16	2-5: $p<0.001$
	5	26.6	2.38	21-34	5	3-5: $p<0.005$

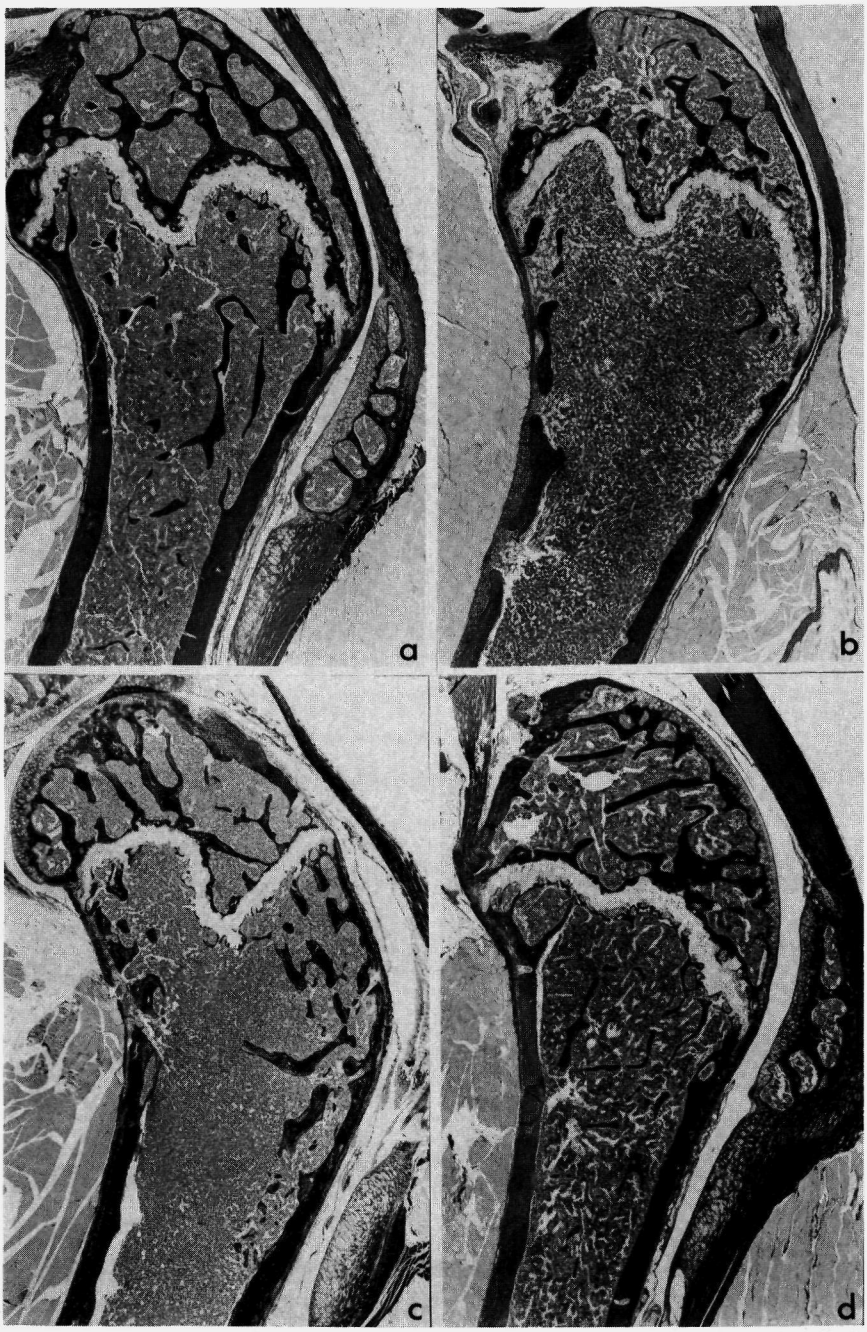
Statistical evaluation: two-tailed Mann-Whitney U-test.

SEM: standard error of the mean.

cortical destruction was most extensive where vessels pass through the cortex, and was most severe in the skull, lumbar vertebrae, rib ends, the proximal metaphysis of the tibia, and the distal metaphysis of the femur. The decrease in trabecular bone could best be quantified in the distal metaphysis of the femur. The number of trabeculae in the ribs of the control mice (Group 1) was too small to allow the recognition of a significant decrease in the mice with 5T2 MM.

The treatment with APD caused a relative increase in the number of trabeculae in the femur and the ribs of the mice with 5T2 MM (Table 3, Figs. 4a-4d and 5a-5d), and seemed to induce a slight increase in the number of trabeculae in the femora and the ribs of normal mice (Group 4). This is most clearly demonstrated in the sternal rib ends (Figs.5a and 5d). In 5T2 MM mice, the increase in the number of trabeculae was greater when they were treated with APD from the day of transplantation (Group 5) than when treated from the moment that the myeloma was in an advanced stage of development (Group 3). An effect of APD treatment on the destruction of cortical bone by 5T2 MM was less obvious. In fact, only in the ribs of the mice of Group 5 was the cortical destruction significantly less than in those of the untreated 5T2 MM mice (Group 2).

Effect of APD treatment on number of osteoclasts (Table 4): Bone destruction in MM is caused by increased osteoclastic resorption (3,4,5). APD inhibits osteoclastic bone resorption (6,7). Therefore, it was investigated whether advanced 5T2 MM or APD treatment was associated with a change in the number of osteoclasts. The osteoclasts in the rib ends were counted and expressed per arbitrary unit of bone marrow space. The mean number of osteoclasts per such arbitrary unit was: Group 1) 3.46±0.53 [standard error of the mean (SEM)], Group 2) 3.31±0.40, Group 3) 3.73±0.60, Group 4) 5.31±0.59, and Group 5) 4.20±0.30. Since the apparent increase in the number of osteoclasts after APD treatment could be a consequence of an increased amount of trabecular tissue within the same area, it was decided to correct for this by dividing the number of osteoclasts, as counted per arbitrary unit in the ribs, by the corresponding number of trabeculae found in the ribs of the same mouse. The resulting values, presented in Table 4 and statistically evaluated by the two-tailed Mann-Whitney U-test, show that APD treatment results in a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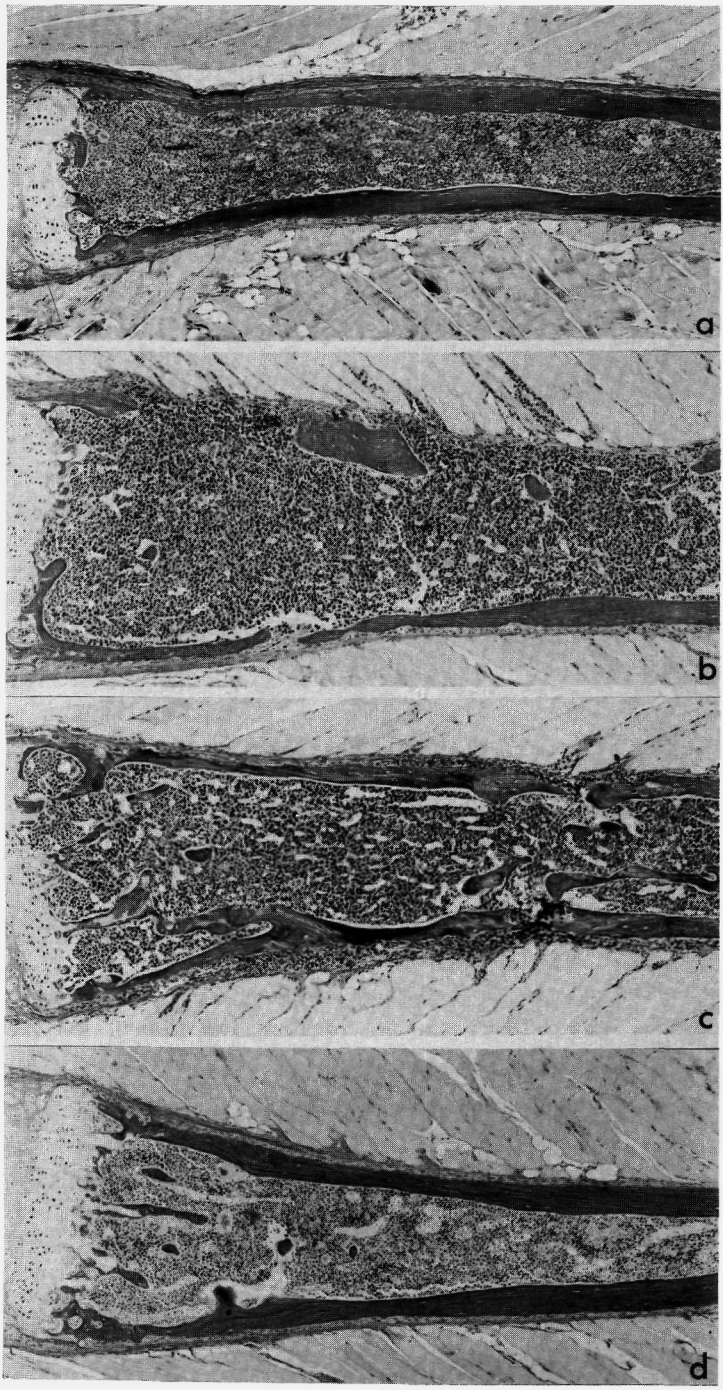
Figs.4a-4d: Histological (sagittal) sections of distal femora of mice of Groups 1-4. Notice (a) the normal appearance of trabeculae and cortex of the femur from a healthy untreated mouse (Group 1) and (d) from an APD-treated control animal (Group 4), also notice (b) the decrease in number of the metaphyseal trabeculae, the multiple areas of cortical thinning and destruction, and the subperiosteal tumour invasion of the femur from an untreated 5T2 MM mouse (Group 2), and (c) the increase in number of the metaphyseal trabeculae cranially in the bone marrow cavity, and focal thinning and destruction of the cortex of the femur from APD-treated myeloma mice (Group 3).
Van Gieson, original magnification x26.

Table 4 EFFECT OF APD TREATMENT ON THE NUMBER OF OSTEOCLASTS IN MICE WITH 5T2 MM AND CONTROL MICE.

Number of osteoclasts* /Number of trabeculae	Experimental group	Mean	SEM	Range	n	Statistical evaluation
bone marrow space	1	2.82	0.52	0.5-7.0	13	1-2:p>0.05
in sternal end of ribs	2	2.55	0.42	1.0-6.0	14	1-4:p>0.05
(arbitrary unit area)	3	1.05	0.23	0.0-3.0	11	2-3:p<0.005
	4	2.00	0.29	0.1-4.0	13	2-5:p<0.005
	5	0.74	0.15	0.2-1.0	5	3-5:p>0.05

* The number of osteoclasts is expressed as the quotient of the absolute number of osteoclasts per arbitrary unit of bone marrow space in the ribs and the corresponding number of trabeculae in the ribs of the same mouse.
Statistical evaluation: two-tailed Mann-Whitney U-test.
SEM: standard error of the mean.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Figs.5a-5d: Histological sections of the sternal ends of the ribs from untreated and APD-treated control and 5T2 MM mice. Notice (c) the presence of many newly formed trabeculae (MM+APD, Group 3), and (d) a slight increase in trabeculae (control+APD, Group 4) as compared with (b) untreated 5T2 MM mice (Group 2) and (a) untreated control mice (Group 1). In addition, severe cortical destruction is seen in (b).
Van Gieson, original magnification x76.

decrease in the number of osteoclasts per unit area of bone surface in 5T2 MM mice as well as in normal mice. The morphological features of the osteoclasts were not different for the various experimental groups, although quantitative parameters such as the number of nuclei per osteoclast were not investigated.

Comparison between histological and radiological results in individual mice: Data from estimates of the surface area of trabeculae of the distal metaphysis in microradiographs of the right femur from individual mice of the different groups were compared with the histologically determined number of trabeculae in the distal metaphysis of the left femur of the same mice. The two estimates of trabecular bone mass were positively correlated, but the individual data scattered widely. Forty-four of the 57 estimations were congruent, 7 were normal by histology and abnormal by microradiography, and 6 were normal by the latter technique and abnormal on histological examination. The combined histological scores for cortical destruction and the number of trabeculae in the distal part of the left femur were compared with the scores on the radiographs of the left femur and the ribs from the same individual mice. The correlation between the histological findings and radiographic data of the femora appeared to be very good; in fact, out of 66 mice available for comparison, 29 were scored as normal and 34 as abnormal by both methods. In three cases, a discrepancy between the two approaches was found. It must be mentioned that the only mouse of Group 3 with a normal appearance on its radiograph and with abnormal histological features was the only one of its group with a moderate tumour involvement of the bone marrow space and a slight involvement of the extramedullary tissues. Comparison of the degree of cortical destruction of the rib ends in 65 mice measured on radiographs with the degree of this destruction determined by histology revealed that 33 and 24

mice were scored, respectively, as normal and abnormal by both methods; in 8 mice, the results showed discrepancies.

1.3 Discussion

A preclinical study in a suitable animal model of MM offers a number of advantages. Within a relatively short period, positive and negative effects of the treatment can be evaluated on a large scale. In addition, if effective, the aspects can be investigated in detail under experimental conditions that would never be possible in the human situation.

Two aspects of the APD treatment of 5T2 MM were evaluated: 1) did the treatment prevent or diminish the bone destruction?, and 2) was there any effect on the growth and distribution of the myeloma? This study established that the treatment with APD protected the mice significantly against the loss of bone. This beneficial effect showed a distinct relationship with the time of initiation and duration of the treatment, as demonstrated by the differences between Groups 3 and 5. The bone destruction in the majority of the treated animals was less severe than observed in the untreated MM mice. It can be expected that this effect of APD may prevent the occurrence of spontaneous fractures or other complications caused by severe bone destruction. The effect of APD was reflected mainly in an increase in the trabecular bone mass, leading to high-normal values of trabecular bone in 5T2 MM mice treated with APD immediately after transplantation (Group 5) and an increased number of trabeculae in APD-treated control mice. The decreased cortical destruction found in APD-treated MM mice may be explained by a decreased endosteal bone resorption. Such an effect of APD (i.e., a decreased endosteal bone resorption) has been observed in normal male but not in normal female rats (8). The bone lesions in MM are due to osteoclasts, which are considered to be activated by an osteoclast-activating factor (OAF) produced by the myeloma cells (3,9,10). The rather crude estimate of the number of osteoclasts per unit of surface area of bone suggests that APD induces a decrease in the number of osteoclasts. This observation should be confirmed by more precise histomorphometric studies. Degenerative changes in osteoclasts caused by the bisphosphonate Cl_2MDP, as described by Rowe and Hausmann (11), were not

observed in the mice in this study.

Treatment with APD had very little, if any, influence on the growth pattern and distribution of 5T2 MM under the experimental conditions of the present study. The tumour load was reflected by the mean serum paraprotein concentration, which was not significantly lowered by the treatment (Groups 3 and 5). The intra- and extramedullary involvement and the histocytological aspects of 5T2 MM in the treated and untreated mice were not substantially different. There might, however, be some indication for a cytotoxic effect of APD treatment on the 5T2 MM cells, which was reflected in a more severe and higher frequency of focal bone marrow necrosis in the treated mice, i.e., 31%, 38%, and 40% in the Groups 2, 3, and 5, respectively . Such necrosis was not observed in the APD-treated control mice. This observation should be confirmed in a different experimental setting, preferably in a follow-up study covering the entire life span of the mice, and by studies in vitro.

Three different methods were used to estimate the effect of APD treatment on bone destruction. The results obtained from skeletal radiographs, microradiographs of the right femur, and from histological study of the ribs and distal metaphysis of the femur all independently led to the conclusion that APD treatment resulted in a decreased bone destruction; even less bone destruction was observed when APD was administered from the time of transplantation of the 5T2 MM bone marrow cells. Comparison of the data obtained by these three methods in individual mice revealed that histological data and radiographic data were generally in agreement. A relatively great discrepancy was found when the number of trabeculae in the distal metaphysis of the femur was compared with the estimate of trabecular bone on the microradiograph of the other femur. This discrepancy could not be explained by a difference between left and right femora, since such a difference was exceptional according to data from radiography. It might be due in part to the fact that the number but not the surface area of the trabeculae was determined by histology, whereas on the microradiographs, the total area of trabecular bone at the cut surface was measured.

For a rapid and relatively easy way of screening for the effect of APD treatment on bone destruction, scoring of the lesions on radiographs of the whole skeleton is clearly superior to both of the above-mentioned methods. The typical effect of APD treatment on the pattern of trabecular bone with a focal

increase in the number of trabeculae was, however, most clearly demonstrated by microradiography and histology. The results of this study indicate that introduction of APD should be considered for the treatment of MM patients in order to reduce the severe complications of their bone disease.

CHAPTER IVB

TREATMENT OF THE BONE LESIONS IN 5T2 MULTIPLE MYELOMA WITH THE BISPHOSPHONATE APD

2. Effects of long-term treatment with the bisphosphonate APD on 5T2 MM

2.1 Introduction and experimental design

When the bisphosphonate APD was orally administered to mice bearing 5T2 MM in an amount of 2,000 ppm, it gave a significant protection against bone destruction. This effect was more prominent if APD treatment was initiated before the 5T2 MM was in an advanced stage. However, no influence of APD was observed on the extent and distribution pattern of 5T2 MM (see chapter IVB.1).

The study in which this was established, was, however, of a short duration, and all mice of the five experimental groups were sacrificed at a predetermined end point of the experiment. Consequently, it was not possible to find out whether APD treatment affected the survival of the mice. Such an effect was not expected to be profound, since APD, in the amount of 2,000 ppm, had no influence on 5T2 MM itself. It may be possible that myeloma mice with a less severe bone disease are in a better general condition. This could be reflected in less morbidity and a prolonged survival of the animals. In addition, further investigation for a possible cytotoxic effect of APD on 5T2 MM cells was indicated from the results of the previous study. Therefore, a separate study was carried out to assess the effects of long-term treatment in which the mice with 5T2 MM were allowed to complete their life span, either dying spontaneously or being killed in extremis.

The experimental design of this study was comparable to that of the previous study. The only differences were that the number of mice in the five groups was larger, that the sex of the animals was female instead of male, and

that the dose of APD was 1,000 ppm instead of 2,000 ppm. At the beginning of the experiment, the mice were three months old. In Fig.1, the experimental design is shown schematically.

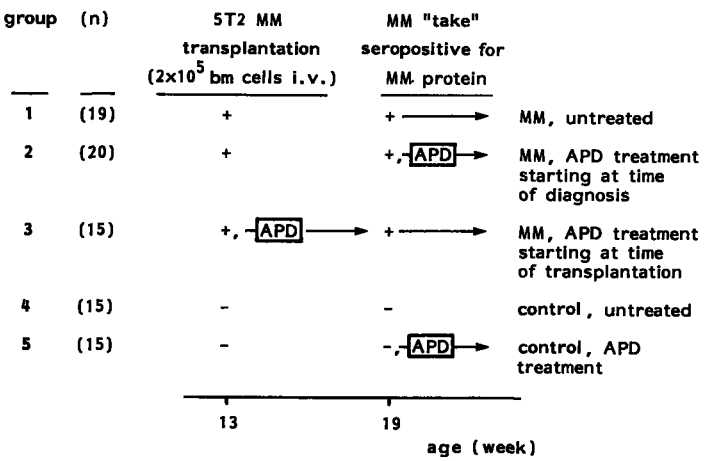

Fig.1: Experimental design. Five groups were included. When they were 13 weeks old, all mice of Groups 1, 2, and 3 received an intravenous injection of 5T2 MM bone marrow cells. APD treatment of the mice of Group 3 was initiated at the time of transplantation. The animals of Group 2 and 5 received APD from the day that the transplanted animals were found seropositive for 5T2 MM immunoglobulin. All mice were allowed to complete their life span.

All mice of the Groups 1, 2, and 3 were injected intravenously with $0.2x10^6$ bone marrow cells from a pool of five 5T2 MM-bearing donor mice. The mice of these groups were regularly examined for "take" and development of 5T2 MM by determinations of the 5T2 MM protein in their sera (chapter II.5). Mice of Group 1 were left untreated. The mice of Group 2 received APD from the time that they all had a well developed myeloma. Treatment of the mice of Group 3 with APD was initiated from the day of transplantation.

The survival times of the mice with 5T2 MM of these three groups were

compared with each other. Dead myeloma mice and MM mice killed when moribund were histologically examined for the extent of the 5T2 MM in intramedullary and extramedullary tissues, and for bone destruction. In addition, a number of different tissues of the 5T2 MM mice was examined for possible side effects of long-term APD treatment.

In the study on an effective and tolerable dose of APD (see chapter IVA), 500 and 2,000 ppm appeared non-toxic for normal C57BL/KaLwRij mice. Since, the experimental groups in that study had been rather small, the present study again contained two control groups (Groups 4 and 5) comprising normal mice which were, respectively, non-treated and treated with APD. A possible toxic effect of APD was investigated by comparison of the survival times of the individual mice of these two groups.

The dose of APD administered to the mice in this study was tentatively determined to be 1,000 ppm. Because 500 and 2,000 ppm of APD were equally effective in inhibition of bone resorption, and 2,000 ppm of APD appeared effective against bone disease due to 5T2 MM, a dose of 1,000 ppm of APD was expected to be sufficient.

2.2 Results

a) Effect of APD treatment on survival of the mice with 5T2 MM

All mice which had been transplanted intravenously with 0.2×10^6 5T2 MM bone marrow cells had a detectable amount of 5T2 MM protein in their sera after about seven weeks.

The survival times of the individual mice of the three 5T2 MM groups (Groups 1, 2, and 3) were expressed in days from transplantation. The differences between the mean and median survival times of the mice of these groups were statistically evaluated with the 2-tailed Mann-Whitney U-test (Fig. 2 and Table 1). The survival times of the mice of Group 3 differed significantly from those of Groups 1 and 2 ($p < 0.001$). No significant difference was observed between the survival times of mice of Groups 1 and 2.

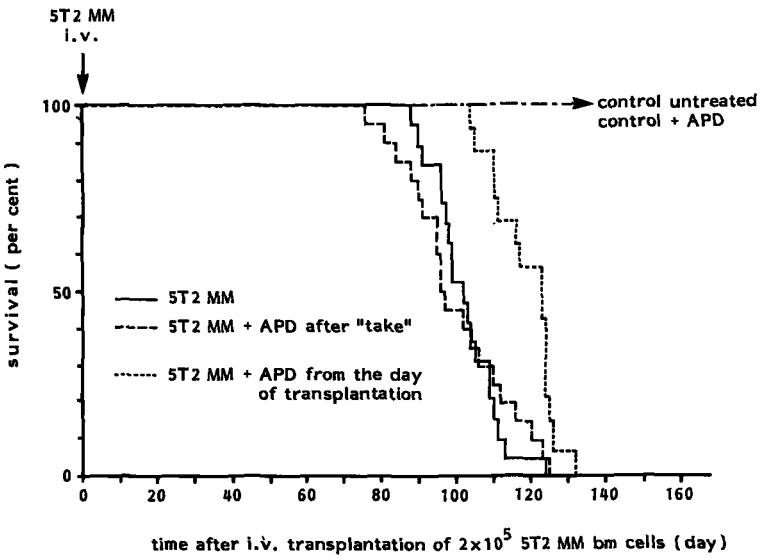

Fig.2: *Survival times of the 5T2 MM mice of the three groups (Groups 1, 2,* and 3) as determined from the day of transplantation. (i.v.: intravenously)

b) Histological examination of the mice with 5T2 MM (Groups 1, 2, and 3)

The various tissues were prepared for histological examination as described in chapter II.10.

The extent of myeloma growth in the individual mice of the three groups was assessed within the bone marrow compartment of different bones and in extramedullary tissues (see chapter II.23). Statistical evaluation of the results was performed with the 2-tailed Mann-Whitney U-test.

Table 1 SURVIVAL VALUES FROM THE DAY OF TRANSPLANTATION OF THE UNTREATED AND APD-TREATED 5T2 MM MICE.

Survival	Experimental group			Statistical evaluation
	1	2	3	
50%	101	96	122	1 vs 2 NS
Range	88-124	76-125	104-132	1 vs 3 $p<0.001$
Mean	102	100	118	2 vs 3 $p<0.001$
SEM	2.06	3.13	2.20	

Statistical evaluation: two-tailed Mann-Whitney U-test.
SEM: standard error of the mean; NS: not significant.

Tumour extent (Table 2): The involvement of the bone marrow cavity of the different bones with myeloma cells was semi-quantitatively scored on an arbitrary scale ranging from 0 to 2 (0: no myeloma cells detected, 1: 0-75 per cent of the total volume contains myeloma cells, 2: 75-100 per cent of the bone marrow space is occupied by MM). At the time the mice with 5T2 MM died, their bone marrow was nearly totally replaced by myeloma cells. No influence of APD treatment was observed on the myeloma growth in the bone marrow compartment.

In the lymph nodes, the myeloma cells were present as small foci or areas in the cortical and medullary regions, or in the marginal sinuses. The degree of myeloma growth in the lymph nodes from each mouse was determined as the quotient of the number of lymph nodes in which myeloma cells were present and the total number of nodes examined. The lymph nodes from the mice which received APD from the day of implantation of 5T2 MM (Group 3) contained significantly more 5T2 MM cells than those from the mice with 5T2 MM which were not treated with APD (Group 1) and those from the mice which had a well developed myeloma when the APD treatment began (Group 2). No significant

difference in tumour involvement of the lymph nodes was observed between the mice of Groups 1 and 2.

In the spleen and the ovaries from the individual mice of Groups 1, 2, and 3, the extent of myeloma growth was graded from 0 (no tumour) to 3 (massive involvement with myeloma). The spleens from the mice not treated with APD (Group 1) were slightly to moderately infiltrated by myeloma cells. The same observation was made in mice of Group 2 (APD treatment from the time the 5T2 MM was well developed). However, on an average, the spleens from the mice which received APD for the longest period (Group 3) contained significantly more myeloma cells.

Of the non-lymphoid extramedullary organs, the ovaries were selected for histological scoring since they were severely affected by myeloma in a large number of the mice of the three groups. The tumour extent in the ovaries had a pattern similar to that observed in the lymph nodes and spleen: APD caused no reduction of the myeloma, and the ovaries from mice that received APD from the day of transplantation (Group 3) contained, in general, more myeloma cells.

A typical feature in a great number of the treated and untreated mice with 5T2 MM was the uninterrupted extension of myeloma from the bone marrow compartment into the muscle tissues directly surrounding the affected bones. In many cases, this growth pattern resulted in the development of a large tumour mass on the exterior surface of the bones (Fig.3). The extent of the myeloma growth in the muscles was graded on an arbitrary scale from 0 to 2. The final score of each group was expressed (in per cent) as the quotient of the total score and the number of mice of which the group consisted. This type of tumour growth was most prominent in the mice treated with APD (Groups 2 and 3). However, the differences between the three groups were statistically not significant.

Bone destruction (Table 2): In this study, the bone destruction was semi-quantitatively scored in the cortex of various bones (see chapter II.23). The total score was derived by adding the scores grading from 0 to 2 that were obtained in individual bones (0: no destruction, 1: moderate destruction, 2: severe destruction). Cortical destruction was severe and most prominent in the bones from the mice which had not been treated with APD (Group 1).

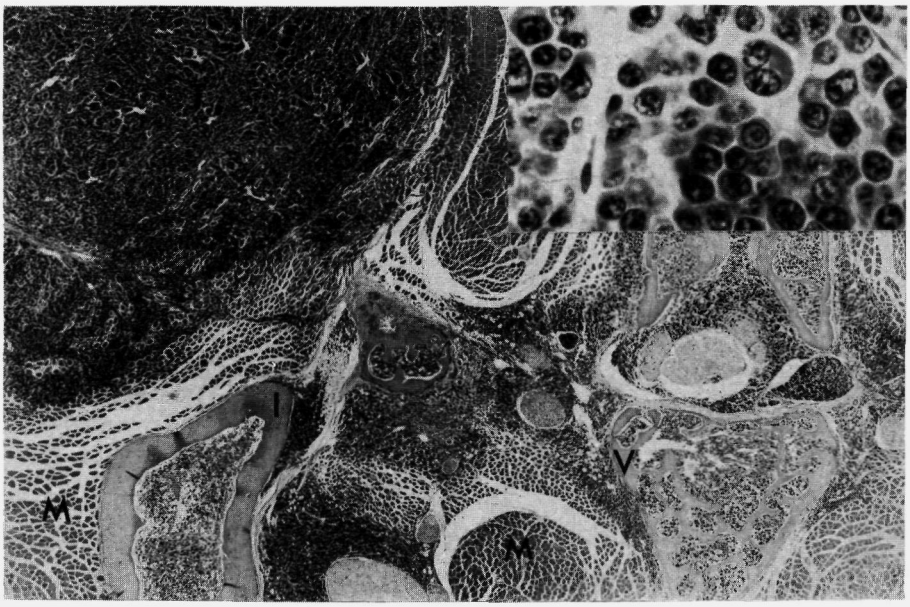

Fig.3: Tumorous growth of 5T2 MM cells infiltrating into muscle tissue adjacent to a vertebra which is affected by the myelomatous growth of 5T2 MM. HPS, 32x.
Inset: higher magnification of myeloma cells in centre of tumour mass. HPS, 700x.
(T: solid 5T2 tumour; M: muscle tissue; V: vertebral bone; I: iliac bone).

Treatment with APD resulted in a significant decrease in the destruction of the bones, as was demonstrated in the mean scores of the mice of Groups 2 and 3. At the time of death, there were no clear-cut differences in bone destruction between the Groups 2 and 3.

Late side effects of APD (Table 2): Histological examination of the various tissues of the mice with 5T2 MM that had been treated with 1,000 ppm of APD did not reveal morphological changes which could be explained by possible side effects of APD.

Table 2 TUMOUR EXTENT, BONE DESTRUCTION, AND LATE EFFECTS IN UNTREATED AND APD-TREATED 5T2 MM MICE AS DETERMINED BY HISTOLOGY.

	Experimental group			Statistical evaluation	
	1	2	3		
Tumour extent					
Intramedullary (0-2)*	2.0	2.0	1.9	1 vs 2: p>0.05	NS
				1 vs 3: p>0.05	NS
				2 vs 3: p>0.05	NS
Extramedullary:					
extension into muscle (%)	33	55	57	1 vs 2: p>0.05	NS
				1 vs 3: p>0.05	NS
				2 vs 3: p>0.05	NS
spleen (0-3)*	0.9	1.0	1.6	1 vs 2: p>0.05	NS
				1 vs 3: p<0.05	
				2 vs 3: p<0.05	
lymph nodes (%)	19	18	29	1 vs 2: p>0.05	NS
				1 vs 3: p>0.05	NS
				2 vs 3: p<0.05	
ovary (0-3)*	1.6	1.4	2.4	1 vs 2: p>0.05	NS
				1 vs 3: p<0.005	
				2 vs 3: p<0.005	
Cortical destruction (0-2*, sum of 8 bones)	7.2	4.2	4.6	1 vs 2: p<0.005	
				1 vs 3: p<0.005	
				2 vs 3: p>0.05	NS
Late effects of APD	none	none	none		

Statistical evaluation: two-tailed Mann-Whitney U-test.

% : per cent of total examined.

* : mean of score in arbitrary units.

NS: non significant.

c) Effect of APD on survival of normal mice

The possibility that treatment with 1,000 ppm of APD during a long period is toxic for mice was investigated by comparison of the survival values of normal C57BL/KaLwRij mice which received APD from the time they were 4.5 months old with those of untreated syngeneic sex- and age-matched mice. Animals that were treated with APD had a slightly shorter mean survival time: 21±1.30 months versus 23.9±1.47 months (mean ± SEM). This difference was, however, not significant (Table 3). The results were statistically evaluated using the Mann-Whitney U-test.

Table 3 SURVIVAL TIMES OF NORMAL FEMALE C57BL/KaLwRij MICE OF GROUP 4 (UNTREATED CONTROLS) AND GROUP 5 (CONTROLS TREATED WITH APD).

	Experimental group		
Survival (mo)*	4	5	Statistical evaluation
50%	23.8	22	
Range	10.8-31,8	11.25-26.5	4 vs 5: P>0.05, NS
Mean	23.9	21.	
SEM	1.47	1.30	

*mo: months.
Statistical evaluation: two-tailed Mann-Whitney U-test.
NS: not significant.
SEM: standard error of the mean.

2.3 Discussion

The bisphosphonate APD did not significantly prolong the median and mean survival of mice bearing 5T2 MM, unless the treatment had been initiated very early in the course of the disease. The mechanism underlying the beneficial effect of prolonged APD treatment on survival is not known. The studies on the influence of APD treatment on the extent of 5T2 MM clearly showed that no inhibition of the growth rate of this neoplasm was obtained. Moreover, the mice of Group 3, i.e., those which had been treated with APD for the longest period, had in general a larger myeloma mass in their different extramedullary tissues than the animals of the other two groups. It might be concluded from that observation that the prolonged life span of the mice of Group 3 allowed a more expansive growth of 5T2 MM. The prolonged survival could perhaps be due to an improvement of the general condition of the mice leading to a later onset of manifest morbidity, which might in turn be a consequence of the decreased bone destruction. In the study on the influence of short-term APD treatment on bone destruction in mice bearing 5T2 MM (see chapter IVB.1), it was demonstrated that the animals treated with APD from the day of transplantation were protected against a loss of bone to a higher degree than those which received APD later in the course of their disease. In contrast, in the present study, the both groups of APD-treated 5T2 MM mice showed similar degrees of bone destruction. Although these results are different, they are not necessarily in conflict with each other. The longer life span allowed a continuous progressive growth of 5T2 MM, as was reflected by the higher tumour extent in the mice of Group 3. This might have led to a prolonged progression of bone destruction. The dose of APD administered to the mice remained unchanged. Therefore, it is not unlikely that an increase in bone destruction occurred during the last few weeks of their life, while the extra-skeletal tumour mass expanded. A multiple cross-sectional study, for example a serial killing experiment, on the effect of APD on the skeleton of myeloma mice should be performed in order to confirm this explanation.

The orally administered dose of 1,000 ppm of APD used in this study could be considered as effective as the 2,000 ppm of the former, short-term, experiment. It should, therefore, be possible to treat mice with oral doses of 1,000 ppm of APD or less in future studies.

The mean life span of both groups of mice that were not transplanted with 5T2 MM (Groups 4 and 5) was less than 24 months. An explanation for this has not been found. APD treatment did not result in a decrease in the life span of the mice; in contrast, the correspondence of mean and median survival between Groups 4 and 5 is rather good. Moreover, no toxic side effects due to APD were observed by histological examination of the different tissues of the mice with 5T2 MM.

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CHAPTER V

GENERAL DISCUSSION

An effective treatment which will result in cure of MM is not yet available. Since the introduction of relatively effective alkylating agents such as melphalan and cyclophosphamide in the 1960's, the possibilities for treatment have not significantly improved. Only some serious complications of MM have since been treated more effectively. For example, serious recurrent infections will often come under control with recently developed, potent antibiotics; life-threatening hypercalcaemia can be treated with saline infusions in combination with bisphosphonates (1,2,3), and formerly intractable pain is often alleviated with modern analgesics. Although the quality of life of many patients may have improved, and survival times somewhat prolonged, the ultimate prognosis remains poor. In order to improve this situation, new ways should be developed in the treatment of MM. It is, therefore, necessary to gain more fundamental insight into the biology of this disease.

The present study has followed two main paths: 1) the selective elimination of the neoplastic clone by immunological means, and 2) the prevention and/or the treatment of the osteolytic bone disease. The various experiments were performed in an animal model. In man, the possibilities for an experimental approach of MM are extremely limited. Studies on new methods of treatment with biological response modifiers or with immune regulation can not be performed in human beings before the efficacy has been determined under appropriate experimental conditions. Even more restrictions apply to cell-biological studies in MM. Human myeloma cells can only be studied in vitro, or after heterotransplantation into nude mice or rats. In vitro systems are, however, artificial and cannot be regarded as a reflection of the situation in vivo. Moreover, the establishment of human myeloma cell lines in vitro has proved extremely difficult. Only a few have been maintained in culture. The establishment of xenografts of myeloma cells in nude mice or rats has even been less successful.

An animal model of MM is a prerequisite for fundamental research on this disease. This study was performed in a new animal model of MM: the mouse 5T2 MM. The 5T2 MM is the first in a series of mouse myelomas (the so-called 5T-MM's), which originated spontaneously in aging C57BL/KaLwRij mice. In chapter IIIA, the different biological characteristics of this mouse myeloma have been described in detail. The 5T2 MM is a unique animal model. It is a genuine MM, resembling the human disease very closely. Since it is transplantable, it offers a nearly unlimited supply of MM material for experimental studies on the biology and the treatment of this malignancy. However, MM in man is always primary. In this respect, it differs fundamentally from the 5T2 MM, which cannot therefore be employed for studies regarding aetiology, site of origin, and pathogenesis of MM. These can only be performed in the primary form.

A new model is only of value if it has advantages compared to already existing models. The transplantable plasma cell tumour models used so far, did not originate as MM, but as a local plasmacytoma. One of these, the Louvain rat immunocytoma, originated spontaneously (4,5); the frequently exploited BALB/c mouse plasmacytomas, however, arose only after an appropriate induction procedure (6,7 and reviewed in 8). These plasmacytoma models have proved to be useful within limits but the 5T2 MM should now be regarded as the model of choice.

A typical feature of B-cell neoplasm in man is its clone-specific determinant: the idiotype of the immunoglobulin which is produced by the tumour cells (reviewed in 9). With immunological methods, one can employ the idiotype for selective detection of the malignant B-cells. Moreover in MM, the presence and the growth of the malignant process can be monitored by measuring the idiotype-bearing immunoglobulin content in the patient's serum (10,11). The use of the idiotype of 5T2 MM as a tumour-specific marker has been described in the second part of chapter IIIA. The serum concentration of 5T2 MM immunoglobulin has appeared an accurate reflection of the growth of the corresponding myeloma. With very sensitive techniques such as ELISA, minute serum concentrations of the 5T2 MM immunoglobulin as detected by its idiotype can be measured. This enables the detection of 5T2 MM in a relatively early stage of development, i.e., shortly after transplantation, or as a minimal persistent disease after treatment. Cytoplasmic immunofluorescence with

anti-idiotype antibodies appeared to result in detection of nearly all myeloma cells, since they are predominantly plasmablasts and/or plasmacytes. With this technique, the 5T2 MM cells within, for example, the bone marrow can be easily visualized. In contrast, surface membrane immunofluorescence with anti-idiotype antibodies did not appear an appropriate method to detect the entire 5T2 MM population. In the experiments described in chapter IIIA, only about fifty per cent of the 5T2 MM cells were shown to be surface membrane idiotype-positive. This observation indicates that 5T2 MM comprises a heterogeneous cell population. Both in MM in man and in plasmacytoma in BALB/c mice, intra-tumour heterogeneity has also been observed (reviewed in 9 and 12,13,14).

The heterogeneity of the 5T2 MM cell population was further investigated in the cell separation experiments. Bone marrow cells from 5T2 MM-bearing mice were separated according to their size, and the resultant subpopulations of cells were subsequently analysed. The prolonged period of time which the small 5T2 MM cells needed to develop into a detectable myeloma after injection into the recipient mice suggested that these cells had a lower proliferation activity than the larger cells. The malignant clone in MM is predominantly composed of more or less mature plasma cells. However, it has generally been accepted that less differentiated precursor cells are also part of the malignant clone (9,12). Whether in human MM, only precursor cells have the capacity to proliferate, or also the more differentiated cells, has not yet been established.

Many neoplasms with an aneuploid content of DNA have been described (15,16). Aneuploidy is also often seen in MM (17). In most instances, aneuploidy represents an excess of DNA. This has also been observed in 5T2 MM. In the DNA distribution curves of 5T2 MM bone marrow cells, one large aneuploid peak, and sometimes, a small peak representing cells with two times the aneuploid DNA content (in G2/M phase of the cell cycle) were observed. If used as a tumour marker for 5T2 MM, the cellular DNA content permits a rapid analysis of the percentage of 5T2 MM cells within the bone marrow compartment by flow cytometry. This facilitates quantitative studies on the 5T2 MM. In addition, the proportion of 5T2 MM cells in cycle (S/G2/M) can also be calculated if a distinct peak representing the aneuploid cells in G2/M phase is present. In various pilot experiments using flow cytometric analysis of the DNA content, the proportion of cycling (S/G2/M) 5T2 MM cells of all myeloma

cells from the bone marrow compartment was assessed to range from twenty to thirty per cent (these preliminary results are not shown). A frequently used, alternative method to calculate the size of the proliferating compartment in MM is the in vitro measurement of the plasma cell labelling index. This procedure is performed by incorporation of tritiated thymidine in the DNA-synthesizing cells, followed by autoradiography, or with an immunofluorescence method, which utilizes a mouse monoclonal antibody reactive with 5-bromo-2-deoxyuridine (18,19). The tritiated thymidine-labelling index of 5T2 MM cells from the bone marrow compartment ranged from 7 to 22 per cent (20). This result is not in conflict with the preliminary results of DNA flow cytometry. However, an essential difference between both methods exists: the DNA flow cytometric method reflects the in vivo situation, whereas the incorporation of tritiated thymidine occurs in vitro.

Considering the different characteristics of 5T2 MM, it is a proper model for MM in man, especially for MM of the immature (plasmablastic) cell type.

Further studies were directed towards the question as to whether this neoplasm is sensitive to immune regulation. This is a prerequisite for the development of an immunological treatment strategy.

Multiple humoral and cellular interactions between idiotypes and anti-idiotypes are important physiologic mechanisms to control the normal immune response (21). Such interactions result in stimulation or suppression of the proliferation of the responding clones. For therapeutic use in B-cell malignancy, only the suppressive effect of immune regulation is of advantage. The sensitivity of induced BALB/c plasmacytomas for idiotype-specific immune regulation has been demonstrated in several investigations (reviewed in 22 and 23). Similar observations were made in studies on the 5T2 MM. A humoral anti-5T2 MM idiotype response could be induced in C57BL/KaLwRij mice. The observed DTH responses to 5T2 MM immunoglobulin or to 5T2 MM cells suggest that cellular immunity to 5T2 MM does also occur. More important was the finding that these immunized animals showed resistance against "take" of 5T2 MM after subsequent intravenous transplantation. Whether humoral or cellular mechanisms (or both) were responsible for this resistance was not clear.

The prevention of "take" of 5T2 MM after establishment of idiotype-specific immunity does not correspond with the clinical situation. Treatment of MM patients is only initiated when their disease is in an advanced stage. Efforts

have been made to treat patients with B-cell lymphoma with anti-idiotype antibodies (24,25). B-lymphoma cells are the malignant counterparts of immature or mature B-lymphocytes, which carry their idiotype on the surface membrane. In general, normal and malignant B-lymphocytes do not secrete large amounts of immunoglobulins. This is in contrast with the situation in MM. The large amount of myeloma idiotype-bearing immunoglobulin in the intra- and extravascular extracellular fluid compartments of patients interferes with successful application of anti-idiotype treatment. An extensive reduction of the tumour load in MM has to precede anti-idiotype therapy. The currently used chemotherapeutic regimens are inadequate for this purpose. In case of remission, the number of persistent myeloma cells is sufficient to maintain a serum myeloma immunoglobulin concentration which exceeds the critical value of 200 µg/ml (25). Attempts to achieve a higher degree of tumour reduction with larger doses of chemotherapy in combination with total body irradiation, and followed by bone marrow transplantation, have resulted in relatively long-lasting remissions in a number of patients (26). However, even in these cases, the residual number of myeloma cells is too high.

The results of the experiments described in the second part of chapter IIIB showed the efficacy of anti-idiotype treatment of 5T2 MM mice when they had an already developed myeloma. In contrast with MM in man, the tumour load in 5T2 MM mice could be reduced to a minimal residual disease. This was achieved by treatment of the mice with cyclophosphamide. 5T2 MM appeared very susceptible to cytotoxic drugs. This sensitivity has also been observed in mouse plasmacytoma (27), but not in MM in man. The favourable outcome of the studies on the effect of anti-idiotype treatment of 5T2 MM offers perspectives for further research into the efficacy and the exact mechanism of idiotype-specific rejection of MM.

As long as no complete cure of MM is possible, the bone disease will remain a major problem. Even after successful remission induction, the number of persistent MM cells is too high, which will result in maintenance of the osteolytic lesions. Bone destruction in MM is caused by an increased osteoclastic activity. Bisphosphonates are inhibitors of osteoclastic bone resorption. One of these compounds, (3-amino-1-hydroxypropylidene)-1,1-bisphosphonic acid (APD), has appeared a potent inhibitor of bone resorption without causing

defects in mineralization. APD has also proved to be effective in the treatment of Paget's disease of bone without being toxic (28,29).

Osteolytic bone destruction is one of the pronounced features of 5T2 MM, and reflects in this respect the situation in man. The bisphosphonate APD was used for treatment of the bone lesions in 5T2 MM mice. In contrast with the untreated mice, APD treatment of the mice with 5T2 MM in an advanced stage resulted in a clear diminution in bone destruction. When APD treatment was initiated before the MM could be detected, i.e., simultaneously with or shortly after transplantation, the results were even better. Moreover, in the long-term study, the mice treated with APD from the day of transplantation had a prolonged median and mean survival. Since APD did not exert any cytotoxic effect on 5T2 MM itself, the longer life span might, at least in part, be considered as a reflection of a decrease in "subjective discomfort" of the mice. The prolonged survival also reflects the lack of toxicity of the administered dose of APD.

The results of these studies indicate that APD treatment should be added to cytotoxic chemotherapy of MM patients in order to improve the quality of their life. Furthermore, it also seems reasonable to treat patients with stationary disease, (i.e., those MM patients who are not considered for cytotoxic chemotherapy) with APD in order to prevent or delay the development of bone disease. This assertion is supported by the increased beneficial effect of APD when it has been administered from the day of transplantation of the mice with 5T2 MM.

The results of the experimental approaches on the treatment of MM were promising. With the introduction of APD treatment in the near future, it will probably be possible to achieve an effective palliation of the serious discomfort of MM patients due to their bone disease. The prevention of a relapse of MM by treatment with anti-idiotype antibodies appears, in essence, to be possible. However, stronger and better treatment regimens with chemotherapy and/or irradiation should be developed in order to achieve a decrease of the serum myeloma immunoglobulin content to minute levels, before anti-idiotype treatment will be of practical value.

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SUMMARY

This thesis describes a new animal model of multiple myeloma and the studies performed in this model in order to find new ways for treatment of this disease. Multiple myeloma (MM) is a B-cell neoplasm characterized by the presence of malignant plasma cells mainly in the bone marrow.

Chapter I gives a description of different aspects of MM such as its morphological, clinical, and laboratory features, and the present status of its therapy.

Multiple myeloma is still an incurable disease. With currently available cytotoxic agents, a remission is induced in many patients, but, eventually, the disease relapses. In addition, the serious osteolytic skeletal destruction, which often complicates MM, remains a major problem. During chemotherapy, bone lesions often persist.

The malignant cells in MM are monoclonal. Their clone-specific marker (the idiotype) may be regarded as a tumour-specific determinant. Idiotypes play an important role in the regulation of the normal immune response. In animal models of plasmacytoma, the malignant clone was also shown to be sensitive to idiotype-specific immune regulation.

APD, which is one of the so-called bisphosphonates, inhibits osteoclastic bone resorption. Its potency has already been demonstrated in the treatment of patients with Paget's disease of bone and with severe hypercalcaemia of malignancy.

These findings stimulated the initiation of the studies described in this thesis. Their aim was 1) to investigate the possibility of treating MM with idiotype-specific immune regulation, and 2) to prevent and/or treat the osteolytic bone disease with APD. These preclinical studies were performed in a unique animal model of MM: the mouse 5T2 MM of C57BL/KaLwRij origin. This transplantable mouse MM resembles the human disease to a high degree.

The materials and methods used in the different experiments are described in chapter II. The first part of chapter IIIA gives a survey of the typical morphological and biological characteristics of the 5T2 MM. The differences between the primary MM and the transplanted progeny have been emphasized.

In addition, 5T2 MM was compared with other models of plasma cell malignancy and with MM in man.

Monoclonal anti-5T2 MM idiotype antibodies were developed for selective detection of the 5T2 MM, and for use in studies on immune regulation of this myeloma. Serial measurements of the serum concentration of "free" 5T2 MM idiotype were shown to be a reliable way to monitor the growth of this malignancy. With sensitive techniques such as ELISA, "take" of the 5T2 MM can be detected fairly soon after its transplantation into recipient mice. Nearly all 5T2 MM cells (which are predominantly plasmablasts or plasmacytes) can be detected with cytoplasmic immunofluorescence. About fifty per cent of the 5T2 MM cells were shown to be surface membrane idiotype-positive. This observation suggests that the 5T2 MM consists of a heterogeneous population. This was confirmed by cell separation experiments using the velocity sedimentation technique. The intra-tumour heterogeneity might be a reflection of differences in maturity of the cells. Aneuploidy has often been observed in MM in man. The 5T2 MM cells showed a hyperdiploid content of DNA. This aberrant DNA content can be utilized as a specific marker for 5T2 MM with flow cytometric analysis (Chapter IIIA).

Chapter IIIB deals with the studies on manipulation of the 5T2 MM clone by immune regulation. A humoral anti-5T2 MM idiotype response was elicited in syngeneic mice after an intensive immunization procedure. These immunized mice were made resistant to subsequently intravenously injected 5T2 MM cells. In addition, a DTH response to 5T2 MM immunoglobulin or to 5T2 MM cells was elicited in C57BL/KaLwRij mice, indicating that 5T2 MM also evokes a cellular anti-idiotype immune response.

In order to investigate immunological treatment of 5T2 MM in a more practical way, mice bearing a well developed 5T2 MM were treated with a cytotoxic agent (cyclophosphamide) in a low dose in order to reduce the myeloma to a minimal residual disease. Subsequently, the mice were treated with monoclonal anti-5T2 MM idiotype antibodies. This treatment resulted in a maintained remission in most of the animals. Further studies have to reveal the mechanism of the cytotoxic effect of passive anti-idiotype therapy.

Chapter IVA and B give results of the studies on the effect of APD. In chapter IVA, the dose-toxicity relationship of orally administered APD is shown. Chapter IVB describes the studies on the treatment of the bone disease

in 5T2 MM mice. Both studies, during the short-term and the long-term, showed the beneficial effect of APD treatment on bone destruction. Moreover, when APD was administered shortly after transplantation of 5T2 MM, this effect was more pronounced. The longer mean and median life span of the mice treated from the day of transplantation suggests that APD *improves* the general condition of the animals. No distinct effect of APD on the MM itself was observed. In the used dosages, APD was not toxic.

SAMENVATTING

Dit proefschrift is gewijd aan een nieuw proefdiermodel voor multipel myeloom (MM) en aan het onderzoek naar nieuwe methoden om de ziekte te behandelen, waarbij van dit model gebruik gemaakt is. Multipel myeloom is een B-cel nieuwvorming, die wordt gekenmerkt door de aanwezigheid, vooral in het beenmerg, van kwaadaardige plasmacellen.

Verschillende aspecten van MM, zoals morfologie, klinische symptomatologie en laboratoriumkenmerken, en de huidige stand van zaken van de behandeling, zijn beschreven in hoofdstuk 1.

Nog steeds is MM een ongeneeslijke ziekte. Met de tegenwoordige cytotoxische medicatie kunnen de ziekteverschijnselen in remissie gebracht worden in een aanzienlijk aantal patiënten, maar na kortere of langere tijd komen de ziekteverschijnselen weer terug. Bovendien blijft de ernstige door osteolysis veroorzaakte aantasting van het skelet, hetgeen een vaak optredende complicatie van MM is, een groot probleem. Gedurende de chemotherapie blijven de botlaesies in veel gevallen bestaan.

De myeloma cellen zijn monoclonaal. De cloon-specifieke determinant, dat wil zeggen het idiotype van de myelomacloon, kan beschouwd worden als tumorspecifiek. Idiotypische determinanten spelen een belangrijke rol in de regulatie van de normale immuunrespons. In diermodellen voor plasmacytoma is aangetoond, dat ook de maligne cloon gevoelig is voor idiotype-specifieke immuunregulatie.

APD, één van de zogenoemde bisfosfonaten, remt de door osteoclasten veroorzaakte botresorptie. De krachtige werkzaamheid van dit middel is inmiddels aangetoond bij de behandeling van patiënten met de ziekte van Paget en patiënten met ernstige tumor-hypercalciaemie.

Deze bevindingen vormden de basis voor het in dit proefschrift beschreven onderzoek. Het doel van deze studie was om 1) de mogelijkheden te onderzoeken om MM te behandelen door middel van idiotype-specifieke immuunregulatie en 2) de botafbraak bij deze ziekte te voorkómen of the behandelen met APD. Dit preklinische onderzoek werd verricht aan de hand van een uniek diermodel voor MM: het 5T2 MM, dat spontaan is ontstaan in een C57BL/KaLwRij muis. Dit transplanteerbare muis MM lijkt in hoge mate op deze

ziekte in de mens.

De materialen en methoden, die in de verschillende experimenten gebruikt werden, zijn beschreven in hoofdstuk II. Het eerste deel van hoofdstuk IIIA geeft een overzicht van de morfologische en biologische kenmerken van het 5T2 MM. Hierbij is aandacht besteed aan de verschillen tussen het primaire 5T2 MM en het 5T2 MM in zijn getransplanteerde vorm. Daarnaast is het 5T2 MM vergeleken met andere diermodellen voor plasmacel-maligniteiten en met MM in de mens.

Monoclonale anti-5T2 MM-idiotype antilichamen werden ontwikkeld en gebruikt voor de selectieve detectie van het 5T2 MM en voor het onderzoek naar de immuunregulatie van dit myeloom. Opeenvolgende bepalingen van het serumgehalte van "vrij" 5T2 MM-idiotype bleken een betrouwbare methode om de groei van deze maligniteit te vervolgen. Met gevoelige technieken, zoals ELISA, kan het "aanslaan" van het 5T2 MM in de ontvangermuis relatief snel na de transplantatie gedetecteerd worden. Vrijwel alle 5T2 MM-cellen zijn plasmablasten of plasmacyten en kunnen aangetoond worden door middel van cytoplasmatische immunofluorescentie. Bij ongeveer vijftig procent van de 5T2 MM-cellen bleek het idiotype ook op de oppervlaktemembraan gelocaliseerd te zijn. Dit is een aanwijzing dat het 5T2 MM uit een heterogene celpopulatie bestaat. Deze veronderstelling werd bevestigd door middel van experimenten, waarin de cellen werden gesorteerd op grond van verschillen in bezinkingssnelheid. De intra-tumor heterogeniteit zou een weerspiegeling kunnen zijn van verschillen in rijping van de cellen. Aneuploidie is vaak waargenomen bij MM in de mens. De 5T2 MM-cellen bezitten een hyperdiploide hoeveelheid DNA. Deze afwijkende hoeveelheid DNA kan als een specifieke determinant voor 5T2 MM gebruikt worden bij analyse met behulp van flow cytometry (hoofdstuk IIIA).

Hoofdstuk IIIB beschrijft het onderzoek naar de door immuunregulatie veroorzaakte effecten op de 5T2 MM-cloon. Een humorale anti-5T2 MM-idiotype-respons werd opgewekt in syngene muizen na een intensieve immunisatieprocedure. Deze geïmmuniseerde muizen bleken resistent tegen de daaropvolgend intraveneus ingespoten 5T2 MM-cellen. Het was ook mogelijk een vertraagd type overgevoeligheid (DTH) reactie tegen 5T2 MM-immuunglobuline en tegen 5T2 MM-cellen op te wekken in C57BL/KaLwRij muizen. Dit duidt erop dat ook een cellulaire anti-idiotype-respons tegen het 5T2 MM opgewekt kan worden.

Om de immunologische behandeling van het 5T2 MM op een meer praktijkgerichte wijze te onderzoeken, werden muizen met 5T2 MM in een ver stadium van ontwikkeling behandeld met een lage dosis van een cytotoxisch middel (cyclophosphamide) om het myeloom te reduceren tot minimale grootte, een zogenoemd "minimal residual disease". Vervolgens werden de muizen behandeld met monoclonale anti-5T2 MM-idiotype-antilichamen. In het merendeel van de dieren resulteerde deze behandelingswijze in een blijvende remissie. Verder onderzoek zal verricht dienen te worden naar het mechanisme van het cytotoxische (of cytostatische) effect van passieve anti-idiotype-therapie.

De resultaten van het onderzoek naar het effect van APD staan beschreven in hoofdstuk IVA en B. Hoofdstuk IVA toont de relatie aan tussen de dosis en de toxiciteit van het oraal toegediende APD. Het onderzoek naar de behandeling van de botziekte in 5T2 MM-muizen staat beschreven in hoofdstuk IVB. Zowel het onderzoek naar korte termijn- als dat naar lange termijn-behandeling laten het gunstige effect van APD op de botdestructie zien. Dit effect was nog meer uitgesproken, indien kort na de transplantatie van het 5T2 MM met de APD-behandeling werd begonnen. De hogere 50% overlevingsduur en gemiddelde leeftijd van de muizen, die vanaf de dag van de transplantatie waren behandeld met APD, suggereert dat APD de algemene toestand van de muizen verbetert. Een duidelijk effect van APD op het myeloom zelf werd niet waargenomen. In de doseringen, die gebruikt werden in deze behandelingsstudies, bleek APD niet toxisch voor de muizen te zijn.

ABBREVIATIONS

APD	:	(3-amino-1-hydroxypropylidene)-1,1-bisphosphonic acid
AvPo	:	avidin conjugated with peroxidase
BSA	:	bovine serum albumin
Cl_2MDP	:	(dichloromethylidene)-bisphosphonate
CFA	:	complete Freund's adjuvant
DNP	:	dinitrophenol
DTH	:	delayed type hypersensitivity
EHDP	:	(1-hydroxyethylidene)-1,1-bisphosphonate
ELISA	:	enzyme-linked immunosorbent assay
FACS	:	fluorescence-activated cell sorter
FITC	:	fluorescein isothiocyanate
HEPES	:	N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid
HBSS	:	Hanks' balanced salt solution
HPS	:	haematoxylin-phloxine-saffron
H+H	:	Hanks' minimal essential medium supplemented with 15 mM HEPES
IFA	:	incomplete Freund's adjuvant
IP	:	idiopathic paraproteinaemia
MM	:	multiple myeloma
OAF	:	osteoclast activating factor
OPD	:	ortho-phenylene diamine
PBS	:	phosphate-buffered saline
PBS-T	:	0.2% (^{v}/v) Tween-20 in PBS
PI	:	propidium iodide
PO	:	peroxidase
ppm	:	parts per million
RIA	:	radioimmuno assay
RT	:	room temperature
SD	:	standard deviation
SEM	:	standard error of the mean
TRITC	:	tetramethyl rhodamine isothiocyanate

CURRICULUM VITAE

De schrijver van dit proefschrift is geboren op 15 oktober 1951 in Amsterdam. Het HBS-B diploma heeft hij behaald in juni 1971 in Breda. In september 1972 is hij met de studie Geneeskunde begonnen aan de Universiteit van Amsterdam. Het doctoraalexamen heeft hij afgelegd in februari 1979, het artsexamen in februari 1982. Daarna is hij gedurende een half jaar werkzaam geweest op de afdeling Immunochemie van het Centraal Laboratorium van de Bloedtransfusiedienst te Amsterdam onder leiding van Dr. T.A. Out. Vanaf november 1982 tot november 1986 is hij als wetenschappelijk medewerker verbonden geweest aan het Instituut voor Experimentele Gerontologie TNO te Rijswijk, waar hij onder leiding van Dr. J. Radl het in dit proefschrift beschreven onderzoek heeft verricht. Momenteel is hij werkzaam als arts in opleiding tot huisarts in een gezondheidscentrum in Amsterdam.

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