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INCREASED 7S ANTIBODY RESPONSE TO SHEEP ERYTHROCYTES IN THE 2-MONTH-OLD NZB MOUSE

J. BAUM

Department of Internal Medicine, Rheumatic Disease Unit, The University of Texas, Southwestern Medical School, Dallas, Texas, U.S.A.

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SUMMARY

The validity of the use of the NZB mouse as a model for systemic lupus erythematosus is supported by a study of the 7S and 19S antibody response to immunization with sheep erythrocytes. In comparison with CBA and C3H mice the 2-month-old NZB animals made significantly more whole serum antibody. DBA, C57BL and A/Jax animals made about the same amount of whole serum antibody. Compared to all the control groups the NZB animals made significantly more 7S antibody in eight of twenty examinations of 2-mercaptoethanol resistant antibody studied from 1 to 8 weeks after immunization. Old NZB animals showed the same degree of 7S antibody production as the 2-month-old animals and were no different from a group of old White Swiss mice. The NZB animals thus show early immunological hyperreactivity most prominently in the 7S immunoglobulin fraction at a time when virtually no autoantibodies are present.

INTRODUCTION

The NZB mouse is considered an excellent model for autoimmune disease (Holmes & Burnet, 1963) and has been considered most closely related to systemic lupus erythematosus (SLE) (Stastny & Ziff, 1967). Since we have previously noted that in SLE most of the antinuclear factor is IgG (7S) (Baum & Ziff, 1962) and more recently that 7S antibody is occasionally prominent in the response to a bacterial antigen in SLE (Baum & Ziff, 1969) we decided to see if in the animal model 7S antibody would also show an increased response to a cellular antigen, the sheep erythrocyte. This light antibody appears to be the one solely concerned in the development of autoimmune disease in the NZB mouse. Recent studies by Lambert & Dixon (1968) of the renal lesion in the NZB/W F_1 hybrids have shown by immunoelectrophoresis that only IgG can be detected in eluates from the affected kidneys. In addition, a study of the serum immunoglobulins producing the antinuclear factor (ANF) in the NZB mouse (Norins & Holmes, 1964a) showed all the reacting antibody to be IgG.

Correspondence: Dr John Baum, Department of Medicine, University of Rochester, School of Medicine and Dentistry, 260 Crittenden Boulevard, Rochester, New York 14620, U.S.A.

MATERIALS AND METHODS

NZB animals maintained and bred in our own colony were used. There were two age groups in this study. One was composed of animals 12 months old and the others were 2 months old at the time of immunization. Both had approximately equal numbers of males and females. The old NZBs were compared to a group matched for age from a colony of outbred White Swiss mice maintained locally. Young Swiss mice of the required matched age were not available from this colony. Controls for the initial group of twelve 2-month-old

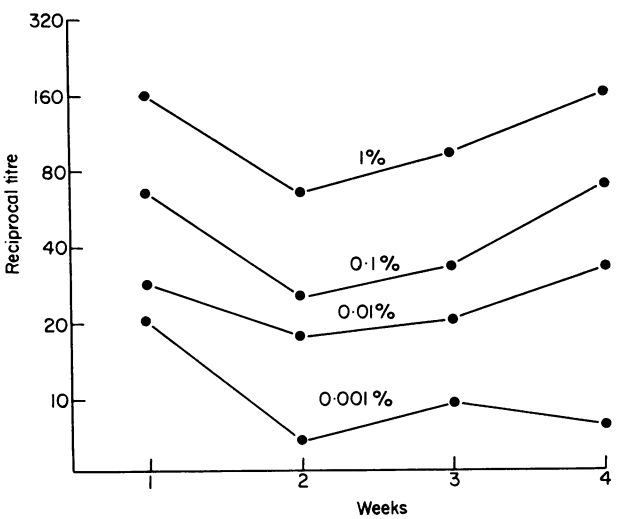

FIG. 1. Whole serum titre response to various concentrations of sheep erythrocytes in a control pool of 2-month-old C57/BL and A/Jax mice. The minimal consistent response showing 2-mercaptoethanol resistant antibody was found at the 0·01% sheep erythrocyte concentration.

NZB mice were six C57BL, six A/Jax and twelve DBA mice obtained from the Jackson Laboratories. Although the results from the initial series appeared to give a definitive answer it was felt that more strains should be studied for additional confirmation. Since some variation between the two groups of young NZB animals was seen (Table 6), each served as the control only in the series where the tests were performed at the same time against the other strains. A second series compared twelve 2-month-old NZB mice with age and sex matched C3H and CBA animals. The initial control groups had fourteen C3H and twenty-seven CBA mice.

Sheep erythrocytes for injection were washed twice in normal saline and then injected intraperitoneally in the appropriate concentration. Preliminary experiments were done to establish an optimum concentration of the injected antigen. It was felt that this should be the minimal dose required to give a good whole serum titre in the normal animals with adequate 7S antibody levels. When 0·01% sheep erythrocytes were used as the antigenic stimulus (Fig. 1) 2-mercaptoethanol (2-ME) resistant 7S antibodies were found. However, all antibody activity was destroyed when the sera from animals given the 0·001% sheep erythrocyte concentration were tested with 2-mercaptoethanol. The group tested for this response

was a pool of 2-month-old C57BL and A/Jax mice. A similar preliminary study of 12-month-old Swiss mice indicated a diminished response in the older animals and a 0·1% concentration of sheep erythrocytes was thus found to be the optimum concentration in the older animals. Therefore, the concentrations used were 0·01% sheep erythrocytes ($4{\cdot}8 \times 10^7$ RBC) for the young mice and 0·1% sheep erythrocytes ($4{\cdot}8 \times 10^8$ RBC) in the old mice. Bleedings were done from the inner plexus of the eye with capillary tubes. Enough blood was obtained by retro-orbital plexus bleeding to test once for total titre and once for mercaptoethanol resistant antibody. All sera were inactivated at 56°C for 30 min before testing. Samples were obtained in most animals in each group at 1, 2, 3, 4 and 8 weeks following antigenic stimulation. The 12-month-old animals were followed for 8 weeks but because of a high mortality rate after the 4th week there was insufficient data for analysis at week 8.

Antibody to sheep erythrocytes was measured by a microtitration method (Wegmann & Smithies, 1966). As indicated above the 7S or IgG antibody was determined by its 2-mercaptoethanol resistance. The sera were diluted 1:5 with a solution of 2-ME to give a final concentration of 0·05 M and incubated at 37°C for 1 hr. The sera was then kept at 4°C overnight before haemagglutination titres were performed.

The mercaptoethanol was not removed by dialysis since we found as did Rowley & Fitch (1965) that the presence of 2-ME in low serum dilutions did not measurably affect the titration of low concentrations of 7S antibody in the haemagglutination test. The efficacy of 2-ME for the determination of IgG antibody by selective elimination of macroglobulin antibody has been established for immunoglobulin separation in mouse serum (Blinkoff, 1966).

Statistical analysis of the first series was done by comparing the percentage of 7S antibody related to total antibody using a two sample Student's *t*-test. For convenience the second series was analysed by Duncan's multiple range test. Both methods were performed by computer analysis. The standard error of the mean was obtained and is listed in the tables (Tables 1–5). The comparison of the *percentage* of 7S antibody was done to eliminate the factor of variation in whole serum titres. These results are shown in Tables 1–5.

RESULTS

In Fig. 2 can be seen the results of comparing the whole serum antibody titre of the 2-month-old NZB group containing twelve animals with a control group of six C57BL and six A/Jax mice. The latter were pooled since there was essentially no difference in their antibody titres during the course of immunization. Whole serum titres were remarkably similar for the entire period of study. However, it can be seen that the mercaptoethanol resistant (MER) titre in the NZB animals was less than in the control group the 1st week after immunization. The NZB animals then rapidly showed an increased titre of 7S antibody until most of the antibody from week 2 to week 8 in this group was 2-ME resistant. The control group showed a comparatively lesser increase of 7S antibody titre at week 2 from higher initial levels which then persisted at similar levels through week 4 and finally reached the same level as the NZB group at the 8th week following immunization.

When the 7S antibody titre is expressed as a percentage of the whole serum antibody titre (Table 1) it can be seen that by the 2nd week 78% of the total antibody in the NZB animals is mercaptoethanol resistant. Though the percentage rises to 85% by week 4 the level reached at the 2nd week essentially flattens out and is maintained throughout the study

period. Not until the 8th week does the percentage of 7S antibody rise to comparable levels in the control group. Thus, the 2-month-old NZB animals appear to generate a higher level

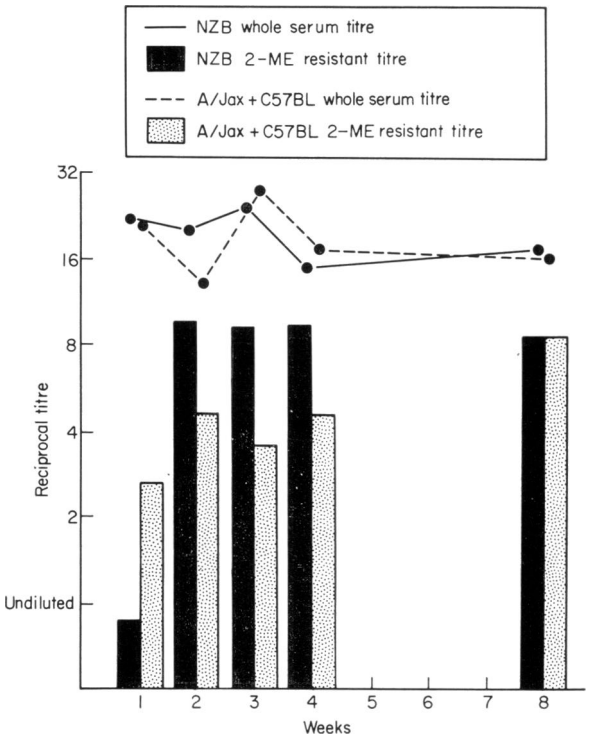

FIG. 2. Comparison of whole serum and 7S (2-mercaptoethanol resistant) antibody titres to sheep erythrocytes in 2-month-old NZB and A/Jax+C57/BL mice. Note the variation in 7S antibody with similar whole serum antibody levels.

TABLE 1. Comparison of relative amounts of 7S antibody in 2-month-old NZB and C57BL + A/Jax mice

Week	$\left(\frac{7S}{\text{whole serum}} \text{ titre} \pm \text{SEM}\right) \times 100$		*P* value
	NZB	C57BL + A/Jax	
1	20 ± 4·9	45 ± 6·1	−0·05
2	78 ± 8·9	68 ± 3·8	NS
3	77 ± 4·1	47 ± 6·5	0·01
4	85 ± 3·5	68 ± 4·1	0·01
8	81 ± 5·7	76·5 ± 6·9	NS

of 7S antibody when compared to C57BL and A/Jax animals. It should be noted that the control group showed a significantly greater amount of 7S antibody production in the 1st week after immunization. This continued to rise the 2nd week to levels that were not significantly

different from the NZBs. However, a relative drop in the production of 7S antibody the 3rd week was significantly different from the NZB animals who persisted with an increased production of 2-ME resistant antibody. This significant difference continued up to week 4 although there was not a steady increase in the amount of 7S antibody produced in the control group. Since the whole serum antibody titres showed their peak at week 3 the total antibody content probably was due to a relatively greater amount of macroglobulin antibody produced at this time in the controls.

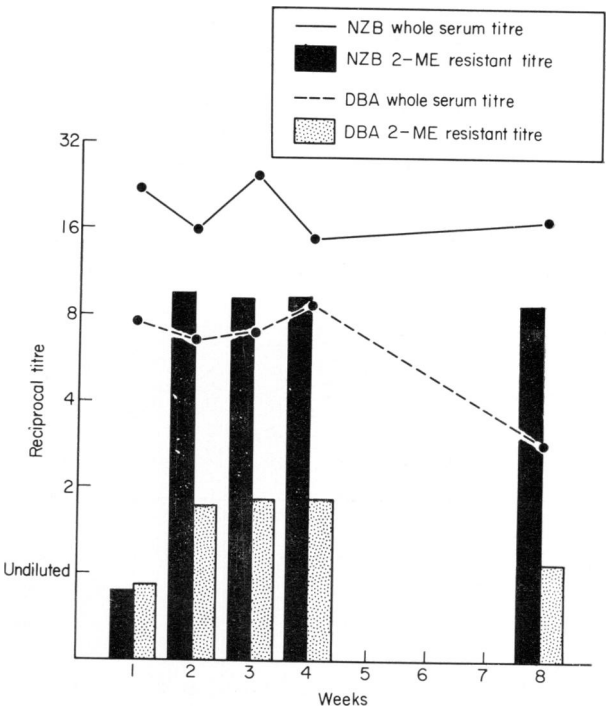

FIG. 3. Comparison of whole serum and 7S (2-mercaptoethanol resistant) antibody titres to sheep erythrocytes in 2-month-old NZB and DBA mice. Note the lower levels of whole serum antibody as well as reduced 7S antibody in the DBA mice.

The DBA mice showed a lesser total antibody response to the sheep cells than the NZB animals as seen in Fig. 3. Though the titres ran roughly parallel to the NZB animals for the first four weeks there was a noticeable divergence at week 8 with a falloff in the antibody response of the control group. The mercaptoethanol resistant (7S) antibody was again low at week 1 and rose to plateau levels at week 2 in both groups. There was some reduction of 7S antibody at week 8 in the DBA animals paralleling the reduced whole serum antibody titre. The relative 7S whole serum antibody fraction (Table 2) was appreciably lower in this group than in the other controls, with the peak value at week 3 only 48%. The levels were significantly lower than in the NZB animals at weeks 2, 4 and 8. There was thus relative agreement with the C57BL and A/Jax mice showing the major significant differences in the 7S antibody titre at week 4.

A subsequent group of sixteen 2-month-old NZB animals were compared to two other

strains of mice, CBA and C3H. All groups had equal numbers of male and female animals. As we had seen in the initial group all the NZB animals responded to the immunization dose of 0·01% sheep cells. Five of the fourteen C3H animals either did not respond or responded so minimally that the data could not be used. Thirteen of the twenty-seven CBA animals, likewise, gave little or no antibody response to this minimal antigenic stimulus. There was, thus, significantly less reactivity in the latter two strains when compared to the NZB mice ($P<0·05$). Subsequent comparison of antibody titres was made only with the responders in the control groups.

There was markedly less total serum antibody to sheep cells produced by the CBA animals than that produced by the NZB animals at 1, 2, 3 and 4 weeks (Fig. 4). There was some

TABLE 2. Comparison of relative amounts of 7S antibody in 2-month-old NZB and DBA mice

Week	$\left(\frac{7S}{\text{whole serum}} \text{ titre} \pm \text{SEM}\right) \times 100$		P value
	NZB	DBA	
1	20±4·9	23 ± 9·9	NS
2	78±8·9	42 ±12·2	0·05
3	77±4·1	48 ±13·5	0·1
4	85±3·5	36 ±11·8	0·01
8	81±5·7	28·5±12·1	0·01

increase in the whole serum titres between week 4 and week 8 in the controls while the antibody level in the NZB animals fell during this period. At week 8, therefore, there was no significant difference between the two groups of animals. The whole serum antibody levels in this part of the study were significantly more different than the relative 7S antibody production. The CBA group made proportionately more 7S antibody than did the previous groups of controls so that a significantly larger percentage of 7S antibody production by NZB animals was seen only at week 4 (Table 3).

Comparison with the C3H strain showed whole serum antibody levels significantly lower during the entire follow-up period after immunization (Fig. 5). Again the production of 7S antibody in these animals was high enough for the first 3 weeks to make the difference in the proportionate amount of 7S to whole serum antibody insignificant (Table 4). However, at 4 and 8 weeks the differences were significant at the 0·01 level. This was due to the persistence of about 50% of the antibody titre as 2-ME sensitive macroglobulin in the control group.

Two-month-old NZB mice show few of the typical autoantibody manifestations (Norins & Holmes, 1964b) that can appear in virtually 100% at 12 months (Mellors, 1965) and since it has been shown that young and old NZB animals have the same degree of reactivity to antigenic stimulation (Evans, Williamson & Irvine, 1968) further comparisons were made with 12-month-old NZB mice and 12-month-old random-bred Swiss mice. As indicated in the methods section, a ten-fold larger dose of antigen was used since our preliminary studies had shown a lesser response in the older group. Fig. 6 shows that the whole serum antibody titres in both groups were similar. There was the expected low level of 7S antibody production

the 1st week with the maximum antibody titres in the NZB animals at 3 weeks and in the Swiss mice at 4 weeks. In general 7S antibody levels were constant from week 2 on in both groups of animals. The older control animals maintained higher relative 7S antibody levels

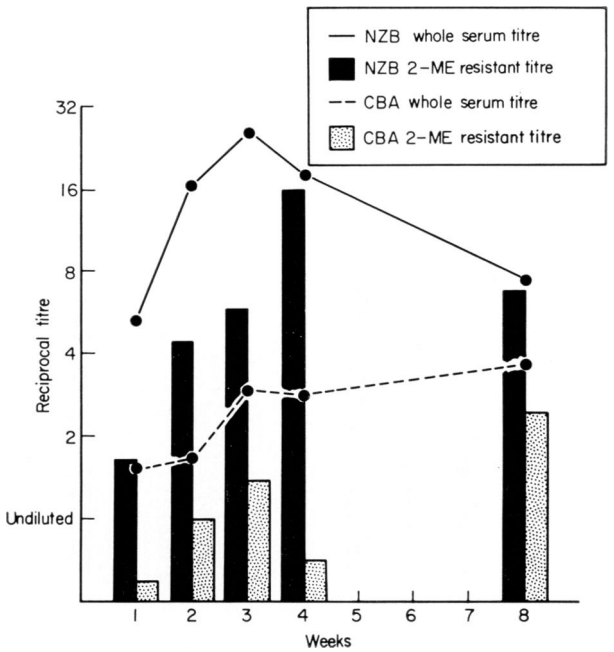

FIG. 4. Comparison of whole serum and 7S (2-mercaptoethanol resistant) antibody titres to sheep erythrocytes in 2-month-old NZB and CBA mice. Note the marked difference in whole serum antibody titres during the first 4 weeks.

TABLE 3. Comparison of relative amounts of 7S antibody in 2-month-old NZB and CBA mice

Week	$\left(\frac{7S}{\text{whole serum}}\text{titre} \pm \text{SEM}\right) \times 100$		*P* value
	NZB	CBA	
1	49±12·6	13 ±13·4	NS
2	62±13·2	57 ±17·6	NS
3	62±13·2	56·5±15·9	NS
4	97± 8·0	20 ±10·6	0·01
8	96±10·9	80 ±15·4	NS

than most of the younger controls (Table 5). The old Swiss mice showed significantly higher levels of 7S antibody at week 1 and significantly less at week 3 when compared to the NZB animals.

The old Swiss mice made more 7S antibody than the young control groups during the

initial 4-week period during which they were followed. Interestingly enough when the relative titres were compared it is noted that the values for the old NZB animals are virtually identical

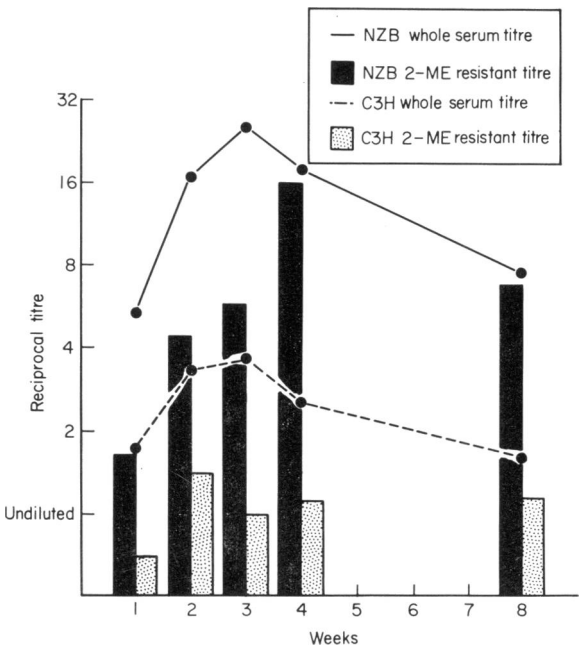

FIG. 5. Comparison of whole serum and 7S (2-mercaptoethanol resistant) antibody titres to sheep erythrocytes in 2-month-old NZB and C3H mice. Note the lower whole serum antibody titres with relatively similar levels of 7S antibody in the C3H animals.

TABLE 4. Comparison of relative amounts of 7S antibody in 2-month-old NZB and C3H mice

Week	$\left(\frac{7S}{\text{whole serum}} \text{ titre} \pm \text{SEM}\right) \times 100$		*P* value
	NZB	C3H	
1	49 ± 12·6	25 ± 12·6	NS
2	62 ± 13·2	54·5 ± 24·8	NS
3	62 ± 13·2	35 ± 19·4	NS
4	97 ± 8·0	50 ± 12·2	0·01
8	96 ± 10·9	60 ± 18·3	0·01

to those previously seen with the 2-month-old animals in group 1 (Table 6). They were also similar to the 7S whole serum antibody percentage found in the second group of young NZB mice (*P* value not significant).

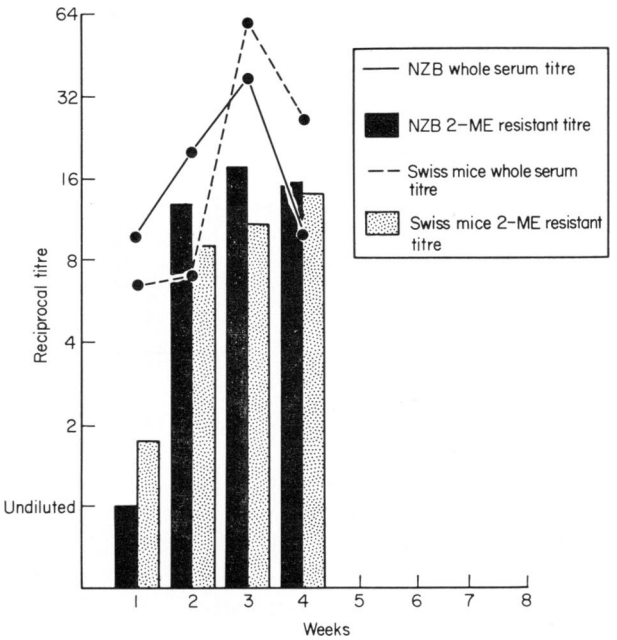

FIG. 6. Comparison of whole serum and 7S (2-mercaptoethanol resistant) antibody titres to sheep erythrocytes in 12-month-old NZB and White Swiss mice. Note the similar levels of whole serum and 7S antibody titres.

TABLE 5. Comparison of relative amounts of 7S antibody in 12-month-old NZB and Swiss mice

Week	$\left(\frac{7S}{\text{whole serum}} \text{ titre} \pm \text{SEM}\right) \times 100$		*P* value
	Old NZB	Old Swiss	
1	20·5 ± 6·1	48 ± 10·6	−0·05
2	81 ± 9·6	89 ± 7·2	NS
3	78 ± 5·9	63 ± 2·7	0·05
4	89 ± 5·9	85 ± 4·2	NS

TABLE 6. Comparison of relative amounts of 7S antibody in 2-month-old and 12-month-old NZB mice

Week	$\left(\frac{\text{7S}}{\text{whole serum}}\ \text{titre}\right) \times 100$		
	Young NZB		Old NZB
	Group 1	Group 2	
1	20	49	20·5
2	78	62	81
3	77	62	78
4	85	97	89

DISCUSSION

The dose response pattern to sheep erythrocytes in this study was similar to the results previously reported by Dietrich & Dukor (1967) with mice. They found that haemagglutination antibodies to sheep erythrocytes showed a distribution (by density gradient centrifugation) of early 19S antibody predominance followed by 7S antibody. However, it has also been noted that 19S antibody tends to persist at higher than background levels indicating continued production (Adler, 1965). This persistence of macroglobulin (2-ME sensitive) antibody was clearly found in this study.

Although there was a ten-fold difference in the amount of sheep erythrocytes used to immunize the two age groups of NZB animals there was little difference in the whole serum antibody response to the sheep erythrocytes. The amount of 2-ME resistant (7S) antibody found was also similar and when the relative amounts of this antibody were compared there was virtually no difference between the two age groups. The percentage of 7S antibody titre to whole serum antibody titre showed essentially the same response to the sheep erythrocyte antigen in young and old NZB mice (Table 6). A similar finding has recently been reported by Evans *et al.* (1968). A contrary finding was reported by Diener (1966) who showed the older mice to be less responsive to antigenic stimulation than controls, while he also found the older animals to be less responsive than the younger mice of the same strain. Much variation is seen among the control strains in this study. In a comparison of all the groups the NZB mice made a higher proportion of 7S antibody ranging from a single significantly higher value when compared to the 12-month-old Swiss and the 2-month-old CBA animals to significantly higher levels when the DBA group was compared (differing at weeks 2, 3, 4 and 8). These findings show the NZB animal to have a greater capacity for the production of 7S antibody and also that this finding is more striking in the younger animal. The appearance of this capability in the young animal before any evidence of autoimmune disease is usually present may indicate either a heightened capability for the production of light antibody or a more rapid conversion from 19S antibody to 7S antibody production.

Though the NZB mouse appears to make more 7S antibody to sheep cells than most of the

other strains studied, the whole serum antibody levels did not appear to be significantly higher compared to DBA, C57BL and A/Jax and White Swiss mice. Similar response patterns have been previously noted by other authors (Warner & Wistar, 1968). Significant differences in whole serum antibody titres were seen with CBA and C3H strains all of which showed less whole serum antibody response than the NZB mice. However, it has been noted that the ability of the mouse to produce specific antibody is strain dependent (Rothberg & Talmage, 1961) as well as antigen dependent (Fink & Quinn, 1953; Barth, McLaughlin & Fahey, 1965).

An increased antibody response of NZB mice to soluble antigens has recently been noted by Weir, McBride & Naysmith (1968). They showed that the NZB mice were more responsive than CBA, DBA/2 and C57BL mice to bovine serum albumin.

The early increase in responsiveness of the 7S antibody may be relative to a decreased production of a specific macroglobulin antibody which we have noted to be present in the human disease SLE (Baum & Ziff, 1969). In another viewpoint the increase in 7S antibody formation production may inhibit the 19S antibody production. This inhibition of 19S antibody production by 7S antibody is said to be highly efficient in the mouse (Möller & Wigzell, 1965). However, there remains the unexpected finding of Warner & Wistar (1968) who, studying the various immunoglobulin levels, found that NZB/BL mice show only an elevation of macroglobulin. A lesser number of macroglobulin antibody producing cells in the NZB mouse is made less likely by their findings. However, if these cells are present in excess and are committed in larger numbers to an unknown antigen or antigens then the *relative* number of macroglobulin antibody producing cells available to react with a new antigen would be diminished. Therefore, the increased total amount of macroglobulin may represent the production of antibody committed at a very early stage. This represents a major immunological discrepancy between these mice and patients with SLE. Most studies of patients with this autoimmune disease have shown them to have a significant elevation of 7S γ-globulin levels with normal or only slightly elevated levels of macroglobulin (Cass *et al.*, 1968). However, most autoantibodies in SLE are 7S (Baum & Ziff, 1962) as well as in the NZB mouse where it has been noted that antinuclear antibodies are all 7S (Norins & Holmes, 1964a) and most of the erythrocyte autoantibodies as well (Norins & Holmes, 1964b). The elevated macroglobulin levels thus could be due to aberrant immunoglobulin not participating in antibody formation. They could also reflect increased sensitivity of the antibody forming system to autogenic stimulation at a low level. Since it is known that low doses of antigen will stimulate 19S antibody but not 7S antibody the elevated macroglobulin level may well reflect the result of many small antigenic insults. We have previously noted an increase in natural antibody to sheep cells in the NZB mouse when compared to the strains used in this study (Baum, 1969). However, this background response of macroglobulin antibody has no relationship to the immune response after stimulation (Biozzi *et al.*, 1968).

The similarity in antibody response between the old White Swiss and the old NZB/BL mice is not apparently related to any decrease in autoimmune antibody activity of the NZB/BL since in one study (Siegler, 1965) they still showed significantly higher levels of autoimmune antibodies to red cells at 200 days of age when compared with Swiss mice of the same age. It indicates that the high levels of 7S antibody production of both the young and old NZB groups may well be a reflection of a continuation of the early levels of antibody-producing activity in the NZB animal which develops later in most other strains (Evans *et al.*, 1968). In that event the release of autoantigens may be a feature of the young NZB

animal at a time when the animal is immunologically highly responsive. The similarity of these findings of increased 7S antibody activity early as well as later when the autoimmune disease is fully developed shows this to be part of the genetically determined antibody response in these animals. Since a viral agent (Mellors & Huang, 1966) has recently been incriminated in the etiology of the autoimmune disease in these animals our findings may indicate that the combination of a highly responsive 7S antibody producer with a viral agent damaging tissue and releasing tissue antigens leads to the production of autoantibodies at a greater rate than is seen with other strains. This finding is analogous to systemic lupus erythematosus which shows increased 7S antibodies to autoantigens and some indication of this when patients with SLE are stimulated with exogenous antigens (Baum & Ziff, 1969). In this disease we have found a decrease in antibody activity especially in the 19S fraction with an occasional increase in 7S antibody production.

It was also noted that both the old Swiss mice and the 2-month-old C57BL and A/Jax showed statistically higher titres of 7S antibody than the NZB mice in the 1st week after antigenic stimulation (Tables 1 and 5). A possible explanation for this finding could be the production of a low affinity 7S antibody initially followed by the later production of larger amounts of antibody with normal affinity as noted by Levine & Levytska (1967). If this hypothesis is true then a corollary could be, as indicated above, a decrease in the early production of macroglobulin antibody in the NZB mouse since the whole antibody titres were the same in the NZB and control mice at week 1. However, the 2-ME resistant antibody titre (7S) may be lower in the NZB/BL in the 1st week because of the presence of mercaptoethanol sensitive 7S antibody which has been said to be present at this time (Adler, 1965) and then disappears after 7 days.

These findings help to confirm the impression that the NZB mouse is a prime model for the human disease systemic lupus erythematosus.

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