RELATIVE INABILITY TO INDUCE TOLERANCE IN ADULT NZB AND NZB/NZW F_1 MICE

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The NZB mouse is genetically predisposed to the development of Coomb's positive hemolytic anemia, splenomegaly, and, to some degree, antinuclear factors and lymphoid malignancies (1). The F_1 offspring of the NZB and NZW cross (B/W) is genetically predisposed to the development of antinuclear factors, anti-DNA antibodies, LE cells, and a fatal lupus-like glomerulonephritis. There is a much lower incidence of Coomb's positivity in this hybrid. Both strains develop lymphoid and plasma cell infiltration of many organs, including the thymus. The heritable factor in these mice may not be any specific disease but rather a propensity to develop autoimmunity.

The natural history of these animals can be divided into three stages: (*a*) a latent phase from birth to about 3 months when the mice appear normal; (*b*) a seropathologic phase from 3 to 5 months when the various autoantibodies begin to appear in the serum; (*c*) a histopathologic phase from 5 months to death when the spleen, kidney, thymus, and other organs show the progressive development of autoimmune lesions and their sequellae. There is no sharp dividing line between these stages. The pathogenesis of tissue destruction in these mice is understood in part. The red cell destruction is related to the antibody molecules coating the erythrocyte surface (2). The nephritis is due to the glomerular deposition of immune complexes, particularly DNA and anti-DNA (3). The mechanism for the formation of these autoantibodies is yet to be explained.

Autoantibody formation may reflect the abrogation of self-tolerance. Since there is a latent phase before autoantibodies appear, self-tolerance may be maintained in NZB mice at birth and then be gradually lost. Very old mice and humans sometimes develop autoantibodies (4), so that a loss of self-tolerance appears to be an age-dependent phenomenon. This process may be greatly accelerated in NZB and B/W mice.

We decided to investigate the potential of these mice to develop immunity and tolerance during the latent phase. Any abnormality discovered at this time, prior to autoantibody formation, could not be a consequence of serologic disease and might be related to some basic causal mechanism. We hypothesized that the progressive abrogation of self-tolerance would be accompanied by an inability to develop or maintain tolerance to exogenously administered protein antigens. Ultracentrifuged bovine and human gamma globulin were selected as

the antigens because they are known to induce tolerance readily and reproducibly in several different mouse strains (5, 6, 7).

Materials and Methods

Animals.—Male and female NZB, NZW, B/W F_1, C57Bl, C3H, and Balb/c mice, 5–8 wk old, were procured from National Institutes of Health (NIH) inbred stock colonies. The New Zealand mouse strains, although currently maintained at NIH, were originally obtained from the University of Otago animal facilities in New Zealand and are in the 60th and 40th generations for the NZB and NZW strains respectively.

C57Bl mice were selected because of their ability to form large amounts of antibody to tetanus toxoid (8) and because they occasionally develop antinuclear factor (9). C3H mice were used because they neither form excessive antibody to tetanus toxoid nor demonstrate antinuclear factor. Finally, Balb/c mice were chosen because they develop plasma cell tumors in response to intraperitoneal Bayol F and because they are known to produce large quantities of antibody (8, 10).

Antigens.—Bovine gamma globulin (BGG) (Armour Pharmaceutical Company, Kankakee, Ill.), human gamma globulin (HGG) (Mann Research Laboratories, Inc., New York), and egg albumin (Ea) crystallized × 2 (Worthington Biochemical Corporation, Freehold, N. J.) served as protein antigens.

Protein Labeling with ^{131}I.—The method of labeling was a modification from McFarlane (11). Assuming a labeling efficiency of 33%, 3 mc of carrier-free and reducing agent-free ^{131}I (E. R. Squibb & Sons, New York) was added to 10 mg of BGG or HGG in 0.5 cc borate buffer, pH 8.0. ICl (0.033 M) was added rapidly to a final concentration of 2.45×10^{-3} mg I/mg. IgG in a final volume of 2.0–2.5 cc. After labeling, dialysis of the sample against five to seven changes of 0.15 M NaCl over 48–72 hr was carried out and the amount of radioactivity precipitable with 20% phosphotungstic acid was shown to be greater than 98%. In this manner, it was possible to label 10 μg of IgG with from 0.3 to 0.7 μc ^{131}I.

Pretreatment.—On day zero, seven to nine mice from each strain received intraperitoneal (i.p.) injections of Ea (12 mg), BGG (0.001, 0.02, 5, 9, 15, or 24 mg), or HGG (1, 12, or 24 mg) dissolved in phosphate buffered saline, pH 7.2. The animals receiving Ea served as specificity controls and developed antibody titers comparable to saline-injected control mice. Prior to injection, proteins were ultracentrifuged in a Spinco 40 rotor at 40,000 rpm (105,000 *g*) for 30 min at 20°C. Subsequent protein determinations were carried out in a Beckman DU spectrophotometer using:

$$E^{1\%}_{280m\mu, pH7.0} = 13.0 \text{ (BGG) (12)}, \; E^{1\%}_{280m\mu, pH7.0} = 14.3 \text{ (HGG) (13)}$$

$$\text{and } E^{1\%}_{287m\mu, 0.1N, NaOH} = 7.39 \text{ (Ea) (13)}.$$

From 6–30% of the starting material was sedimented by the ultracentrifugation.

Challenge.—On day 12, mice were challenged with either BGG or HGG, 10 mg/cc, emulsified with an equal volume of complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.). Each animal received 0.05 cc into each of four footpads for a total dose of 1.0 mg per mouse. A control group of mice was left unchallenged.

^{131}I Immune Elimination.—Starting on day 22, the elimination of an i.p. tracer dose of 10 μg of ^{131}I-labeled BGG or of 20 μg of ^{131}I-labeled HGG was followed for each mouse in a whole body Baird Atomic Counter. 3 days prior to the administration of the radioactive tracer all mice were given 0.1% KI in their drinking water. Final elimination curves were determined after correction for background, radioactive decay, and machine variability.

Hemagglutination.—Animals given HGG were bled on days 28 and 40; animals given BGG

were bled on days 12 and 40. Hemagglutination titers were determined by standard microhemagglutination methods using a formalinized tanned sheep cell assay (14, 15). All sera from a single bleeding were run on the same day; positive sera of known titer and known negative sera were included with each assay.

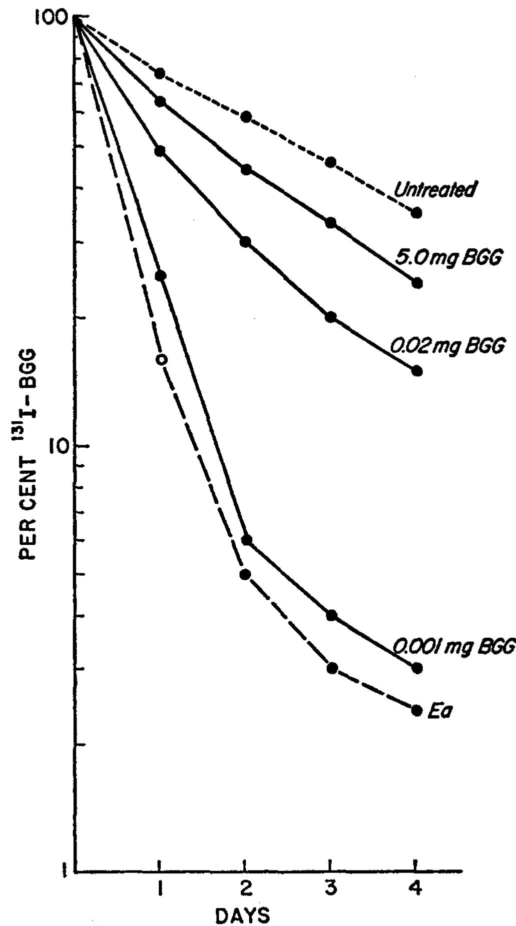

FIG. 1. Elimination of 10 μg ^{131}I-BGG by C3H mice 10 days postchallenge. Data are plotted as per cent retained radioactivity against time in days for varying pretreatment doses of BGG. Each point in this and other figures represents average values for five to seven mice.

Purification of Antigen for Hemagglutination.—Because of the well-known contamination of commercial BGG by small amounts of a β-globulin (16), the BGG used to coat tanned sheep red blood cells was first purified by starch block electrophoresis (17). Crude BGG, 200–400 mg, was added to a 1 × 46 × 30 cm starch block and electrophoresed in barbital buffer, pH 8.6, for 48 hr at a potential of 450 v. Protein was then eluted from 1 cm strips of starch and Lowry protein determinations (18) carried out. The protein in the slowest two-thirds of the

γ-region was dialyzed against cold 0.85% NaCl for 48 hr. and concentrated to 10 mg/cc. Immunoelectrophoresis against a rabbit anticrude BGG antiserum showed that the β-globulin present in the crude BGG preparation had been entirely eliminated.

HGG (Mann Fine Chemicals, Inc., New York), certified to be chromatographically pure, was also tested at 10 mg/cc against a known rabbit antiwhole human serum and was found

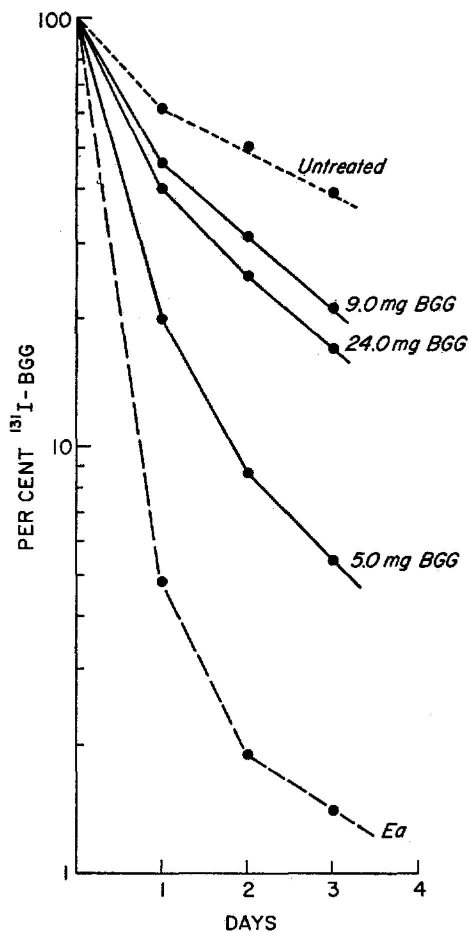

FIG. 2. Elimination of 10 μg ¹³¹I-BGG by C57Bl mice 10 days postchallenge.

to contain no demonstrable contaminant. Therefore, it was used directly, without further purification, to coat sheep red blood cells.

RESULTS

¹³¹I-BGG Elimination.—In the first experiment, NZB, B/W, NZW, C57Bl, and C3H mice were pretreated with 0.001, 0.02, or 5 mg of soluble BGG. In a

second experiment, NZB, B/W, and C57Bl animals were given 9, 15, and 24 mg of soluble BGG.

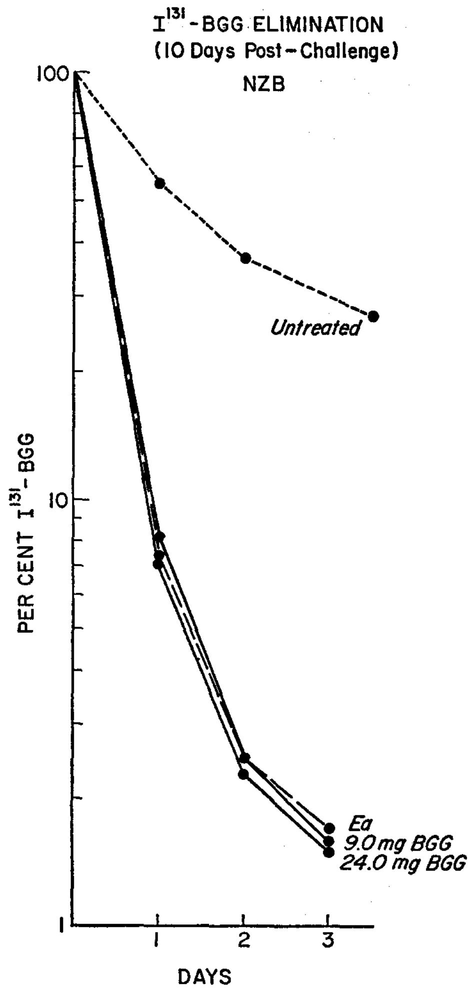

FIG. 3

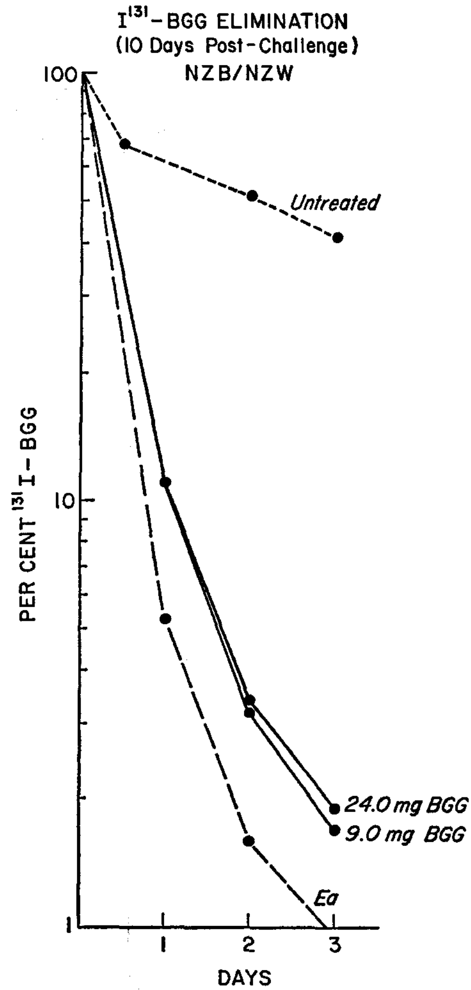

FIG. 4

FIGS. 3. and 4. Immune elimination 10 days postchallenge for NZB (left) and B/W F_1 (right) mice given maximum pretreatment doses of soluble BGG.

Fig. 1 shows elimination data (plotted as per cent retained radioactivity) for C3H mice studied 10 days postchallenge. When pretreated with 1 μg BGG, these animals showed immune elimination comparable to that seen in Ea-pretreated controls. However, 0.02 and 5 mg of BGG pretreatment led to pro-

gressive nonimmune elimination approaching that of untreated mice. Nonimmune elimination was also observed in C57Bl mice (Fig. 2). Here, however, higher pretreatment doses of 9 and 24 mg BGG were required to obtain nonimmune elimination curves comparable to that seen in the C3H mice.

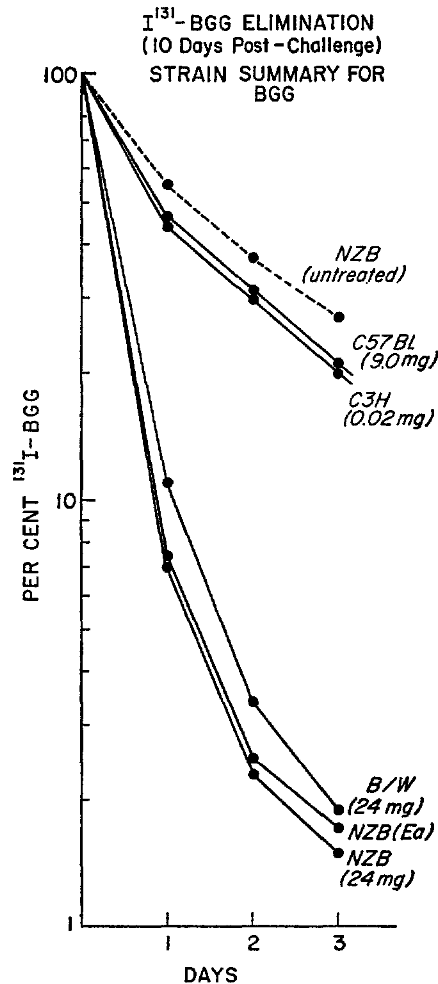

FIG. 5. Comparative strain data showing nonimmune elimination 10 days postchallenge for C3H and C57Bl mice vs. immune elimination following 24 mg soluble BGG in NZB and B/W animals.

By contrast, Figs. 3 and 4 show results of ^{131}I-BGG elimination for the NZB and B/W F_1 strains given maximum pretreatment doses of 9 and 24 mg. All animals showed rapid immune elimination comparable to NZB and B/W mice pretreated with Ea. There was no evidence for high dose tolerance. Lower

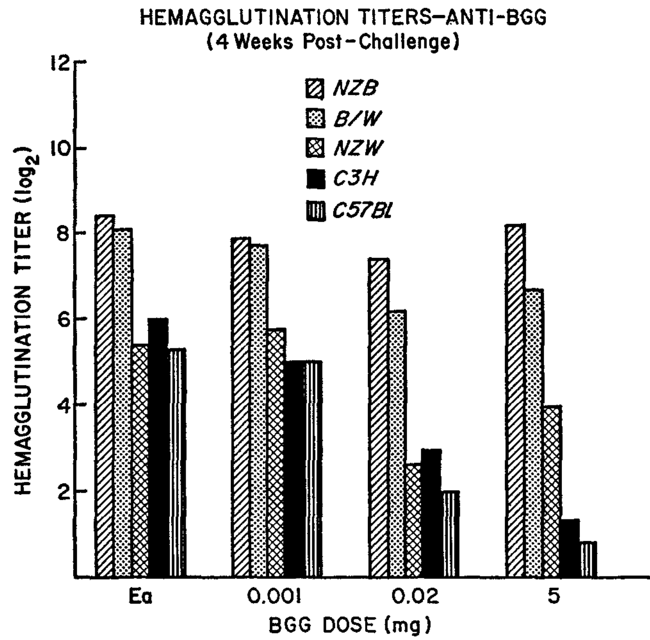

FIG. 6. Hemagglutination titers ($\log_2$) 4 wk postchallenge for increasing pretreatment doses of BGG in five strains of mice.

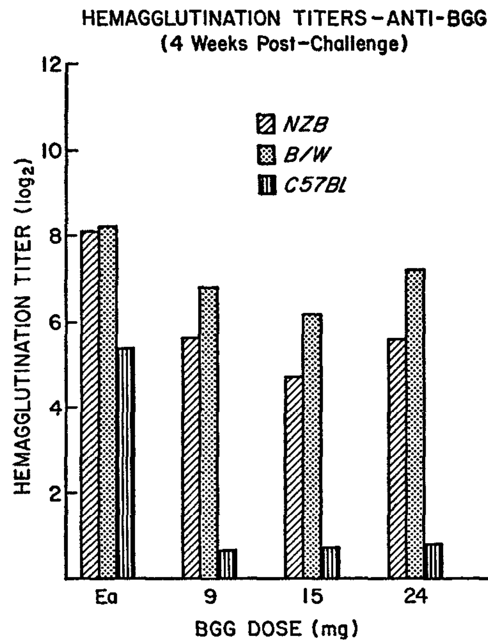

FIG. 7. Hemagglutination titers ($\log_2$) 4 wk postchallenge for 9, 15, and 24 mg soluble BGG.

pretreatment doses of 0.001, 0.02, and 5 mg BGG (not shown) resulted in no evidence for low dose tolerance. NZW mice given 5 mg BGG (also not shown) showed partial nonimmune elimination curves (30 and 18% retained radioactivity at 1 and 2 days) but were not tested at higher dose levels.

TABLE I
4 Wk Hemagglutination Titers (log₂) for Individual Mice
Experiment 1

Dose	Antigen	Strain	Hemagglutination titers*												Mean titers‡
			0	1	2	3	4	5	6	7	8	9	10	11	
mg															
5.0	BGG	NZB								1	3	2			8.2
		B/W						1	2	1	2				6.7
		NZW	2					1		1	1				4.0
		C57B1	3	1	2										0.8
		C3H	2	2	1		1								1.3
0.02	BGG	NZB							1	2	4				7.4
		B/W					1		1	3					6.2
		NZW	2			1		2							2.6
		C57B1	2		1	1	1								2.0
		C3H	3					4							2.9
0.001	BGG	NZB						1		1	3	3			7.9
		B/W								2	4				7.7
		NZW						1	3						5.8
		C57B1				1	1	2			1				5.0
		C3H			1		1	2	2	1					5.0
5.0	Ea	NZB								1	2	1	1		8.4
		B/W								2	3	1	1		8.1
		NZW					1	2	1	1					5.4
		C57B1					1	2	3						5.3
		C3H						3	2	1	1				6.0

* Number of mice having individual (log_2) titers.
‡ Average (log_2) titer by strain.

Comparative strain data are summarized in Fig. 5 in which the minimal doses of antigen which resulted in tolerance in the C3H and C57Bl mice are contrasted with the higher and maximal doses of antigen which resulted only in nontolerance in NZB and B/W mice.

BGG–Hemagglutination.—Assays of serum obtained just prior to challenge with BGG in adjuvant were negative for all strains, indicating no immune response to the pretreatment doses of BGG. However, assays 4 wk postchal-

lenge confirmed the lack of tolerance induction in the NZB and B/W mice. As shown in Fig. 6, increasing pretreatment doses of ultracentrifuged BGG led to a generally progressive suppression of antibody titers in the C3H, C57Bl, and NZW strains. This was most striking in C3H and C57Bl mice, and especially so in those animals receiving the highest dose of BGG (5 mg). By contrast, there was no significant fall in antibody titers in NZB or B/W mice.

Fig. 7 shows similar results for the experiment in which maximum pretreatment doses of BGG were used while Tables I and II summarize the hemagglutination data for individual mice for all doses. Once again, the C57Bl mice were fully tolerant at this time, showing virtually no antibody formation at

TABLE II

4 Wk Hemagglutination Titers (log_2) for Individual Mice
Experiment 2

Dose	Antigen	Strain	Hemagglutination titers*												Mean titers‡
			0	1	2	3	4	5	6	7	8	9	10	11	
mg															
9, 15, and 24	BGG	NZB			1	1	2	5	7	1	1				5.3
		B/W					1	1	4	5	5				6.7
		C57B1	12	2	2	1									0.7
14	Ea	NZB								2	1	3			8.1
		B/W							1		1	3			8.2
		C57B1					1	2	3						5.4

* Number of mice having individual (log_2) titers.

‡ Average (log_2) titer by strain.

any pretreatment dose. On the other hand, NZB and B/W mice showed only a mild depression of antibody formation at 9, 15, and 24 mg, this depression being most marked in the NZB pretreated with 15 mg.

An additional observation was that consistently higher titers (two to three tubes) were observed in Ea-pretreated, immunized NZB and B/W mice, as compared with the other three strains. This suggests that, in addition to a relative lack of tolerance induction, NZB and B/W mice may produce quantitatively more antibody to a standard 1 mg BGG challenge and to a 10 μg booster (the ^{131}I-BGG) than do other mouse strains. While these higher titers for standard challenge in the NZB mouse might reflect only an increased amount of γ_M antibody, this possibility was ruled out by incubating positive NZB and B/W sera with 2-mercaptoethanol and retesting. No ME-sensitive antibody was found.

^{131}I-HGG Elimination.—In an attempt to confirm the lack of tolerance induction in NZB and B/W mice, a second protein antigen (ultracentrifuged HGG)

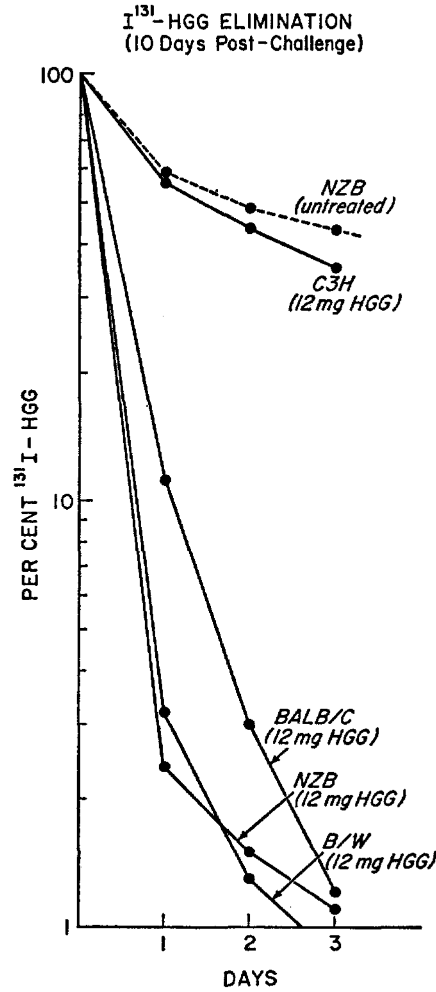

FIG. 8. Elimination of 20 μg ^{131}I-HGG 10 days postchallenge in mice pretreated with 12 mg soluble HGG.

was studied. Pretreatment doses of 1.0, 12, and 24 mg were given to NZB, B/W, C3H, and Balb/c mice.

Fig. 8 presents the 10 day postchallenge elimination data for all strains given 12 mg HGG and is representative of results seen with both the lower 1.0 mg and the higher 24 mg antigen doses. C3H mice showed a nonimmune elimination,

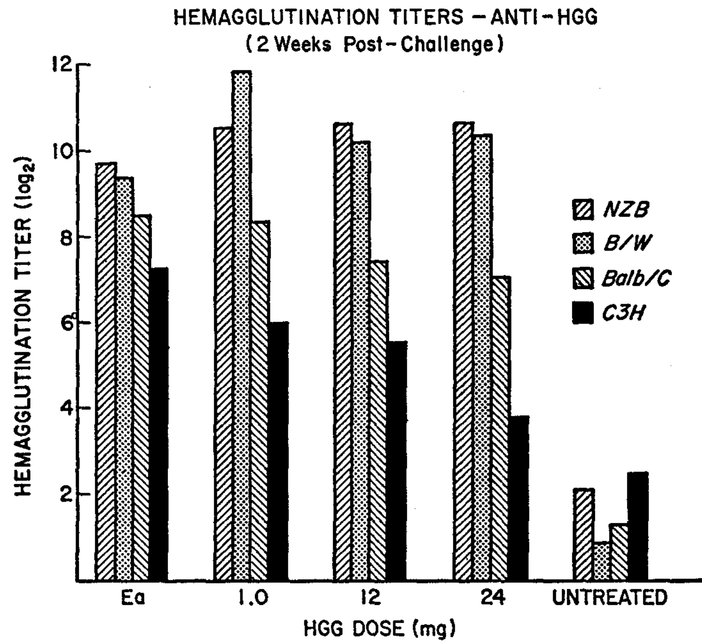

FIG. 9. Hemagglutination titers ($\log_2$) 2 wk postchallenge for pretreatment doses of 1, 12, and 24 mg soluble HGG.

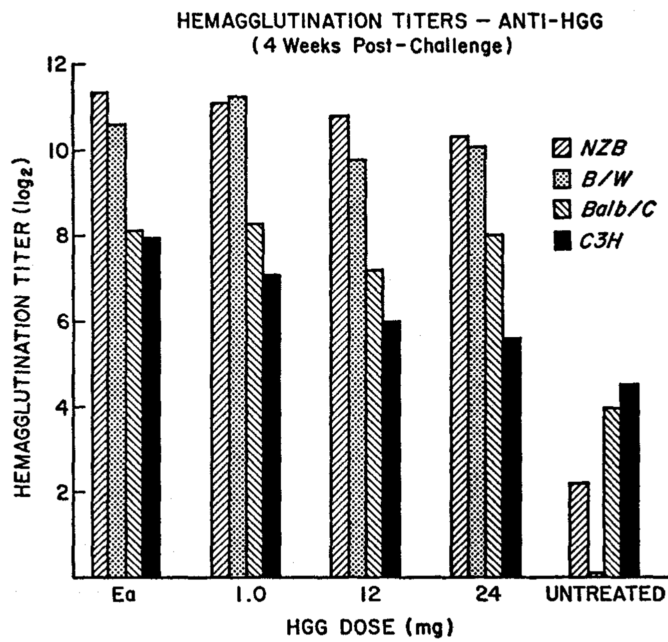

FIG. 10. Hemagglutination titers ($\log_2$) 4 wk postchallenge. Animals are the same as those of Fig. 9.

extending Golub and Weigle's observations[1] of a similar ease of tolerance induction with 0.5–1.0 mg soluble HGG in C57Bl, A/Jax, and DBA/2 strains. In contrast, NZB and B/W mice showed striking immune elimination curves even when pretreated with 24 mg HGG. However, the Balb/c strain also showed

TABLE III
2 Wk Hemagglutination Titers (log_2) *for Individual Mice*
Experiment 3

Dose	Antigen	Strain	Hemagglutination titers*														Mean titers‡
			0	1	2	3	4	5	6	7	8	9	10	11	12	13	
mg																	
24	HGG	NZB										1	2	3			10.6
		B/W										1	4	1	1		10.3
		Balb/c						1		3	2						7.0
		C3H			2		1	3									3.8
12	HGG	NZB										1	1	3	12		10.6
		B/W										2	1	1	1		10.2
		Balb/c							1	1	3						7.4
		C3H					4	1	1								4.5
1	HGG	NZB											3	3			10.5
		B/W												2	4	1	11.9
		Balb/c								2	1	4					8.3
		C3H					1	2	1	2	1						6.0
12	Ea	NZB									1	1	4	1			9.7
		B/W										3	2				9.4
		Balb/c									3	3					8.5
		C3H								4	2						7.3
Untreated		NZB	1		2	3											2.1
		B/W	4		1	1											0.9
		Balb/c	3		1	3											1.3
		C3H			2	2											3.5

* Number of mice having individual (log_2) titers.
‡ Average (log_2) titer by strain.

fairly prompt elimination at all pretreatment doses and in this respect was more like the NZB and B/W strains than any other mouse strain tested thus far.

HGG-Hemagglutination.—Hemagglutination data (Figs. 9 and 10) obtained 2 and 4 wk postchallenge revealed several important differences from the data presented in the previous BGG studies.

[1] Golub, E. S., and W. O. Weigle. 1967. Unpublished observations.

Most control mice neither pretreated nor challenged with HGG made a weak antibody response to the 20 μg ^{131}I-HGG booster. This, taken with the fact that antibody titers were two to three tubes higher in Ea-injected control mice immunized with HGG than were antibody titers of control mice immunized with BGG, suggested that HGG was a better immunogen and therefore perhaps a weaker tolerogen than was BGG.

A second difference was that although both Balb/c and C3H mice pretreated with 12 and 24 mg HGG appeared to show a slight depression of antibody formation, there was never the marked depression of antibody production seen in BGG-pretreated control strains (cf. Fig. 6). This again indicated that HGG may be a stroger immunogen than BGG.

The major finding, however, was that NZB and B/W mice showed no evidence of tolerance to a second protein antigen. As individual data from Table III show, *no* NZB or B/W mouse pretreated with HGG had an antibody titer of less than nine. This was true both 2 and 4 wk postchallenge.

Finally, nonpretreated, immunized (Fig. 9 and 10) NZB and B/W mice were again found to have antibody titers two to three tube dilutions higher than comparably immunized Balb/c and C3H mice. Moreover, NZB and B/W mice pretreated with 1 mg of HGG and studied at 2 wk showed greater quantities of antibody when compared to their Ea-treated counterparts.

DISCUSSION

We studied six mouse strains and found considerable variation in their ability to produce antibody and develop tolerance to BGG and HGG. Three strains (C3H, C57Bl, and NZW) made a comparable amount of antibody to a standard antigenic challenge and could be rendered tolerant readily. Two strains (NZB and B/W) made significantly greater quantities of antibody and were extremely resistant to tolerance induction. Finally, a sixth strain (Balb/c) resembled the C3H, C57Bl, and NZW with respect to amount of antibody produced but resembled the NZB and B/W with respect to difficulty of tolerance induction.

The lymphoid system has the capacity for the simultaneous development of immunity and tolerance (19), but which event ultimately predominates depends on many factors. These include the nature, dose, and route of administration of antigen, as well as the immunologic maturity, number of both antigen-processing and antibody-forming cells, and species of animal used. The net result of antigen administration is probably a balance between antibody production and tolerance, yet it may be difficult to achieve or detect tolerance under conditions which greatly favor antibody production.

The relative lack of tolerance observed in the NZB and B/W mice could reflect an abnormality in either the afferent or the efferent portion of the immune system, tending to favor an immune response to an antigen which, in other strains, was tolerogenic. The antigens used in our studies were ultracen-

trifuged heterologous gamma globulins which produce a state of unresponsiveness because they are probably poorly phagocytized by cells of the reticuloendothelial system (20). The nonphagocytized antigen may then interact directly with receptors on the lymphocyte to induce a state of immunologic unresponsiveness.

If the reticuloendothelial system of the NZB and B/W mice was unusually active and capable of phagocytizing soluble protein antigens which would not ordinarily be ingested because of their physical state, then the antigen would be processed normally and presented to the lymphoid cells in a way leading to an immune response. This explanation would seem to apply to the Balb/c strain, for Golub and Weigle have produced tolerance in this strain after salt fractionation of HGG to yield a greater concentration of monomers (21).

On the other hand, the relative lack of tolerance in the New Zealand strains might be due to an abnormality of the efferent arc of the immune response. Viewed in this way, soluble antigen might, upon contact with immunologically competent but abnormal small lymphocytes, either have no effect at all, prime them for subsequent challenge, or actually commit them to a low grade immune response. We could detect no antibody following treatment with ultracentrifuged BGG, although a small amount of antibody could have escaped detection. The increased amounts of antibody formed in response to pretreatment with 1 mg of HGG and later challenge with that antigen may mean that the mice were primed by the ultracentrifuged HGG. This point is worthy of further study.

The concept that immunologically competent cells might be committed in an immune rather than an unresponsive direction could explain the development of autoimmunity as a consequence of immunization of lymphocytes by small amounts of self-antigen over time. This would imply that immunocytes either become capable of initiating an autoimmune response autonomously or that the influence of some regulatory mechanism necessary for suppression or modification of potentially autoreactive cells is defective. A potential site for the generation of self-tolerant cells and for the elimination of autonomous, autoreactive cells is the thymus, a central lymphoid organ known to be necessary for tolerance induction (22) as well as escape from tolerance (23).

A functional abnormality of the NZB thymus may be related to lack of tolerance induction. DeVries and Hijmans (24) described changes in thymic epithelial cells and Hassall's corpuscles in young NZB and B/W mice during the latent phase of their disease prior to the onset of serologic abnormalities. Neonatal thymectomy in NZB mice did not delay the onset of autoimmune hemolytic anemia or Coomb's positivity but rather hastened them (25, 26), while thymectomy in rabbits (27) and Swiss mice (28) led to the development of Coomb's positive reactions and antinuclear autoantibodies respectively. It is therefore tempting to speculate that the thymic histologic changes in NZB and

B/W mice have as a functional counterpart the loss of a thymic function necessary for the induction of both experimentally induced and self-tolerance.

Of the three mouse strains relatively resistant to tolerance induction, two (NZB and B/W) are predisposed to autoimmune disorders and two (NZB and Balb/c) to lymphomas (29) and plasmacytomas (10). Thus it is worth considering that the immunologic hyperresponsiveness in the NZB and B/W strains and the relative lack of tolerance to protein antigens in all three strains reflect an altered immunologic mechanism(s) ultimately responsible for the development of lymphoid malignancies. Viral particles resembling the C-particles of murine leukemia are present in NZB mice (30). Mice infected with lactic dehydrogenase virus produce antibody to a normally tolerogenic dose of HGG (31). Thus the virus of NZB mice, if it is an etiologic agent in NZB disease, may be acting in a manner analogous to LDH virus to alter the balance between immunity and tolerance.

Although we have demonstrated that adult NZB and B/W mice do not become tolerant by conventional methods to soluble BGG or HGG, this does not imply that NZB mice are incapable of being rendered tolerant by other methods or to other antigens. The fact that we and others have induced tolerance to heterologous gamma globulins in six strains of mice (see reference 7 and footnote 1) suggests that additional strains, when studied, may also become tolerant more readily than NZB and B/W mice.

Since autoantibodies in the NZB are absent at birth and appear after a 3 month latent period, NZB and B/W mice may initially develop self-tolerance which is gradually lost. If so, it should be possible to induce tolerance to exogenous antigens in newborn or very young NZB mice, then follow the rate at which this tolerance disappears. Such experiments are currently in progress and it will be interesting to see if the relative inability to induce tolerance as described in this report will correlate with a rapid loss of possible preexisting tolerance.

SUMMARY

Immunologic tolerance to ultracentrifuged bovine gamma globulin could not be induced in 6-wk old NZB or B/W mice, but developed readily in C3H, NZW, and C57Bl mice. NZB and B/W mice, as well as Balb/c mice, failed to become tolerant to ultracentrifuged human gamma globulin. The NZB and B/W mice also showed higher antibody titers to a standard antigenic challenge. This immunologic hyperreactivity and lack of experimental tolerance may be related to the lack of self-tolerance, autoimmunity, and lymphomas that develop in these mice at a later age.

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