IMMUNOGLOBULINS IN NZB/BL MICE

I. Serum Immunoglobulin Levels and Immunoglobulin Class of Erythrocyte Autoantibody*

By NOEL L. WARNER,‡, § Ph.D., and RICHARD WISTAR, Jr.,§ M.D.

(From the Departments of Genetics and Medicine, Stanford University School of Medicine, Palo Alto, California 94304)

(Received for publication 25 August 1967)

Mice of the inbred strain NZB/Bl spontaneously develop several parameters of autoimmune disease, namely autoimmune hemolytic anemia (1–3), thymic germinal centers (4–7), and kidney lesions similar to those seen in systemic lupus erythematosus (8–10). These mice accordingly serve as a unique and invaluable model for a study of the underlying causes of autoimmune disease. Although at least one genetic component is involved in the development of this syndrome (11, 12), its precise mode of action is at present unknown.

Hypergammaglobulinemia is found in most human autoimmune diseases (13–15), and usually consists of an elevation of the γG-immunoglobulin class. The immunoglobulins in mice have been divided into five classes on the basis of antigenic (16, 17), functional (18, 19), and chemical (20, 21) properties. Quantitative assays have been developed for the measurement of each of these classes and the results obtained with the sera of NZB and other mouse strains are presented in this report.

It has previously been reported that a classical γ-globulin forms the major component of the protein coating on NZB erythrocytes leading to the direct Coombs'-positive reaction (22). This aspect has been examined with reagents capable of discriminating between the five mouse immunoglobulin classes, and a preliminary attempt was made to quantitate the amount of autoantibody on the red blood cells.

Materials and Methods

Mice.—The NZB mice used were obtained from two separate colonies. One of these was maintained in The Walter and Eliza Hall Institute, Melbourne, Australia, and the other in the Department of Medicine, Stanford University School of Medicine, Palo Alto, Calif. Both

* Supported by research Grant GM-12075 to Dr. L. A. Herzenberg, and Helen Hay Whitney Fellowship Grant F109.

‡ Fellow of the Helen Hay Whitney Foundation.

§ Present address: The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia. (Address for reprints).

colonies were originally derived from breeding pairs obtained from the colony of Dr. Marianne Bielschowsky. We are indebted to Dr. Margaret Holmes and Dr. Halsted Holman for providing the NZB mice or sera used in this study. Mice of the other inbred strains used and hybrids of NZB mice were obtained through the kindness of Dr. L. A. Herzenberg from colonies maintained in the Department of Genetics, Stanford University School of Medicine.

Mouse Serum Pools.—The various mouse sera used in this study were obtained by tail bleeding of the appropriate mice. Each serum pool consisted of equal volumes of 6–10 individual sera from mice of identical strain, age, and sex.

Estimation of Immunoglobulin Levels.—The basic assay used involves the inhibition of precipitation of ^{125}I-labeled immunoglobulins by the test serum as compared to a purified immunoglobulin standard using specific antisera for precipitation. The methodis similar to that reported by Weiler et al. (23), and Fahey and Lawrence (24) for the determination of human

TABLE I

The Antigen-Antibody Combinations Used to Quantitate Mouse Immunoglobulin Classes

Immunoglobulin class	Purified labeled immunoglobulin	Antiserum
γM	γM isolated from normal C57B1/10 serum	Rabbit anti-mouse (C57B1/10) γM absorbed with pooled normal mouse γG
γA	MPC-1 myeloma protein	Rabbit anti–MPC-1 ribosomes absorbed with pooled normal mouse γG
γG_1	MPC-25 myeloma protein	Rabbit anti–MOPC-21 myeloma protein absorbed with pooled normal mouse γG
γG_{2a}	RPC-5 myeloma protein	Rabbit anti–normal mouse γG absorbed with MPC-25 conjugated to PAPS* *or* C57B1/10 anti-C3H (antiallotype serum)

* Polyamino polystyrene.

immunoglobulin concentrations. Four different precipitating assays were developed, each specific for one of the five mouse immunoglobulin classes. (γG_{2b}-levels were not quantitated, see Results.) The four antigen-antibody combinations used are listed in Table I; each assay was shown to be specific for the designated immunoglobulin class by the failure of proteins of other classes to inhibit the precipitation of the labeled protein.

The serum concentration of each particular immunoglobulin class was determined by testing three to four serial twofold dilutions of the test serum for inhibitory capacity in the particular assay. The degree of inhibition was compared with that obtained with known amounts of a purified immunoglobulin preparation of the appropriate class. The results are plotted as the reciprocal of per cent–labeled antigen precipitated against the amount of inhibitor protein or serum used. The complete methodology used in these assays, i.e. amounts of reagents, buffers, conditions of precipitation, etc., was identical to that previously described in detail (25, 26).

Immunization of Rabbits.—Rabbits were immunized with purified mouse myeloma proteins or various fractions of normal immunoglobulins by methods previously described (26). When necessary, the antisera obtained were absorbed with either lyophilized mouse γ-globulin or mouse myeloma proteins.

Isolation and Iodination of Mouse Immunoglobulins.—The methods used for isolation of these proteins have been previously described in detail (26). Labeling with ^{125}I was performed by the method of Greenwood et al. (27). Many of the antigens and rabbit antisera used in this work were prepared in the course of other studies on mouse immunoglobulin antigens by Dr. L. A. Herzenberg and Dr. N. L. Warner. We are extremely grateful to Dr. Herzenberg for making these available for the studies reported in this paper.

Direct Antiglobulin (Coombs) Tests.—Mice were bled from the tail into citrate-saline and

TABLE II

Serum Immunoglobulin Concentrations in Mice

Strain	Age	Serum concentration, mg/ml			
		γM		γA	γG_1	γG_{2a}
	months					
NZB/B1	1	1.1	0.7			
	2	3.3	2.0	0.16	1.0	6.2
	3	3.3	3.5			
	4	4.4	2.7	0.23	1.2	9.5
	5	5.5				
	6	5.9	3.6	0.59	1.5	10.8
	7	5.5				
	8	8.0		0.56	1.3	12.8
	9		5.4	0.50	1.5	10.5
(NZC × NZB) F_1	4	1.8		0.12	1.3	11.5
(C57B1 × NZB) F_1	4.5	1.9		0.25	1.8	11.5
(C3H × NZB) F_1	6	1.4		0.16	2.7	5.1
NZC	4.5	1.4		NT	1.1	5.2
NZW	4	0.7		0.30	1.4	5.9
Balb/c	4	0.9		0.29	1.3	7.7
RIII/J	5	0.7		0.18	1.6	5.7
CE/J	7	0.6		0.23	1.1	9.0
DBA/2	8	0.9		NT	1.6	9.0
C3H.SW/HZ	5	1.5		1.10	2.5	20.6

NT, not tested.

the erythrocytes were washed four times in large volumes of isotonic saline at 37°C. The cells were then brought to a 20–30% suspension for the Coombs' test. Each suspension was tested as described by Long et al. (28), using various rabbit antisera at several dilutions.

Quantitation of Immunoglobulins on Mouse Erythrocytes.—Four times-washed mouse erythrocytes were brought to a certain volume in saline, a sample was counted in a hemocytometer, and the suspension then adjusted to a known cell concentration (usually around 4.0×10^9/ml). Samples of the cell suspensions were then placed in inhibition assays to determine the content of immunoglobulins of each class. The results are expressed as micrograms of immunoglobulin protein per 10^{10} red cells.

RESULTS

Serum Immunoglobulin Concentrations:—All determinations of serum immunoglobulin concentration given in Tables II and III were performed on pools of mouse sera. The values for NZB mice of ages 2–9 months from two different colonies are presented in Table II together with the values for several different inbred strains and their hybrids.

Recently it has been found that heterologous rabbit anti-mouse immunoglobulin sera may contain antibodies directed to allotypic determinants (29). These antisera cannot be used in assays to determine immunoglobulin concentration since sera from strains lacking the particular allotypic antigen will not be capable of completely inhibiting precipitation. A linear relationship will, therefore, not be obtained in the plot of reciprocal per cent precipitation. In

TABLE III

Serum Immunoglobulin Concentrations in NZB and Normal Mice

Strains	Age range	γM	γA	γG_1	γG_{2a}
	months				
NZB/B1	4–6	4.4*	0.43	1.3	9.9
NZB hybrids‡ (3)	4–6	1.7	0.18	1.9	9.3
Normal strains§ (7)	4–8	0.9	0.25	1.5	9.0

* Each value represents the mean immunoglobulin concentration for the respective strains in mg/ml.

‡ Hybrids listed in Table II.

§ Normal strains listed in Table II.

the γG_{2b}-assay it was found that all rabbit anti-mouse γG_{2b}-sera used, detected an allotypic antigen present in all strains except those carrying the Ig-3^d and Ig-3^e alleles (30) which include NZB. Values for γG_{2b}-levels in NZB have not, therefore, been included. Some inhibition was still obtained, however, with NZB sera in the γG_{2b}-assay, due to antibodies in the rabbit antiserum directed to (nonallotypic) γG_{2b}-class-defining specificities, and it appeared that the level of γG_{2b} was *not* elevated in NZB sera. Allotypic antigens were not detected in any of the other four assays, since all mouse sera used completely inhibited precipitation of the labeled antigen when sufficient inhibitor was used.

The results of immunoglobulin class quantitation from various strains have been grouped, and a mean value determined. This is presented in Table III for mice between 4–6 months of age. In view of the consistent elevation of γM-immunoglobulins observed in pooled NZB sera, individual mouse sera were also analyzed. It was found that most NZB mice tested had elevated

γM-levels (10/14 8 months over 2 mg/ml), whereas only an occasional normal mouse showed an elevation over 2 mg/ml (2/20 C3H and BDP 1-yr-old mice). Old C57Bl/10 mice were also found to have slightly elevated γM-levels (mean 1.8 mg/ml at 1 yr) although it should be stressed that younger C57Bl/10 mice had normal levels (mean 0.9 mg/ml at 8 months).

Antiglobulin Tests with NZB Mouse Erythrocytes:—Washed erythrocyte suspensions from 39 NZB mice ranging in age from 7–12 months old were tested by the direct Coombs' test with various antiglobulin reagents. 35 of the 39 mice gave a positive direct Coombs' test with a polyvalent rabbit anti-mouse whole serum (previously absorbed with C57B1-washed red cells). Four other antisera were then tested for their ability to agglutinate many of the NZB erythrocyte suspensions. Each of these antisera had been appropriately

TABLE IV

Immunoglobulin Class of Erythrocyte Autoantibody in NZB/Bl Mice

Age of mice	Direct Coombs' test with specific Anti-immunoglobulin sera			
	γM	γG_1	γG_{2a}	γA
months				
7–9	50* (6/12)‡	91 (10/11)	50 (5/10)	20 (2/10)
10–12	65 (15/23)	83 (15/18)	70 (14/20)	50 (7/14)

* Per cent of mice with direct Coomb's-positive test.

‡ No. positive/No. tested.

absorbed where necessary to render them specific for only one of the mouse immunoglobulin classes. Each antiserum was titrated against each of the five ^{125}I-labeled purified immunoglobulin preparations (indicated in Table I with MPC-31 for γG_{2b}), and was found capable of precipitating only one of these proteins. Three of the four antisera used were absorbed rabbit anti-mouse immunoglobulin sera, the other being an alloantiserum containing antibodies directed to γG_{2a}–allotypic specificities present in NZB. Although the specific rabbit antisera were not absorbed with normal mouse red cells no agglutination of washed erythrocytes from any normal strain was ever detected with these sera.

The results of antiglobulin tests are presented in Table IV as the number and percentage of mice tested that give positive Coombs' tests with the various antisera. The group of mice tested included approximately equal numbers of both sexes, and the Coombs' results were similar for both. The mice were divided into two age ranges, 7–9 and 10–12 months. (These are different mice

and not repeated tests on the same mice.) Although the results for the two groups are basically similar, a higher percentage of positive Coombs' tests was obtained in the older mice with all reagents except anti-γG_1. Nearly all mice have γG_1-autoantibodies on their red cells, and a very strong agglutination was usually obtained with the anti-γG_1-reagent. The results from both groups show that 86% of the mice had γG_1-autoantibodies on their red cells. The other immunoglobulin classes were encountered less frequently. The autoantibody population can therefore consist of antibodies of any of the immunoglobulin classes (γG_{2b} has not been tested), and furthermore most of the mice

TABLE V

Immunoglobulin Class of Erythrocyte Autoantibody in NZB/Bl Hybrid Mice

Strain	Age	Direct Coombs' test with specific Anti-immunoglobulin sera				
		Poly-valent	γM	γG_1	γG_{2a}	γA
	months					
(NZC × NZB)F_1	7–8	0/5*	—	—	—	—
	8–9	10/15	2/10	7/10	1/10	0/10
	9½–12	12/14	5/12	11/12	2/12	0/12
	13–16	11/11	11/11	10/11	2/10	NT
	21	9/9	NT	NT	4/9‡	NT
NZB Hybrids with C57B1/10(6) DA/Hu(6) & FZ/Di(4)	12	1/16	—	—	—	—

NT, not tested.

* No. of Coombs'-positive mice/No. tested.

‡ 4/9 Coombs'-positive with Balb/C anti-NZB alloantiserum; 0/9 NZB anti-NZC alloantiserum.

simultaneously have autoantibodies of more than one class. 8 of the 35 positive mice simultaneously had autoantibodies of all four immunoglobulin classes. There did not seem to be any pattern of association of classes in the other mice; i.e., some mice positive for γM were positive for γG_{2a} and some were not, etc. With only one exception, however, γA-antibodies were only found when all other classes were present.

Antiglobulin Tests with (NZB × NZC)F_1 Hybrid Mouse Erythrocytes:— Erythrocytes from 15 (NZB × NZC)F_1 hybrids and 16 hybrids of NZB with other strains were Coombs' tested with various antiglobulin sera. The tests were repeated on the same mice at different ages. By 8–9 months many of the (NZB × NZC)F_1 hybrids were Coombs' positive, whereas even by 12 months, only 1/16 of the other hybrids was Coombs' positive. The 15 (NZB × NZC)F_1 hybrids included six males and nine females, and the degree of agglutination

obtained with the erythrocytes from the female mice was usually much greater than from males. Although the numbers of mice are small, there was a definite tendency for the female mice to become Coombs' positive before the males.

The results are presented in Table V and show that the incidence of γM-autoantibodies rises with increasing age of the mice, whereas the incidence of γG_1-autoantibodies is nearly maximal at all ages tested. The incidence of both γG_{2a}- and γA-autoantibodies is much lower than that observed in the NZB mice. The four mice which had γG_{2a}-autoantibodies were also Coombs' positive with an alloantiserum specific for the NZB parental type.

Quantitation of Immunoglobulins on Mouse Erythrocytes:—In an attempt to quantitate the amount of autoantibody of each immunoglobulin class present on the NZB erythrocyte, we have taken advantage of the inhibiton of precipitation assays used for determining serum immunoglobulin levels. Mouse erythrocytes were washed four times in saline, and samples of the red cell suspension (of known concentration) were placed in various immunoglobulin-inhibition assays. Inhibition of precipitation of the labeled immunoglobulin should only be given by virtue of the presence of the immunoglobulin (anti-erythrocyte autoantibody) on the red cell surface. By comparing the degree of inhibition obtained with the "coated" red cells with that given by a purified immunoglobulin a value was obtained for the amount of immunoglobulin per given number of red cells.

Unfortunately, washed mouse erythrocytes from normal strains have always given a slight degree of inhibition. This is not due to a failure to wash out all the serum proteins, since when sheep red cells were incubated in normal mouse serum and then washed in an identical fashion no inhibition at all was observed (whereas when mouse anti-sheep erythrocyte serum is used with sheep red cells, marked inhibition of precipitation is subsequently observed).

Erythrocytes from 16 normal mice of ages between 6 and 12 months (4 at 6 months, 8 at 10 months, 4 at 12 months) and representing seven different strains (three C57Bl/10; three C3H.SW; one SEA/J; one A.SW; one LP; one BDP/J; six (C3H $\times$ DBA/2)F_1) were washed four times and tested in the different inhibition assays, which were specific for γM-, γG_{2a}-, and a γG-assay in which γG_1, γG_{2a}, and γG_{2b} were all detected (but not discriminated).

Erythrocytes from 13 NZB/B1 mice were similarly tested. (Ages: one at 3 months; one at 6 months; three at 8 months; four at 10 months; three at 12 months, and one at 18 months.)

All the normal mice were Coombs' negative, and all of the NZB mice (except the two at 3 and 6 months) were direct Coombs' positive with anti-γG- and anti-γM-antisera.

The results of inhibition assays with the washed erythrocytes are given in Table VI, as the micrograms of immunoglobulin per 10^{10} red blood cells. Although the normal cells have given some inhibition, a significant elevation of

γM and total γG with only slight elevation of γG_{2a}, was observed for the NZB erythrocytes. The values for the NZB erythrocytes less those of the normals would approximately correspond to 400 molecules of γM and 5000 molecules of γG per red cell (taking mol wt of γM at 900,000 and γG at 160,000).

DISCUSSION

Inbred New Zealand black mice spontaneously develop a broad spectrum of immunological aberrations which range from the production of autoantibodies of several specificities (28, 31, 32) to lymphoma development (33). These animals accordingly provide us with an eminently suitable model for examination of possible genetic components involved in autoimmune disease. This report is concerned with the expression of the immunoglobulin H chain

TABLE VI

Quantitation of γM- and γG-Immunoglobulins on NZB Mouse Erythrocytes

Mice	No.	Micrograms of immunoglobulin per 10^{10} erythrocytes		
		γM	Total γG	γG_{2a}
Controls*	16	1.7‡(0.7-2.7)§	3.1(2.0-8.6)	2.5(1.8-4.0)
NZB	13	8.3(2.8-16.5)	15.9(4.8-85.0)	5.5(1.7-12.8)

* Eight Coombs'-negative mouse strains represented.

‡ Median value.

§ Range of values.

genes in NZB mice as determined by measuring serum immunoglobulin class levels, and the class(es) of erythrocyte autoantibodies.

Determination of serum immunoglobulin concentrations has shown that NZB mice have a specific elevation of only one immunoglobulin class, γM (macroglobulin). The degree of γM-elevation is by no means as high as that observed in classical Waldenström's macroglobulinemia (34), but is still particularly striking in that it is both specific for γM and is detectable many months prior to any previously recognized symptoms in these mice. The quantitative results presented in this report for all the immunoglobulin classes are in agreement with immunoelectrophoretic studies (35) which also demonstrated apparent increases in γM, and with several reports of serum protein concentrations as determined by paper (10) or cellulose acetate electrophoresis (36) which demonstrated elevations in the γ-globulin region in sera of NZB mice. These latter observations however did not distinguish between the different immunoglobulin classes.

The specific elevation of γM in NZB mice is quite different from the hyper-

gammaglobulinemia of γG-type which is frequently observed in human autoimmune disease (15), and the marked hypergammaglobulinemia in experimental aleutian mink disease (37).

Several possible explanations for the elevation of γM in these mice might be advanced. A decrease in catabolism of this protein would lead to increased serum levels without requiring any increased synthesis. Alternatively, an increased synthesis leading to elevated serum levels could be due to increased production per cell or to an increase in the number of γM-synthesizing cells. Recent observations in man have shown that some of the human immunoglobulin H chain genes appear to be associated with different rates of synthesis of the protein (38). It is therefore possible that the genetic type (allotype) of NZB γM is also associated with an elevated rate of synthesis. However, in view of the well established lymphoproliferative changes observed in these mice (2, 33, 35), an increase in γM-synthesizing cells probably occurs. This could represent either a general immunological hyperreactivity or specific antibody responses.

Several groups have indeed speculated that autoimmunity may be associated with (perhaps dependent upon) immunological hyperreactivity (35, 39). There are, however, several difficulties with this concept as applied to NZB mice. Firstly, it would be unlikely that such a general hyperreactivity would not involve the γG-immunoglobulin classes; and secondly, preliminary observations have indicated that the response of NZB mice to direct immunization is not significantly elevated as compared with the range of reactivity observed in normal mouse strains.[1] One report has, however, stated that NZB mice do show increased hemolysin plaque production to sheep red cells as compared with one normal mouse strain (40). This is difficult to evaluate, as there is a wide variation in the response to any given antigen by different inbred mouse strains (41, 42).[2] This question will need critical examination with attention being paid to an analysis of γM- and γG-responses. Such studies are at present in progress.

The second alternative mentioned above implies that the increased γM represents specific antibody production. The known autoantibodies in NZB mice do not account for this elevated γM since the antinuclear factors are γG (31) and although some of the erythrocyte autoantibodies are γM-type, γG-components (particularly γG_1) are also involved without being accompanied by an apparent increase in serum level.

Several recent reports relating to human macroglobulinemia might provide some clues for this increased γM. Seligmann et al. (43) have made a systematic

---

[1] Warner, N. L., M. Davis, and H. H. Fudenberg. Unpublished observations.

[2] Warner, N. L., N. M. Vaz, and Z. Ovary. 1967. Immunoglobulin classes in antibody responses in mice. I. Analysis by biological properties. Submitted for publication.

survey of the sera of family members of Waldenström macroglobulinemic patients. Three main abnormalities were observed in these patients: (*a*) presence of a paraprotein of γM-type; (*b*) abnormal immunoglobulin levels without detectable paraproteins; and (*c*) abnormally high incidence of anti–γ-globulin factors in relatives less than 60 yr of age. These observations show an association of macroglobulinemia with some autoimmune manifestations, in a similar fashion to the well recognized association between acquired agammaglobulinemia and autoimmunity (44). The similar association of autoimmunity and elevated macroglobulin levels in NZB mice may well indicate an underlying genetic defect linking these various aberrations of the immune system.

Furthermore, it is pertinent to note that the three hybrids of NZB mice tested showed a lesser but still detectable elevation of γM. The elevation of all immunoglobulin classes in the C3H.SW mice is probably accounted for by their being under constant excessive antigenic stimulation, as this colony showed repeated infections and numerous early deaths.

Analysis of the immunoglobulin coating on red blood cells of old (8–12 month) NZB mice has demonstrated that all immunoglobulin classes can be represented in the autoantibody population. In a previous study by Norins and Holmes (22), it was found that the major coating of red cells in direct Coombs'-positive NZB mice was a γ-globulin. Our results are in agreement with this observation in identifying the major component to be γG_1-globulin; however, other immunoglobulin classes were also detected in the autoantibody population (notably γM). This was probably not detected in the previous study (22) since the antisera used apparently did not recognize γM-globulin. A report on the serum of one NZB mouse (giving indirect Coombs'-positive test) does describe γM-autoantibody, but not γG (10). Examination of more mice would probably have also demonstrated γG.

Our observations are therefore in agreement with other reports (22, 28, 45) in indicating that in the NZB mice, as for warm erythrocyte autoantibodies in acquired human hemolytic anemia (46), the bulk of the autoantibody response is of a classical γG-globulin.

Antierythrocyte autoantibodies of several specificities have been described in NZB mice (28, 32). The results presented in this paper do not discriminate between these possible antibody populations.

From intensity of agglutination patterns, incidence in the mice, and preliminary quantitative data, it appears that a marked preponderance of the antibodies are γG_1. Expression of the various immunoglobulin classes in a given antibody population can be markedly altered by the form of the antigen administration (47). Of particular relevance is the preponderance of γG_1-antibodies in mice immunized with an antigen in the absence of complete Freund's adjuvant.[2] Further analysis of this restriction in immunoglobulin class of autoantibody is in progress in NZB mice. Of particular importance is

the expression of the parental types in the autoantibody population. This can be examined in (NZB × NZC)F_1 hybrids, since the two parental types belong to different alleles at each of the mouse immunoglobulin H chain loci (25). Unfortunately, antigenic markers for γG_1-allotypes have not been detected, even though a polymorphism of this heavy chain gene has been found by analysis of electrophoretic mobility (48). In the four (NZB × NZC)F_1 mice that eventually developed γG_{2a}-autoantibody, NZB components were clearly demonstrated, although NZC immunoglobulin was not detected. However, in view of the lower titer of antibody used to detect NZC (as judged by precipitation of ^{125}I-labeled globulin) further tests are needed to determine the incidence of the parental types.

It should be stressed that sequential examination of individual mice has not been done and these results do not provide any further information on the question of possible γM- to γG-conversion (49) within a specific antibody-forming clone.

Restriction of erythrocyte autoantibodies in human-acquired hemolytic anemia has been observed in some instances by examination of L chain types, and electrophoretic mobility of the eluted antibodies (50). We do not have any comparative data on L chain types at present. However, it is interesting to note that most of the restriction in electrophoretic mobility of human erythrocyte autoantibody was in the fast migrating region, analogous perhaps to γG_1 in mice. Examination of human H chain subclasses might also indicate restriction in erythrocyte autoantibodies.

The expression of red cell autoantibodies in NZB hybrid mice is clearly influenced by genetic factors (11, 12). As previously reported (51), (NZB × NZC) F_1 hybrids appear to be relatively unique amongst NZB hybrids in that development of Coombs' positivity occurs at a similar age to the NZB parent strain. However, as demonstrated by expression of the immunoglobulin classes, the full qualitative development of erythrocyte autoantibodies observed in NZB mice is not realized in most of the (NZB × NZC) F_1 hybrids.

Preliminary attempts to quantitate the amount of autoantibody of various classes on the NZB red cell has been partly hampered by the persistent background inhibition of precipitation given by washed normal mouse red cells. This background level is probably not due to immunoglobulin, since inhibition was still given in assays detecting an allelic form not represented in the genotype of the normal red cell donor. Calculation of the approximate number of immunoglobulin molecules per NZB red cell gives the value of 400 γM-molecules and 5000 γG-molecules.

Previous studies of the amount of antierythrocyte antibody on red blood cells have shown a wide variability between the number of molecules required to saturate the available antigen sites and the number required for the detection of the biological activity of the antibody. Greenbury, Moore, and Nunn

(52), using rabbit antisera and human A red cells, found that the maximum number of molecules which could be bound per cell was 8.3×10^5 for γG and 1.7×10^5 for γM. In contrast the number of molecules required at the 50% agglutinating concentration was 1.9×10^4 for γG and 2.5×10^1 for γM per cell. In the case of human anti-Rh antibody, perhaps more analogous to our experimental situation, Mollison and Hughes-Jones (53) showed that as few as 10 molecules of antibody per Rh-positive cell would significantly decrease the in vivo half-life of the coated cells. The data reported in the present paper concerns the actual measurement of the number of antibody molecules fixed in vivo to the red cells rather than a minimum number needed for detecting antibody activity or the maximum number capable of being bound to the red cell. Thus it is apparent that for a full understanding of the antibody-mediated anemic state we must take into account the quantity of antibody present on the red cells and the biologic activity of the immunoglobulin class of erythrocyte-bound autoantibody.

SUMMARY

Determination of serum immunoglobulin levels in NZB and normal mice has indicated that an elevation of the γM-globulin level occurs in NZB mice. This can be demonstrated in young NZB mice well before other autoimmune manifestations are observed. A slight elevation of γM-globulin was also observed in NZB hybrid mice.

Erythrocyte autoantibodies were analyzed by the direct Coombs' test with specific antiimmunoglobulin reagents. Autoantibodies in NZB mice can be of all immunoglobulin classes, although predominantly of γG_1-class. In (NZB $\times$ NZC)F_1 hybrid mice, although Coombs' positivity develops around the same age as in NZB mice, there is a greater restriction of the autoantibodies into γM- and γG_1-classes.

Preliminary attempts to quantitate immunoglobulins coating NZB red cells have shown that there are approximately 400 molecules of γM and 5000 molecules of γG-globulin per NZB mouse red cell.

This work was carried out in the laboratory of Dr. L. A. Herzenberg to whom we are most grateful for advice and discussion. Excellent technical assistance was given by Mr. G. M. Iverson.

BIBLIOGRAPHY

1. Bielschowsky, M., B. J. Helyer, and J. B. Howie. 1959. Spontaneous haemolytic anaemia in mice of the NZB/B1 strain. *Proc. Univ. Otago Med. School.* **37**:9.
2. Holmes, M. C., and F. M. Burnet. 1963. The natural history of autoimmune disease in NZB mice. *Ann. Internal Med.* **59**:265.
3. Helyer, B. J., and J. B. Howie. 1963. Spontaneous autoimmune disease in NZB/B1 mice. *Brit. J. Haematol.* **9**:119.
4. Burnet, F. M., and M. C. Holmes. 1964. Thymic lesions associated with autoim-

mune disease in mice of strain NZB. *In* The Thymus in Immunobiology. R. A. Good and A. E. Gabrielsen, editors. Hoeber Medical Division, Harper and Row, New York. 656.

5. East, J., and M. A. B. de Sousa. 1966. The thymus autoimmunity and malignancy in New Zealand black mice. *J. Natl. Cancer Inst. Monograph No. 22.* 605.
6. Siegler, R. 1965. Pathogenesis of thymic changes in NZB mice with hemolytic anemia. *J. Exptl. Med.* **122:**929.
7. de Vries, M. J., and W. Hijmans. 1967. Pathological changes of thymic epithelial cells and autoimmune disease in NZB, NZW and (NZB × NZW) F_1 mice. *Immunology.* **12:**179.
8. Helyer, B. J., and J. B. Howie. 1963. Renal disease associated with positive lupus erythematosus tests in a cross bred strain of mice. *Nature.* **197:**197.
9. Burnet, F. M., and M. C. Holmes. 1965. The natural history of the NZB/NZW F_1 hybrid mouse. A laboratory model of systemic lupus erythematosus. *Australian Ann. Med.* **14:**185.
10. Mellors, R. C. 1965. Autoimmune disease in NZB/B1 mice. I. Pathology and pathogenesis of a model system of spontaneous glomerulonephritis. *J. Exptl. Med.* **122:**25.
11. Holmes, M. C., and F. M. Burnet. 1964. The inheritance of autoimmune disease in mice: A study of hybrids of the strains NZB and C3H. *Heredity.* **19:**419.
12. Burnet, F. M., and M. C. Holmes. 1965. Genetic investigations of autoimmune disease in mice. *Nature.* **207:**368.
13. Roitt, I. M., P. N. Campbell, and D. Doniach. 1958. The nature of the gamma-globulins in lymphadenoid goitre. *Biochem. J.* **69:**248.
14. Kunkel, H. G., and E. M. Tan. 1964. Autoantibodies and disease. *Advan. Immunol.* **4:**351.
15. Mackay, I. R., and F. M. Burnet. 1963. Autoimmune Diseases: Pathogenesis, Chemistry and Therapy. Charles C Thomas, Springfield, Ill.
16. Fahey, J. L., J. Wunderlich, and R. Mishell. 1964. The immunoglobulins of mice. I. Four major classes of immunoglobulins $7S_{\gamma 2}$-, $7S_{\gamma 1}$-, γ_{1A} (β_{2A})-, and $18S_{\gamma 1m}$-globulin. *J. Exptl. Med.* **120:** 233.
17. Fahey, J. L., J. Wunderlich, and R. Mishell. 1964. The immunoglobulins of mice II. Two sub classes of mouse 7S γ_2 - globulins: γ_{2a} -, and γ_{2b} - globulins. *J. Exptl. Med.* **120:**243.
18. Nussenzweig, R. S., C. Merryman, and B. Benacerraf. 1964. Electrophoretic separation and properties of mouse anti-hapten antibodies involved in passive cutaneous anaphylaxis and passive hemolysis. *J. Exptl. Med.* **120:**315.
19. Ovary, Z., W. F. Barth, and J. L. Fahey. 1965. The immunoglobulins of mice III. Skin sensitizing activity of mouse immunoglobulins. *J. Immunol.* **94:**410.
20. Potter, M., and E. L. Kuff. 1964. Disorders in the differentiation of protein secretion in neoplastic plasma cells. *J. Mol. Biol.* **9:**537.
21. Potter, M., E. Apella, and S. Geisser. 1965. Variations in the heavy polypeptide chain structure of gamma myeloma immunoglobulins from an inbred strain of mice and a hypothesis as to their origin. *J. Mol. Biol.* **14:**361.
22. Norins, L. C., and M. C. Holmes. 1964. Globulins on NZB mouse erythrocytes. *J. Immunol.* **93:**897.

23. Weiler, R. J., D. Hofstra, A. Szentivanyi, R. Blaisdell, and D. W. Talmage. 1960. The inhibition of labelled antigen precipitation as a measure of serum γ-globulin. *J. Immunol.* **85**:130.
24. Fahey, J. L., and M. E. Lawrence. 1963. Quantitative determination of 6.6S γ-globulins, β_{2A} globulins, and γ_1-macroglobulins in human serum. *J. Immunol.* **91**:597.
25. Herzenberg, L. A., N. L. Warner, and L. A. Herzenberg. 1965. Immunoglobulin isoantigens (allotypes) in the mouse. I. Genetics and cross reactions of the 7S γ_{2A}-isoantigens controlled by alleles at the Ig-1 Locus. *J. Exptl. Med.* **121**:415.
26. Herzenberg, L. A., and N. L. Warner. 1967. Genetic control of mouse immunoglobulins. *In* Symposium on Regulation of the Antibody Response, Toronto, Canada, 1966. In press.
27. Greenwood, F. C., W. M. Hunter, and J. S. Glover. 1963. The preparation of I^{131} labelled human growth hormone of high specific radioactivity. *Biochem. J.* **89**: 114.
28. Long, G., M. C. Holmes, and F. M. Burnet. 1963. Autoantibodies produced against mouse erythrocytes in NZB mice. *Aust. J. Exp. Biol. Med. Sci.* **41**:315.
29. Warner, N. L., and L. A. Herzenberg. 1966. Immunoglobulin isoantigens (allotypes) in the mouse. III. Detection of allotypic antigens with heterologous antisera. *J. Immunol.* **97**:525.
30. Warner, N. L., L. A. Herzenberg, and G. Goldstein. 1966. Immunoglobulin isoantigens (allotypes) in the mouse. II. Allotypic analysis of three γG_2-myeloma proteins from (NZB × Balb/c) F_1 hybrids and of normal γG_2-globulins. *J. Exptl. Med.* **123**:707.
31. Norins, L. C., and M. C. Holmes. 1964. Antinuclear factor in mice. *J. Immunol.* **93**:148.
32. Holborow, E. J., R. D. S. Barnes, and M. Tuffrey. 1965. A new red-cell autoantibody in NZB mice. *Nature.* **207**:601.
33. Mellors, R. C. 1966. Autoimmune disease in NZB/B1 mice. II. Autoimmunity and malignant lymphoma. *Blood.* **27**:435.
34. Waldenström, J. 1965. Macroglobulinaemia. *Advan. Metab. Disorders.* **2**:116.
35. East, J., M. A. B. de Sousa, and D. M. V. Parrott. 1965. Immunopathology of New Zealand Black (NZB) mice. *Transplantation.* **3**:711.
36. Kamm, D. P., F. Miyasato, and V. E. Pollak. 1966. Serum proteins in NZB/B1 and related strains of mice. *Proc. Soc. Exptl. Biol. Med.* **122**:903.
37. Henson, J. B., J. R. Gorham, R. W. Leader, and B. M. Wagner. 1962. Experimental hypergammaglobulinemia in mink. *J. Exptl. Med.* **116**:357.
38. Yount, W. J., H. G. Kunkel, and S. D. Litwin. 1967. Studies of the Vi (γ_{2c}) subgroup of γ-globulin. A relationship between concentration and genetic type among normal individuals. *J. Exptl. Med.* **125**:177.
39. Zingale, S. B., J. C. S. Avalos, J. A. Andrada, S. G. Stringa, and J. A. Manni. 1963. Appearance of anticoagulant factors and certain autoimmune antibodies following antigenic stimulation with blood group substance in patients with systemic lupus erythematosus. *Arthritis Rheumat.* **6**:581.
40. Diener, E. 1966. The immune response in NZB and NZB × C3H hybrid mice as

measured by the haemolysin plaque technique. *Intern. Arch. Allergy Appl. Immunol.* **30**:120.

41. Fink, M. A., and V. A. Quinn. 1953. Antibody production in inbred strains of mice. *J. Immunol.* **70**:61.
42. Barth, W. F., C. L. McLaughlin, and J. L. Fahey. 1965. The immunoglobulins of mice. VI Response to immunization. *J. Immunol.* **95**:781.
43. Seligmann, M., F. Danon, C. Mikaesco, and H. H. Fudenberg. 1967. The occurrence of immunoglobulin abnormalities in families of patients with Waldenstrom macroglobulinemia. *Am. J. Med.* **43**:66.
44. Fudenberg, H. H., J. L. German, and H. G. Kunkel. 1962. Occurrence of rheumatoid factor and other abnormalities in families of patients with agammalobulinemia. *Arthritis Rheumat.* **5**: 565.
45. Giltinan, P. J., L. C. Norins, and M. C. Holmes. 1965. Relation of reticulocytosis to positive Coombs tests in NZB and other mice. *Blood.* **26**:790.
46. Dacie, J. V. 1962. The Haemolytic Anemias. Congenital and Acquired Part 2. The Autoimmune Anaemias. J. & A. Churchill Ltd., London.
47. Coe, J. E. 1966. $7S_{\gamma 1}$ and $7S_{\gamma 2}$ antibody response in the mouse. I. Influence of strain, antigen and adjuvant. *J. Immunol.* **96**:744.
48. Minna, J. D., G. M. Iverson, and L. A. Herzenberg. 1967. Identification of a gene locus for γG_1 immunoglobulin H chains and its linkage to the H chain chromosome region in the mouse. *Proc. Natl. Acad. Sci. U. S.* **58**:188.
49. Nossal, G, J. V., A. Szenberg, G. L. Ada, and C. M. Austin. 1964. Single cell studies on 19S antibody production. *J. Exptl. Med.* **119**:485.
50 Leddy, J. P., and R. F. Bakemeier. 1965. Structural aspects of human erythrocyte autoantibodies. I. L chain types and electrophoretic dispersion. *J. Exptl. Med.* **121**:1.
51. Bielschowsky, M., and F. Bielschowsky. 1964. Observations on NZB/B1 mice. Differential fertility in reciprocal crosses and the transmission of the autoimmune hemolytic anemia to NZB/B1 and NZC/B1 hybrids. *Australian J. Exptl. Biol. Med. Sci.* **42**:561.
52. Greenbury, C. L., D. H. Moore, and L. A. C. Nunn. 1963. Reaction of 7S and 19 S components of immune rabbit antisera with human groups A and AB red cells. Immunology. **6**:421.
53. Mollison, P. L., and N. C. Hughes-Jones. 1967. Clearance of Rh-positive red cells by low concentrations of Rh antibody. *Immunology.* **12**:63.