MONOCLONAL MACROGLOBULINEMIA IN NZB/NZW F_1 MICE

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New Zealand Black (NZB) and NZB/NZW F_1 hybrid mice are genetically predisposed to an immunologic disorder characterized by autoantibody formation, hemolytic anemia, lymphoid infiltration of many organs, immune complex nephritis, and lymphoid malignancies (1–3). They have been studied as a laboratory model for systemic lupus erythematosus (4) and Sjögren's syndrome (5). Immunologic and viral factors have been implicated in their disease (1, 2, 4).

Certain immunologic abnormalities, such as excessive antibody responses to some antigens (6, 7) and relative resistance to tolerance induction (7–9), are present in these animals before any signs of disease. Other abnormalities, such as impaired cell-mediated immunity (10–13), develop after autoimmunity is present. An antibody cytotoxic for thymus-derived lymphocytes (T cells) is present early in life (14) and may contribute to a progressive loss of T cell effector functions and slight decrease in θ-positive lymphocytes (15–18). Their general immune status can be summarized as an imbalance between excessive antibody responses and diminished cellular immunity (4).

New Zealand mice harbor a Gross-type leukemia virus (19, 20) and make antibodies to viral antigens (21). Whether this is the ubiquitous natural virus common to AKR and many other mouse strains or whether it is a unique agent is an important unresolved question. Recent evidence suggests that the NZB virus does have some unusual properties (22, 23).

Serum IgM concentrations are increased in New Zealand Black mice (24, 25). East and her colleagues could observe no correlation between serum IgM concentration and sex, clinical status, thymic infiltration, or kidney disease. One NZB serum showed a "local increase" of IgM on immunoelectrophoresis suggestive of a paraprotein (25). A high IgM is not prevented by neonatal thymectomy or by maintenance in a germ-free environment (3). Another group found IgM cryoglobulins, some with immunoelectrophoretic characteristics suggestive of a monoclonal disorder, in about 22% of NZB mice (26).

The autoantibodies that can be detected on the erythrocyte surface in Coomb's positive NZB mice are heterogeneous. Warner and Wistar found greater amounts of IgG than of IgM coating these erythrocytes (24). Likewise, IgG is the major immunoglobulin present in the immune complexes that deposit in the glomeruli of NZB/NZW (B/W) F_1 mice (27).

NZB mice over 1 yr of age have an increased incidence of lymphoid malignancy (3, 28, 29). Generalized reticulum cell sarcoma, thymoma, and lymphocytic lymphoma have been observed. Mellors described a "pleomorphic malignant lymphoma" that, in one mouse, was associated with a peak in the β-γ-globulin region on paper electrophoresis (29). A narrow electrophoretic peak was also observed in serum of old NZB mice by Bhoopalam et al. (30).

Malignant lymphoma in NZB mice is potentiated by administration of the immunosuppressive drug azathioprine (31). High-dose cyclophosphamide given to B/W mice for 2 yr resulted in an increased incidence of lymphomas and other neoplasms (32). Two mice developed paraproteins.

We now report the presence of electrophoretically restricted immunoglobulins in over 30% of hybrid B/W mice greater than 11 mo of age. 20 of these proteins have been characterized and every one belongs to the IgM immunoglobulin class. Only a single light chain type was detected in 10 proteins studied. These monoclonal macroglobulins are often associated with a generalized lymphoproliferative disorder that in some mice shows features of malignancy.

Materials and Methods

Mice.—B/W mice were from our colony maintained at the Vivarium of the University of California at San Francisco. They were housed at the Veterans Administration Hospital and examined monthly under ether anesthesia for palpable tumors. Mice with palpable tumors were bled and serum proteins studied by agarose gel electrophoresis. Age- and sex-matched B/W mice without palpable tumors were also bled for serum protein studies.

Serum Proteins.—Electrophoresis and immunoelectrophoresis were performed in 0.5% agarose buffered with 0.05 Veronal (pH 8.6). A constant current of 15 mA per 1 cm of agarose gel was maintained for 30 min. Immunoglobulins were precipitated with monospecific goat antisera that were kindly furnished by Dr. Richard Asofsky. The following antisera were used: anti-IgM (G135A), anti-IgA (G134A), antiIgGγ_1 (G155), anti-IgGγ_2 (G180), and anti-λ (G135A2A). A rabbit anti-κ antiserum was furnished by Dr. Rose Mage. Proteins and immunoprecipitin lines were stained with amido black 10B. Double diffusion studies were also performed in 0.5% agarose buffered with 0.05 Veronal (pH 8.6).

Isolation of IgM.—IgM was isolated from B/W mouse serum by gel filtration on Sephadex G-200 columns. To eliminate α2-macroglobulin, these filtrates were subjected to agarose or Pevikon preparative electrophoresis in 0.05 Veronal (pH 8.6). A constant current of 20 mA per 1 cm was maintained for 6 h. The IgM was recovered by elution from Pevikon or by freeze-thawing from agarose. It was concentrated and studied for light chain type by double diffusion in agarose.

Preparation of Anti-Idiotypic Antisera.—The isolated IgM from two sera was emulsified in complete Freund's adjuvant and used to immunize rabbits. Each rabbit received two 1 ml injections into multiple footpad, subcutaneous, and intramuscular sites. Rabbits were bled 2 wk after the second injection. 1 ml of antiserum was absorbed with 0.4 ml of pooled electrophoretically normal B/W mouse serum.

Sucrose Gradient Ultracentrifugation.—Selected mouse sera were subjected to ultracentrifugation in a 10–35% sucrose gradient (0.15 M NaCl, pH 8.0) using an SW 40 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The centrifugation was at 39,000 rpm for 20 h. The 13.5 ml gradient was collected dropwise into fractions and analyzed for antibodies to DNA and RNA using a cellulose ester filter radioimmunoassay, and for immunoglobulin content by electrophoresis and immunoelectrophoresis. The radioactive nucleic acids studied were

[^{3}H]reovirus double-stranded RNA (33) and [^{14}C]KB cell DNA (34). The radioactive antigen-antibody complexes were collected on cellulose ester filters (Millipore Corp., Bedford, Mass.) and assayed for radioactivity in a liquid scintillation counter as previously described (33).

Cytotoxicity Assay.—Cells from the enlarged lymph nodes were teased in RPMI 1640 medium (Microbiological Associates, Bethesda, Md.). Cells were washed three times in medium and adjusted to a concentration of 10^7 cells/ml. Viability, determined by trypan blue dye exclusion, was 90% or greater. Surface antigens were studied in a cytotoxicity assay (14) using antimouse immunoglobulin sera and a heterologous antimouse T cell serum. The latter was prepared by immunizing rabbits against mouse brain followed by absorption with mouse liver cells. Its specificity for T cells was confirmed by reactivity with mouse thymocytes and lack of reactivity with bone marrow cells (35). 50 μl of the cell suspension were mixed with 50 μl of appropriately diluted antisera and incubated with rocking for 45 min at 37°C. The cells were washed twice and 50 μl of diluted rabbit complement (Hyland Div., Travenol Laboratories, Costa Mesa, Calif.) was added. After incubation for 30 min at 37°C, trypan blue was added and cell viability determined.

Tumor Transplantation.—Enlarged lymph nodes, spleen, and other organs were routinely examined by light microscopy. Certain specimens were fixed in glutaraldehyde and examined by electron microscopy. Many attempts were made to establish a transplanted tumor line by injecting 5×10^6 to 5×10^8 spleen or lymph node cells into syngeneic newborn or adult recipients.

RESULTS

Electrophoretically Restricted Proteins.—The sera of B/W mice were studied by agarose gel electrophoresis for the presence of electrophoretically restricted proteins. In male mice 8–11 mo old, only 1 of 13 sera (8%) had this finding (Table I). Between 11 and 17 mo of age, 37 of 117 mice (31.6%) showed such

TABLE I

Incidence of Electrophoretically Restricted Proteins and Palpable Tumors in B/W Mice

Sex	Age	Electrophoretically restricted proteins	Palpable tumor
	mo		
Male	8–11	1/13 (8%)	13/136 (10%)
Male	11–17	37/117 (31.6%)	117/362 (32.4%)
Female	11–13	4/11 (36.4%)	11/47 (23.4%)
Male	12–18	1/29 (3.4%)	0/29 (0%)

proteins in the γ-region. The frequency in female mice 11–13 mo old was 4 out of 11 (36.4%). The proteins varied in amount but many had the appearance of typical M-spikes (Fig. 1).

Physical examination showed that these electrophoretic abnormalities were common in mice with palpable tumor masses. For example, only 1 of 29 age-matched male mice without tumors had an M-spike (Table I). The tumors were generally subcutaneous nodules in the cervical or inguinal region that histologically proved to be very enlarged and somewhat atypical lymph nodes.[1]

[1] Greenspan, J. S., S. Sugai, and N. Talal. Data to be published

Immunochemical Characterization.—20 sera containing relatively large amounts of these electrophoretically restricted proteins were further studied by immunoelectrophoresis with specific anti-immunoglobulin antisera. In every instance, the protein belonged to the IgM immunoglobulin class. Many of these immunoglobulins had typical features of monoclonal immunoglobulins (Fig. 2).

The macroglobulin nature of these IgM proteins was confirmed by gel filtration on Sephadex G-200, by analytical ultracentrifugation, and by sucrose gradient ultracentrifugation. These indicated a high molecular weight immunoglobulin with an approximate s_{20} value of 17.

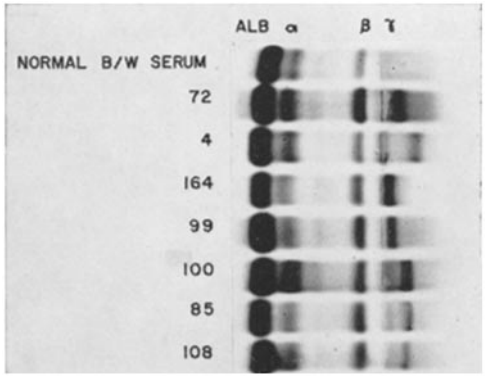


FIG. 1. Agarose gel electrophoresis of normal B/W mouse serum and individual sera from older B/W mice showing electrophoretically restricted proteins.

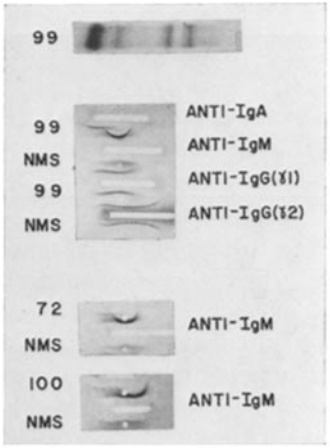


FIG. 2. Immunoelectrophoresis of serum 99 developed with monospecific antisera to mouse immunoglobulins indicating that the restricted protein is an IgM. Two other sera with restricted IgM proteins (72 and 100) are also shown.

10 of these IgM proteins were isolated by gel filtration on Sephadex G-200 and then studied by immunodiffusion with specific anti-kappa and anti-lambda antisera. In every instance, only a single light chain type was found (Fig. 3). Five of them were κ-type and five were λ-type (Table II).

Idiotype Characteristics.—Two of the IgM proteins (108, 141) were isolated by gel filtration on Sephadex G-200 and further purified by Pevikon or agarose

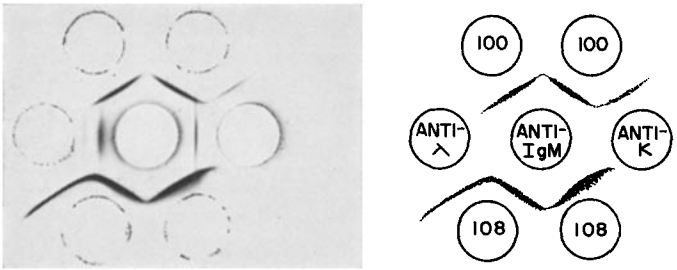


FIG. 3. Double diffusion in agarose of serum 100 and 108 with monospecific antisera to IgM (μ-chain), κ- and λ-chains. Serum 100 is an IgM κ-type and serum 108 is an IgM λ-type. The actual experiment is shown on the left and schematically on the right. Reactions between antisera are due to materials added during absorption.

TABLE II

Light Chain Type of Monoclonal IgM

Number	Kappa	Lambda
4	+	
28		+
39	+	
72	+	
85		+
99	+	
100	+	
108		+
141		+
164		+

block electrophoresis. They were then used to immunize rabbits for the preparation of anti-idiotypic antisera. After absorption of the rabbit antisera, typical idiotypic reactivity was observed (Fig. 4). The absorbed antisera to 108 reacted strongly with the homologous IgM and with one of 20 other IgM proteins (164) but no longer had the characteristics of an anti-IgM serum. The significance of this limited cross-reaction is under study. This experiment indicates that the IgM proteins contain individually specific determinants and supports their monoclonal nature.

Antibodies to Nucleic Acids.—The spontaneous appearance of antibodies to double-stranded RNA and DNA are hallmarks of the autoimmune disease that develops in B/W mice (27, 36). DNA immune complexes are involved in the pathogenesis of nephritis (27). We studied whether the monoclonal IgM proteins in these mice were antibodies to nucleic acids.

The incidence of antibodies to DNA and RNA in older B/W mice without monoclonal IgM was 96.4% and 67.8%, respectively (Table III). The incidence in age- and sex-matched B/W mice with monoclonal IgM was 50% and 40%, respectively. Thus, the monoclonal IgM seems to be associated with a reduced incidence of antibodies to nucleic acids.

This question was further studied by fractionating two sera containing anti-

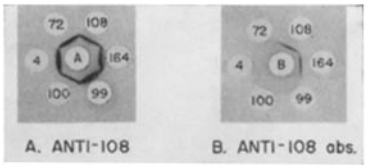


FIG. 4. Double diffusion in agarose with antiserum prepared against the monoclonal IgM 108. Before absorption (*A*) the antiserum reacted with all IgM proteins. After absorption (*B*) IgM's 108 and 164 were still positive but the other sera were no longer reactive.

TABLE III

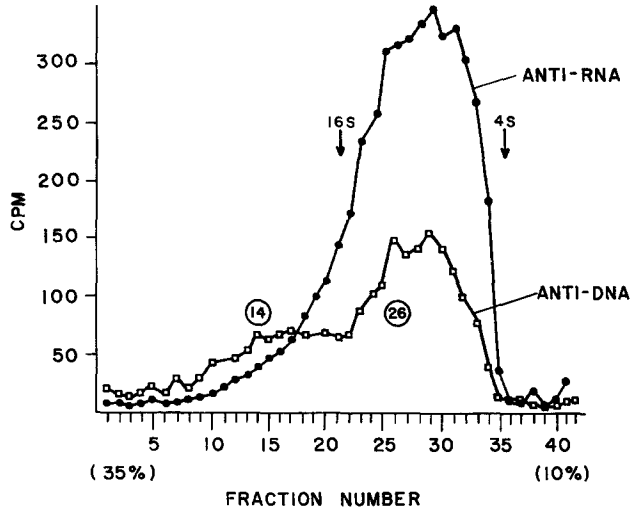
Incidence of Antibodies to DNA and RNA in Old B/W F_1 Male Mice

Monoclonal IgM	Antibody to DNA	Antibody to RNA
No	27/28 (96.4%)	19/28 (67.8%)
Yes	10/20 (50%)	8/20 (40%)

bodies to nucleic acids and monoclonal IgM by sucrose gradient ultracentrifugation. The results (illustrated for one serum in Fig. 5) show that the antibodies to DNA and RNA are predominantly in the 7S region of the gradient, clearly separated from the heavier monoclonal IgM. Thus, the monoclonal IgM appears not to be an antibody to DNA or RNA. Further search for other antibody activities is continuing.

T and B Cells in Enlarged Lymph Nodes.—Although the enlarged cervical lymph nodes presented as tumor masses, histologically many lacked certain characteristics of malignant neoplasms. A detailed report on this aspect is beyond the scope of this communication and will appear separately.[1]

We studied whether there was an increased number of B cells with surface IgM in these enlarged nodes, such as one finds in the blood of patients with Waldenström's macroglobulinemia (37). To do this we employed a cytotoxicity assay and specific antisera to mouse immunoglobulins and to mouse T cells.



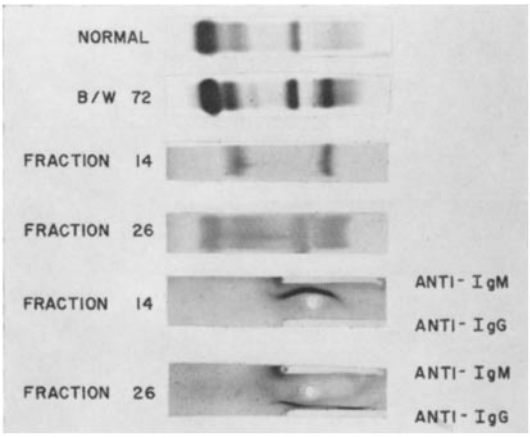


FIG. 5. *Top*: Sucrose gradient ultracentrifugation of serum 72 performed as described in Materials and Methods. Each of 42 fractions was assayed for antibodies to DNA and RNA by radioimmunoassay (33). The peak of antibody activity was in the 7S region of the gradient. Fractions 14 and 26 were studied by electrophoresis and immunoelectrophoresis. *Bottom*: Fraction 14 (from the 19S region of the gradient) contained the monoclonal IgM (as well as α2-macroglobulin). Fraction 26 contained predominantly IgG and other serum proteins.

Antiserum to mouse IgM killed about 30% of the lymphocytes, and anti-T cell serum was cytotoxic for approximately 40% of the cells. These results indicate neither an abnormal distribution of T and B lymphocytes in the enlarged lymph nodes nor any great increase in cells with surface IgM.

Transplantable Tumor Producing Monoclonal IgM.—Many attempts to transplant cell suspensions from the enlarged cervical lymph nodes into syn-

geneic newborn or adult mice were unsuccessful although continued observation of the recipients is in progress. However, one mouse (141) was producing a λ-type monoclonal IgM and had an enlarged spleen. Cell suspensions from the spleen were injected subcutaneously into newborn B/W mice and intraperitoneally into adult B/W mice. Within 1 mo, the newborn recipients developed subcutaneous lymphoid tumors and a monoclonal IgM that reacted with the anti-idiotypic serum prepared against 141 (Fig. 6). The tumor cells had the characteristics of medium to large lymphocytes (Fig. 7) and could be further transplanted to second and third generation newborn and adult recipients as solid tumors.

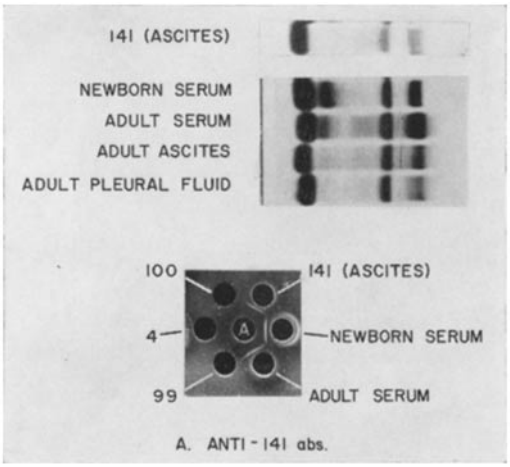


FIG. 6. Agarose gel electrophoresis of 141 ascites fluid, newborn recipient serum and adult recipient serum, ascites, and pleural fluid showing electrophoretically restricted IgM proteins. Double diffusion in agarose (*below*) shows reactivity with anti-idiotype serum (anti-141 abs.), whereas three other monoclonal IgM proteins fail to react.

The adult recipients of the 141 splenic lymphocytes developed ascites and pleural effusions within 3 wk of transplantation. The serum, peritoneal, and plural fluids all contained a prominent λ-type IgM paraprotein that reacted with the anti-141 idiotypic antiserum (Fig. 6).

DISCUSSION

This report establishes the presence of monoclonal macroglobulinemia in a large number of B/W mice over 11 mo of age. The monoclonal nature of the IgM is shown by restricted electrophoretic mobility, characteristic appearance on immunoelectrophoresis, restriction to a single light chain type, and ability to induce anti-idiotypic antisera.

These monoclonal macroglobulins often appeared in mice with enlarged

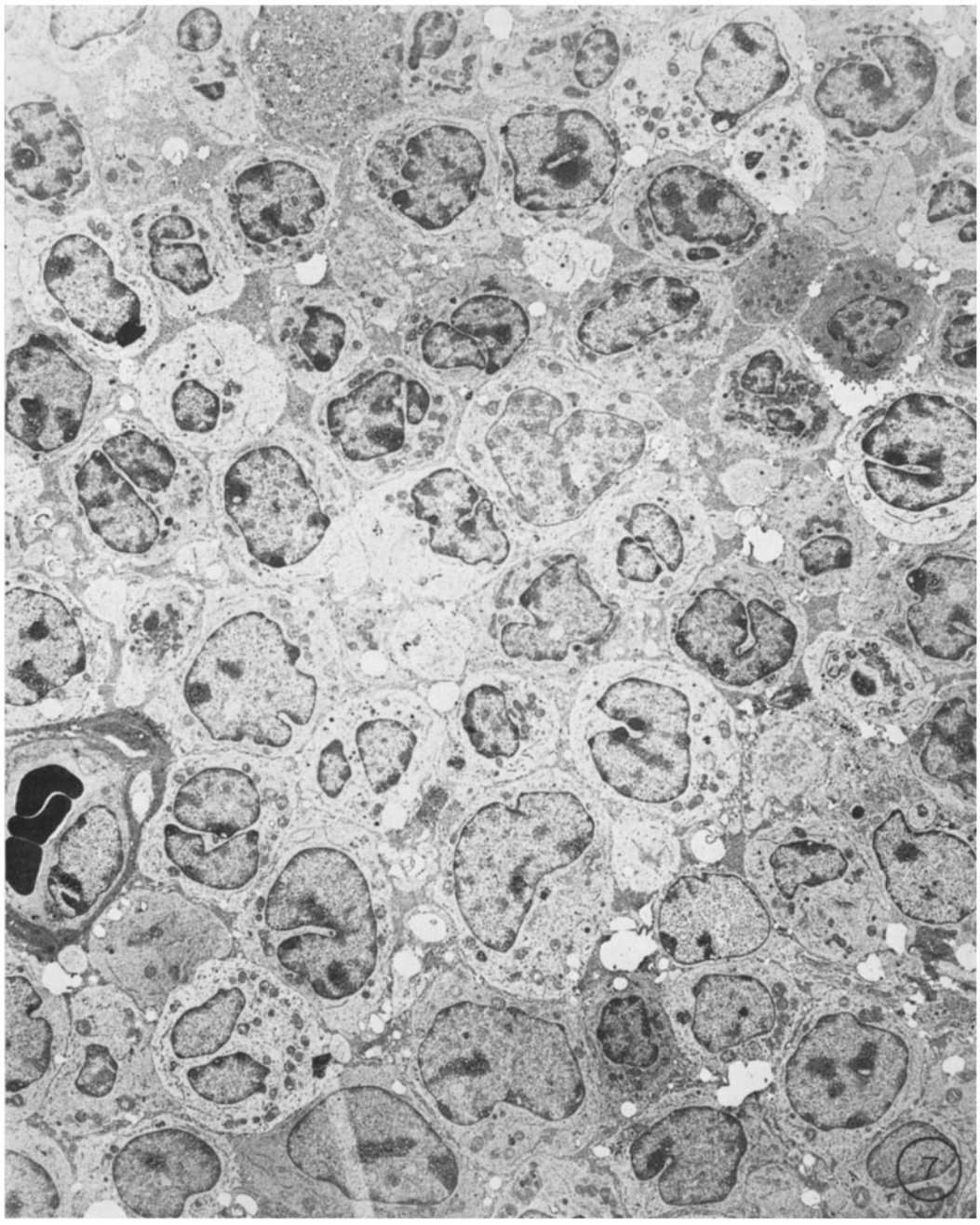


FIG. 7. Electron micrograph of lymphoid tumor transplanted into newborn B/W recipients. × 3,000.

cervical or inguinal lymph nodes as part of their generalized lymphoproliferation (1, 2). Initially, we suspected that the lymphoid masses were synthesizing the monoclonal IgM. However, some mice had neck tumors without M-spikes and others had faint M-spikes with no apparent tumors. Moreover, surgical excision of enlarged cervical lymph nodes in three mice caused no change in the amount of monoclonal IgM.

We also suspected that the lymph node enlargement was due to malignant lymphoma. However, as commented upon by East (3), it is almost impossible by morphologic criteria alone to determine whether malignancy is present in such lymph nodes. Furthermore, cytotoxicity testing for T and B cells indicated a mixed lymphoid population in proportions expected for ordinary lymph nodes. This mixed cell population was further confirmed by immunofluorescent studies employing antisera to T and B cells.[1] Finally, repeated attempts to establish a transplantable neoplasm by injecting lymphocytes from the enlarged nodes into syngeneic recipients were negative.

By contrast, a transplantable line producing a monoclonal IgM was established by transfer of spleen cells into newborn and adult recipients. This result suggested that cells in the original donor spleen were producing the monoclonal IgM and, because of their transplantability, had malignant characteristics. This line, now in its third transplant generation, seems analogous to IgM-producing tumors induced in BALB/c mice by mineral oil injection (38). It is noteworthy that NZB mice share with BALB/c the ability to develop plasmacytomas after mineral oil injection (39).

Further evidence that the monoclonal IgM was produced in the spleen came from studies of immunoglobulin synthesis using another mouse with an enlarged cervical lymph node and a monoclonal IgM. Immunoautoradiographic analysis after culture of lymphoid organs with radioactive amino acids indicated that the IgM paraprotein was produced in the spleen and not in the enlarged cervical lymph node.[2] Further experiments with additional mice are underway.

Although λ-light chains are rare in BALB/c IgG and IgA myelomas, 5 of 10 B/W monoclonal IgM proteins that we studied were λ-type. The best known IgM-producing Balb/c plasmacytoma (104E) is also λ-type (38). Statistically it seems likely that normal B/W mouse IgM may not show a marked predominance of κ-light chains.

Experience with animals immunized to certain types of antigens suggests that the B/W monoclonal macroglobulins may have antibody activity (38). For example, Braun and Krause found that homogeneous antibodies were produced by rabbits immunized with streptococcol antigens (40). Genetic factors influenced production of these proteins that showed idiotypic cross-reactivity (41).

[2] Sugai, S., N. Talal, and R. Asofsky. Data to be published.

About 5% of mouse myeloma proteins have antigen-binding activity (38). These monoclonal immunoglobulins, generally of the IgA class, react with specific antigens such as nitrophenyls, phosphorylcholine, and specific sugar linkages such as $\alpha 1 \rightarrow 3$ dextrans and $\beta 1 \rightarrow 6$ galactose. For example, 11 IgA myeloma proteins precipitate with pneumococcus C polysaccharide because of their specificity for choline or phosphorylcholine (38). Several of these myeloma proteins also share a common idiotypic specificity.

In humans, certain IgM proteins that are cold agglutinins have properties of monoclonal immunoglobulins and share cross-specificity (42). Recently, a similar cross-idiotypic specificity has been found for monoclonal IgM proteins that are anti-γ globulins (43). Such monoclonal "rheumatoid factors" are frequently associated with "mixed cryoglobulinemia" (44) or Sjögren's syndrome (45).

The possible antibody activity and the significance of idiotypic cross-reactivity seen with the B/W monoclonal IgM proteins are under study. The sucrose gradient fractionation experiments indicate that they are not antibodies to DNA or to RNA.

This finding of monoclonal macroglobulinemia is particularly remarkable when one considers that New Zealand Black and hybrid mice are models for human autoimmune diseases such as systemic lupus erythematosus and Sjögren's syndrome. An association between autoimmunity and lymphoid neoplasia has been observed (46, 47). Monoclonal macroglobulinemia occurs particularly in Sjögren's syndrome (48). A generalized lymphoproliferative disorder of variable malignant potential is sometimes seen in such patients and has been termed pseudolymphoma (48). The New Zealand mice with salivary gland lymphoid infiltrates, generalized lymphoproliferation, autoantibodies, and now monoclonal IgM seem remarkably analogous to this human condition.

A possible contributing pathogenetic factor is latent virus infection. Stimulation of lymphocytes by graft-vs.-host reaction can activate latent murine leukemia virus (49). Leukemia virus is very prominent in the New Zealand mice and is probably implicated in their development of lymphoma and reticulum cell sarcoma (3, 28). The exact contribution of viral or immunologic factors to the various pathologic events in these animals may become clearer through further investigation. Recent attention has focused on a possible deficiency of suppressor T cells normally capable of suppressing autoantibody-producing B cell clones. A lack of such suppressor function, it is argued, permits the emergence of autoimmunity. This concept can now be extended to explain the emergence of monoclonal macroglobulins as well as autoantibodies in New Zealand mice. By analogy, it may also explain the production of autoantibodies and monoclonal IgM in human diseases such as Waldenström's macroglobulinemia and chronic lymphocytic leukemia.

SUMMARY

Serum monoclonal macroglobulins were detected in over 30% of NZB/NZW F_1 mice greater than 11 mo of age. The monoclonal nature of the IgM was shown by restricted electrophoretic mobility, characteristic appearance on immunoelectrophoresis, restriction to a single light chain type, and ability to induce anti-idiotypic antisera. The monoclonal macroglobulins were separated from antibodies to DNA and RNA that migrated in the 7S region of sucrose gradients. Enlarged lymph nodes were often present in mice with monoclonal IgM, and a transplantable IgM-producing lymphoid tumor was established from the spleen of one animal.

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