PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE, **147** 425-429 (1974)

Effect of Con A on Tolerance to BGG in NZB/W Mice (38355)

M. ERIC GERSHWIN AND ALFRED D. STEINBERG

Arthritis and Rheumatism Branch, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014

New Zealand mice develop spontaneously disease characterized by multiple autoantibodies and excessive lymphoid proliferation (1). Genetic, viral and immunologic factors have been implicated in the pathogenesis of these disorders (1–4). Several papers have presented evidence suggesting that New Zealand mice cannot easily be made tolerant to protein antigens such as BSA (5), HGG (6), and BGG (6–8). The response to ultracentrifuged BGG has been studied extensively. NZB and NZB/W mice under three weeks of age readily were made tolerant. However, the tolerance was transient in contrast to that in C57BL/6, C3H/He and BALB/c mice (6, 7). Older New Zealand mice, about 4–6 weeks of age, could not easily be made tolerant (6, 7). These abnormalities could be overcome by administering ultracentrifuged BGG repeatedly or by treating pregnant mothers with ultracentrifuged BGG (7).

The abnormality in tolerance to BGG has been associated with a generalized thymic regulatory abnormality associated with loss of self tolerance and autoimmunity (8). Recent studies have provided further support for a loss of thymic regulatory or suppressor function in the pathogenesis of autoimmunity in New Zealand mice (9–11). These functional abnormalities may be related to histological defects described in the thymus of New Zealand mice (12).

The plant lectin Concanavalin A (Con A), has been found to depress both "cellular" (13–15) and "humoral" (16–19) immune reactions *in vivo* and *in vitro*. Of particular interest was the observation by Dutton that small numbers of Con A treated spleen cells could inhibit the response of untreated cells to antigen (16). Although the mechanism by which Con A suppresses immune responses is still incompletely understood, the data are compatible with stimulation of a suppressor T cell population.

We herein report investigations of the relationship between Con A administration and tolerance to BGG in NZB/W mice. We reasoned that if the loss of BGG tolerance in NZB/W mice were due to loss of suppressor cells and if Con A could induce suppressor cells, then Con A might restore more normal BGG tolerance.

Materials and Methods. Animals. NZB × NZW F_1 (NZB/W) mice were obtained from colonies maintained at the National Institutes of Health.

Materials. Bovine γ globulin (BGG) as Cohn Fraction II obtained from Mann Research Laboratories, New York, was prepared for tolerance induction as previously described (6, 7). Each mouse received 10 mg ultracentrifuged BGG intraperitoneally (ip) within 30 min of preparation.

Antibody assays were performed using chromatographically pure 7S-BGG purchased from Mann Research Laboratories, New York. This material gave only a single precipitin line at a concentration of 10 mg/ml after immunoelectrophoresis with rabbit anti-whole bovine serum.

Concanavalin A (Con A) was purchased from Miles Laboratories, Kankakee, Illinois and dialyzed against phosphate buffered saline (PBS), pH 7.2, and the optical density read at 260 nm.

Treatment protocols. Two groups of mice are distinguished on the basis of age at onset of treatment. In the first group, 14 day old mice of both sexes were injected with 60 μg of Con A. This was repeated 4 days later at 18 days of age and twice weekly until 28 days of age, at which time the Con A dose was increased to 150 μg per

Printed in U.S.A.

injection. Thereafter, injections were continued for the duration of the experiment at a dose of 150 μg twice weekly. These mice received 10 mg ultracentrifuged BGG at 20 days of age. Ten days later (age 30 days) they were challenged ip with 1 mg BGG in PBS, pH 7.2, emulsified with an equal volume of Freund's Complete Adjuvant (CFA) purchased from Difco Laboratories, Detroit, Michigan. Littermates housed in the same cage were treated identically with the exception that they did not receive Con A. Additional NZB/W mice received no pretreatment, but were immunized with BGG in CFA at 30 days of age as above. All mice were bled by orbital sinus puncture every fortnight for 3 mo at which time the experiment was terminated. The second group was treated at 25 and 29 days of age with 150 μg Con A, and thereafter twice weekly with 150 μg of Con A for the duration of the experiment. When they were 28 days old they were injected with 10 mg of ultracentrifuged BGG. Ten days later, at 38 days of age, they were immunized with 1 mg BGG in CFA. Mice were then bled every fortnight for 3 mo at which time the experiment was terminated. Control littermates were treated identically except some randomly received (a) no Con A, (b) no ultracentrifuged BGG, or (c) neither Con A nor ultracentrifuged BGG.

Antibody assay. Mice were bled by orbital sinus puncture and the blood allowed to clot at room temperature after which sera were separated and cellular debris removed by centrifugation. The sera were then stored at −20° until used. Prior to assay, the sera were heated to 56° for 30 min and absorbed once with approximately one-half volume of packed washed sheep erythrocytes (SRBC). Antibody titers to BGG were determined in micro titer agglutination plates (Cooke Engineering Company, Alexandria, Virginia) by a standard hemagglutination method (20) using tanned fresh SRBC coated with 7S-BGG (see Materials above). All sera were run on a single day. Serial two-fold dilutions were made in phosphate buffered saline containing 1% normal rat serum previously heated to 56° for 30 min and absorbed with SRBC. Known positive reference standards and known negatives were included. These gave the appropriate previously observed titers.

Results. Mice treated with Con A starting at 14 days of age and ultracentrifuged BGG at 20 days (Group A) made very little antibody following challenge with BGG in CFA until 8 weeks after the challenge (Table I). Higher antibody titers were observed following challenge in mice receiving ultracentrifuged BGG but no Con A (Group B), and peak titers were achieved by six weeks after challenge. The responses of males and females were the same in each group and have therefore been combined. The antibody response of mice pretreated with ultracentrifuged BGG and Con A was significantly lower than that of mice receiving ultracentrifuged BGG alone at 2, 4, 6 and 8 weeks after challenge (Table I). There was no difference at 10 or 12 weeks. The titers in the BGG and Con A group were significantly lower at 4 and 6 weeks than in the BGG group at 2 and 4 weeks, respectively. There was, therefore, at least a two week difference in the rise in antibody titers in the two groups. The control mice who received no pretreatment (Group C, Table I) had a rapid rise in antibody to BGG demonstrating that tolerance had been achieved in the mice pretreated with ultracentrifuged BGG.

A different result was observed when animals were treated at 28 days of age with ultracentrifuged BGG. These mice had a more rapid and greater antibody response to challenge with BGG in CFA (Table II). Partial suppression of this response was observed in animals receiving both ultracentrifuged BGG and Con A. At two and four weeks post challenge, the BGG plus Con A pretreatment group had antibody levels significantly lower than those of all other groups (Table II). However, Con A pretreatment alone had a small but significant suppressive effect. There was no suppression by pretreatment with ultracentrifuged BGG alone. At 6 weeks and after, the titers were similar in all four groups; however, there was still a small but significant reduction at 6 weeks in the BGG plus Con A pretreated group when compared with no pretreatment.

Discussion. In this study we have confirmed that NZB/W mice treated with ultracentrifuged BGG are rendered tolerant when injected at less than 3 weeks of age, but that tolerance is not induced when treatment with ultracentrifuged BGG begins at 4 weeks of age or later. We have not employed salt fractionation or multiple injections of BGG so as to render the older NZB/W mice tolerant. Rather we tried to take advantage of the relative age differences observed with ultracentrifuged BGG to study the effect on Con

TABLE I. Antibody Response of NZB/W Mice to Challenge with BGG in CFA following Pretreatment with Ultracentrifuged BGG at 20 Days of Age with or without Multiple Injections of Con A (see Methods).

Week after challenge	Group A[d] Pretreatment with BGG + Con A	Group B[e] Pretreatment with BGG	Group C[f] No Pretreatment	Statistical Comparison between Group A and B (Student's t-test)	
		Mean Hemagglutination titer ± SEM[a]			
2	0.4 ± 0.18	1.8 ± 0.28[b]	4.9 ± 1.0	t = 4.103	$P < 0.001$
4	0.6 ± 0.26[b]	3.0 ± 0.33[c]	7.9 ± 0.42	t = 5.655	$P < 0.001$
6	1.3 ± 0.18[c]	8.3 ± 0.37	10.0 ± 0.86	t = 16.239	$P < 0.001$
8	7.0 ± 0.43	8.5 ± 0.43	10.3 ± 0.65	t = 2.236	$P < 0.05$
10	8.2 ± 0.58	8.1 ± 0.46	9.9 ± 0.91	t = 0.102	$P > 0.1$
12	8.5 ± 0.65	9.3 ± 0.25	10.4 ± 0.73	t = 1.083	$P > 0.1$

[a] Reciprocal of $\log_2$ dilution.
[b] $t = 2.993$, $P < 0.01$.
[c] $t = 4.382$, $P < 0.001$.
[d] Group A contained eight mice.
[e] Group B contained nine mice.
[f] Group C contained eight mice.

A upon BGG tolerance in NZB/W mice. Mice pretreated with ultracentrifuged BGG at 20 days of age were rendered tolerant, but rapidly escaped producing maximum antibody titers at 6 weeks post challenge as was observed previously (6). In contrast, littermates pretreated with ultracentrifuged BGG and in addition treated with Con A had very low anti-BGG titers 6 weeks postchallenge. Peak antibody levels were not achieved in these mice until 10 weeks after challenge. We concluded that Con A prolonged significantly the tolerant state of these mice. The NZB/W mice given ultracentrifuged BGG at 4 weeks of age showed no suppression of the response to BGG-CFA challenge unless Con A also was administered. Although Con A itself had a modest suppressive effect, the reduction in antibody response was significantly and dramatically greater when ultracentrifuged BGG and Con A were both given. It was of interest to find that the curve for mice given Con A plus BGG at 28 days was similar to that for mice given BGG alone for 20 days.

These results are subject to a number of in-

TABLE II. Antibody Response of NZB/W Mice to Challenge with BGG in CFA following Pretreatment with Ultracentrifuged BGG at 28 Days of Age With or Without Multiple Injections of Con A (see Methods). Control Littermates Received Either no Ultracentrifuged BGG or Neither Ultracentrifuged BGG nor Con A.

Weeks post challenge[a]	Mean hemagglutination titer ± SEM[b] for each treatment			
	BGG + Con A	Con A	BGG	Neither
2	1.8 ± 0.24[c]	5.0 ± 0.27[d]	6.1 ± 0.29	5.8 ± 0.25[d]
4	5.0 ± 0.31[c]	10.4 ± 0.29[e]	12.5 ± 0.29	13.2 ± 0.36[e]
6	12.3 ± 0.29[f]	12.7 ± 0.39	13.2 ± 0.38	13.3 ± 0.32[f]
8	13.4 ± 0.36	12.5 ± 0.38	13.1 ± 0.38	13.4 ± 0.20
10	13.0 ± 0.38	13.5 ± 0.33	12.8 ± 0.42	13.2 ± 0.30
12	13.0 ± 0.91	13.8 ± 0.63	12.0 ± 0.71	12.5 ± 0.29

[a] 15 mice in each group.
[b] Reciprocal of $\log_2$ dilution.
[c] $P < 0.0001$ compared to each of the other three groups, Student's t-test.
[d] $t = 2.104$, $P < 0.05$.
[e] $t = 5.983$, $P < 0.001$.
[f] $t = 2.343$, $P < 0.05$ only significant difference in six weeks.

terpretations. We may merely be observing the combination of a marginally effective tolerance regimen and an immunosuppressive agent (Con A). The minimal antibody reduction with Con A alone argues against that interpretation. Alternatively, Con A may be increasing the number or function of thymic regulatory cells which participate in tolerance control. Finally, Con A may effect nonlymphoid cells which are involved, directly or indirectly, in tolerance to BGG such as the macrophages of the reticuloendothelial system. In the present study Con A could cause a nonspecific suppressive effect upon macrophage function, reducing the immunogenicity of aggregates in the ultracentrifuged BGG. Similarly, a nonspecific immune enhancer could be interfering with tolerance induction and/or maintenance in New Zealand mice, and might be counteracted by Con A treatment.

Over the past several years evidence has accumulated to support the notion of thymic regulatory ("suppressor") control of immune responses (21–26) which may be responsible for certain forms of antigenic competition and which may play a role in some forms of tolerance. Suppressor cells in the BGG response have been suggested by the finding that the PHA response of spleen cells given soluble BGG was suppressed in the presence of BGG (27). Furthermore, a thymic suppressor cell subpopulation recently has been described for BGG tolerance in the rat (28).

New Zealand mice appear to lose normal thymic regulatory (suppressor) function early in life and later develop autoimmunity (1, 8–11). It is possible that in the present experiments Con A is capable of activating thymic regulatory ("suppressor") cells so as to prolong and facilitate tolerance to BGG, but that ultimately the regulatory cells are either lost or inadequate. The loss of normal tolerance regulation appears to occur in association with a subpopulation of T cells since escape from tolerance, which apparently depends upon intact helper T cell function, is quite effective.

A recent study has presented evidence that a nonspecific B cell mitogen and specific antigen can cooperate in a specific immune response (29). Similarly, a nonspecific T cell mitogen (Con A) plus a tolerogen may cooperate in the induction and/or maintenance of tolerance. The present experiments suggest that further study of Con A may help elucidate some of the mysteries of normal regulatory control of the immune response. Similar studies in situations where there is defective regulatory control may shed further light on the causes of the immunological aberrations.

Summary. NZB/W mice 4 weeks or older are not easily made tolerant by ultracentrifuged BGG. Younger mice can be made tolerant, but they escape rapidly. Recent studies have suggested a loss of regulatory or suppressor cells in the pathogenesis of immunologic abnormalities early in life in NZB/W mice. Several reports indicate that Concanavalin A (Con A) is capable of activating suppressor cells in normal mice. We found that Con A could prolong the period of tolerance in NZB/W mice when ultracentrifuged BGG was given at 20 days of age. NZB/W mice given ultracentrifuged BGG at 4 weeks of age were not tolerant to subsequent challenge with BGG in adjuvant unless they also received Con A. These studies are compatable with the hypothesis that Con A activates suppressor cell activity which is deficient in NZB/W mice. Alternative explanations of these phenomena are discussed.

1. Talal, N., and Steinberg, A. D., "Current Topics in Microbiology and Immunology." Springer–Verlag, New York (1974).

2. Burnet, F. M., and Holmes, M. C., Nature **207,** 368 (1965).

3. Mellors, R. C., Aoki, T., and Huebner, R. J., J. Exp. Med. **129,** 1045 (1969).

4. Steinberg, A. D., Baron, S., and Talal, N., Proc. Nat. Acad. Sci. USA **63,** 1102 (1969).

5. Weir, D. M., McBride, W., and Naysmith, J. D., Nature **219,** 176 (1968).

6. Staples, P. J., and Talal, N., J. Exp. Med. **129,** 123 (1969).

7. Staples, P. J., Steinberg, A. D., and Talal, N., J. Exp. Med. **131,** 1123 (1970).

8. Steinberg, A. D., Law, L. W., and Talal, N., Arthritis Rheum. **13,** 369 (1970).

9. Chused, T. M., Steinberg, A. D., and Parker, L. M., J. Immunol. **111,** 52 (1973).

10. Barthold, D. R., Kysela, S., and Steinberg, A. D., J. Immunol. **112,** 9 (1974).

11. Steinberg, A. D., Arthrit. Rheum. **17,** 11 (1974).

12. de Vries, M. J., and Hijmans, W., J. Pathol. Bacteriol. **91,** 487 (1966).

13. Markowitz, H., Person, D. A., Gitnick, G. L., and Ritts, R. E., Science **163,** 476 (1969).

14. Leon, M. A., and Schwartz, H. J., Proc. Soc. Exp. Biol. Med. **131,** 735 (1969).

15. Nirmul, G., Severin, C., and Taub, R. N., Transplant. Proc. **14,** 91 (1972).

16. Dutton, R. W., J. Exp. Med. **136,** 1445 (1972).

17. Rich, R. R., and Pierce, C. W., J. Exp. Med. **137,** 205 (1973).

18. Barth, R. F., and Singla, O., Cell. Immunol. **9,** 96 (1973).

19. Egan, H. S., Reeder, W. J., and Ekstedt, R. D., J. Immunol. **112,** 63 (1974).

20. Stavitsky, A. B., and Arquilla, E. R., Int. Arch. Allergy Appl. Immunol. **13,** 1 (1958).

21. Gershon, R. K., Cohen, P., Hencin, R., and Liebhaber, S. A., J. Immunol. **108,** 586 (1972).

22. Baker, P. J., Stashak, P. W., Amsbaugh, D. F., Prescott, B., and Barth, R. F., J. Immunol. **105,** 1581 (1970).

23. Droege, W., Nature **234,** 549 (1971).

24. Okumura, K., and Tada, T., J. Immunol. **107,** 1682 (1971).

25. Kerbel, R. S., and Eidinger, D., Eur. J. Immunol. **2,** 114 (1972).

26. Herzenberg, L. A., Chan, E. L., Ravitch, M. M., Riblet, R. J., and Herzenberg, L. A., J. Exp. Med. **137,** 1311 (1973).

27. Gershon, R. K., Gery, I., and Waksman, B. H., J. Immunol. **112,** 215 (1974).

28. Ha, T-Y, Waksman, B. H., and Treffers, H. P., J. Exp. Med. **139,** 13 (1974).

29. Kreisler, J. M., and Moller, G., J. Immunol. **112,** 151 (1974).

Received May 8, 1974. P.S.E.B.M., 1974, Vol. 147.