Immunosuppression of Anti-Sheep Erythrocyte Responses by Glycerol Teichoic Acid Immune Complexes

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Sprague-Dawley rats which produce "naturally occurring" antibodies to glycerol teichoic acid (GTA) displayed immunosuppression of anti-sheep erythrocyte plaque-forming cell and serum antibody responses when a single dose of lipid-free GTA was administered 24 h before immunization. Such suppression was enhanced by administering GTA complexed with anti-GTA immunoglobulin G. Animals fed a GTA-free diet produced no anti-GTA immunoglobulins and failed to show GTA-mediated immunosuppression under similar experimental conditions. However, when those animals were given GTA-anti-GTA complexes, suppression was evident. The results suggested antigenic competition mediated by immune complex-activated suppressor T cells. Lipid-free GTA did not stimulate serum antibody responses under the experimental conditions employed.

Modulation of immune responses by bacterial products has received considerable attention in recent years. Such products would be expected to have considerable impact on host-parasite relations and possibly have some immunotherapeutic potential (reviewed in 28). The modulatory effects of several bacterial products such as lipopolysaccharide (16), dextran (2), cholera toxin (8), and pyrogenic exotoxin (18) have been investigated; however, the actual mechanism(s) of suppression or enhancement remains unclear. In fact, the nature of the modulatory effect of some of these materials depended on dosage, route, and time of administration.

Several investigators have reported immunosuppression mediated by glycerol teichoic acid (GTA). Miller and Jackson (21) found that GTA from *Streptococcus pyogenes* suppressed the anti-sheep erythrocyte (SRBC) response of mice. They later showed that the lipid moiety of their GTA preparation was necessary for the observed suppression and that their GTA did not suppress, but enhanced the antibody response to lipopolysaccharide (22). Chorpennig et al. (11) found both suppression and enhancement of plaque-forming cells (PFC) in mice when lipid-free GTA from a *Bacillus* species was administered 24 h before immunization. Their response kinetics data suggested that GTA merely delayed the formation of PFC by 24 h and actually produced enhancement rather than suppression.

The mechanism of immunomodulation by GTA remains unclear. GTA has been shown to have potent membrane-binding ability (3, 4, 7, 13), to activate the alternate complement path-

way (29), and to be a T-lymphocyte mitogen (4), yet no studies have elucidated the mechanism of immunosuppression. Results of the previous studies have been clouded by the presence of environmental GTA. Laboratory animals raised in a conventional manner are exposed to GTA via their Laboratory Chow diet (26), and many produce "naturally occurring" anti-GTA immunoglobulins (14). Therefore, dosage of GTA in such animals cannot be accurately controlled since environmental stimulation by GTA will possibly mask the effect of parenterally administered material. However, rats fed a GTA-free liquid diet produce no naturally occurring anti-GTA (26) and provide a more desirable model for investigation of GTA-mediated immunomodulation. In the present investigation GTA-mediated modulation of anti-SRBC responses was examined in rats fed a GTA-free diet. Environmental GTA and antibody responses to it appeared to suppress anti-SRBC responses in rats. Antibodies specific for GTA appeared to play a critical role in immunosuppression mediated by parenterally administered lipid-free GTA.

MATERIALS AND METHODS

Animals. Sprague-Dawley rats aged 20 weeks were utilized for all experiments. Some were reared on Purina Laboratory Chow (Ralston-Purina, St. Louis, Mo.), and others were raised from 14 days of age on a sterile Similac liquid infant formula which has been reported free of GTA (26). Rats raised on this diet produce no naturally occurring antibodies to GTA, but are fully immunocompetent (26).

Antigens. The GTA used in this investigation was a gift from F. W. Chorpennig. It was extracted from *Bacillus* sp. ATCC 29726 with aqueous phenol and

purified as described elsewhere (13, 14). This material was free of lipid and peptidoglycan.

SRBC suspensions were prepared from defibrinated sheep blood. Cells were washed three times in phosphate-buffered saline and adjusted to a 10% concentration (vol/vol) for injection and for use in hemolytic plaque assays.

Hemolytic plaque assay. The hemolytic plaque assay of Jerne and Nordin (19) was used, with slight modification, for evaluation of splenic antibody-forming cells. SRBC were spectrophotometrically standardized to a 10% concentration, and the incubation time was extended to 2.5 h. All tests were performed in triplicate using an agarose-Hanks balanced salt solution medium in 50-mm petri dishes.

Serology. Serum antibodies specific for GTA were measured by passive hemagglutination (PHG) using GTA-coated rat erythrocytes as previously described (12). Anti-SRBC antibodies were evaluated in a similar manner by hemagglutination using a 1% SRBC suspension. All sera were heat inactivated at 56°C for 30 min before testing. Close-interval parallel dilutions were used, and each sample was examined in triplicate.

Preparation of immune complexes. Adult rats were given three intraperitoneal injections of 10^9 *Bacillus* sp. ATCC 29726 at 1-week intervals and were exsanguinated 1 week after the final injection. The pooled immune serum was then absorbed 3× with 20% (vol/vol) washed SRBC to remove natural agglutinins. The serum was then characterized by Sephadex G-200 gel filtration as described previously (5). Fraction 2 (immunoglobulin G) was dialyzed and concentrated back to the original serum volume. This material had a PHG titer of 256 when GTA-coated rat erythrocytes were used as antigen. No SRBC-agglutinating activity was present.

Soluble GTA was diluted in phosphate-buffered saline in concentrations ranging from 0.01 to 25 µg/ml. To each dilution an equal volume (0.05 ml) of the purified Sephadex fraction 2 anti-GTA immunoglobulin was added, and the mixture was incubated for 30 min at room temperature. Each sample was then tested for PHG inhibition ability (10) using GTA-coated rat erythrocytes and a standardized antiserum. The undiluted fraction 2 antiserum could completely block the PHG inhibiting ability of as much as 5 µg of GTA (normally 100% inhibition), but not 7 µg or more. Therefore, the GTA-anti-GTA complexes were prepared by diluting each milliliter of the purified immunoglobulin with 100 µg of GTA dissolved in 1 ml of phosphate-buffered saline, followed by 30 min of incubation before injection.

Experimental protocol. Base-line serum samples were obtained from rats aged 20 weeks. Each animal was then given a single intraperitoneal injection of GTA or GTA-anti-GTA. The dosage was adjusted to 0.32 µg of GTA/g of body weight (11). Control rats received only phosphate-buffered saline. After 24 h, all rats received an intraperitoneal injection of 0.5 ml of 10% SRBC. Rats were sacrificed 4 or 5 days later, and splenocytes were examined for PFC. Sera were evaluated for anti-SRBC and anti-GTA by hemagglutination and PHG. Equal numbers of Similac-fed and Laboratory Chow-fed rats were immunized, with a total of 96 rats for the entire experiment.

RESULTS

GTA significantly suppressed the anti-SRBC response in rats when administered 24 h before immunization (Table 1). Both serum antibody titers and splenic PFC showed significant degrees of suppression (ca. 80%) at 4 and 5 days postinjection. Administration of GTA-anti-GTA complexes rather than GTA alone also produced significant immunosuppression and to a somewhat higher degree (ca. 90%). Again, both serum immunoglobulin and PFC responses were suppressed. All 48 animals examined produced anti-GTA antibodies.

Rats fed a sterile GTA-free diet and not producing anti-GTA antibodies showed no immunosuppression at 4 or 5 days after immunization with SRBC (Table 2). However, those GTA-free rats which received GTA-anti-GTA complexes displayed significant suppression of anti-SRBC responses at 4 and 5 days after immunization.

Numbers of PFCs and hemagglutinin titers of serum anti-SRBC immunoglobulin were generally higher in those rats free of anti-GTA (Table 2) than in animals producing such antibodies (Table 1). No GTA-mediated enhancement of anti-SRBC responses was observed in any of the 96 animals examined. The low dosage of lipid-free GTA administered to the rats appeared non-immunogenic within the time frame of this investigation since anti-GTA levels did not rise above base-line titers in any animal.

DISCUSSION

The results of the present study indicated that antibodies to GTA play an important role in GTA-mediated immunosuppression of anti-SRBC responses. Anti-GTA induced by environmental GTA as well as parenterally administered GTA-anti-GTA complexes suppressed anti-SRBC responses, whereas rats producing no anti-GTA and injected with GTA alone showed no suppression.

There are several hypotheses that may be considered in explanation of the reported GTA-mediated immunosuppression. Examination of naturally occurring anti-GTA levels in control rats (Table 1) revealed an increase from day 4 to day 5 after SRBC immunization. This suggests that GTA may cross-react with SRBC. If such cross-reactivity did exist, GTA-mediated immunosuppression of SRBC responses could be explained by "blocking" of antigen receptors. However, there have been no reports of such cross-reactivity, and extensive hemagglutination and immune hemolysis inhibition tests performed in my laboratory have failed to detect any cross-reactivity (unpublished data). A more acceptable explanation for the variability in nat-

TABLE 1. *GTA-mediated immunosuppression in rats producing naturally occurring anti-GTA antibody*

Expt ^a	Rats ^b	PFC per 10 ⁶ spleen cells ^c	Suppres- sion (%)	Serum anti- SRBC ^d	Suppres- sion (%)	Serum anti- GTA ^e
Day 4	Control	647 ± 22	—	56.0 ± 3.4	—	8
	GTA injected	97 ± 16	85	11.6 ± 3.2	77	4.6
	GTA-anti-GTA injected	39 ± 7	94	6.6 ± 1.4	88	6.8
Day 5	Control	1,042 ± 31	—	79.0 ± 8.7	—	11
	GTA injected	208 ± 29	80	13.4 ± 2.1	83	7.4
	GTA-anti-GTA injected	93 ± 37	91	8.7 ± 3.2	89	3.2

^a Days after immunization with SRBC.^b Each group included eight animals.^c ±Standard error of the mean.^d Mean hemagglutination titer.^e Mean PHG titer.TABLE 2. *GTA-mediated immunosuppression in rats producing no naturally occurring anti-GTA antibody*

Expt ^a	Rats ^b	PFC per 10 ⁶ spleen cells ^c	Suppres- sion (%)	Serum anti- SRBC ^d	Suppres- sion (%)
Day 4	Control	1,128 ± 27	—	160 ± 17.0	—
	GTA injected	1,245 ± 80	—	200 ± 13.4	—
	GTA-anti-GTA injected	209 ± 24	81	57 ± 11.5	64
Day 5	Control	1,845 ± 89	—	213 ± 13.1	—
	GTA injected	1,810 ± 83	—	192 ± 13.8	—
	GTA-anti-GTA injected	692 ± 62	62	37 ± 6.8	83

^a Days after immunization with SRBC.^b Eight animals per group.^c ±Standard error of the mean^d Mean hemagglutination titer. PHG titer was 0 for each group.

urally occurring anti-GTA levels is antibody cycling, which I have previously reported to occur in these animals (6).

A possible explanation for the increased PFC responses in rats fed the Similac diet (Table 2) is that there may be some type of immunopotentiator, possibly a plant lectin, present in Similac and absent in the Laboratory Chow. The two diets certainly differ in composition to a much greater extent than merely GTA content; however, the observation that injection of GTA immune complexes produced immunosuppression in the Similac-fed rats (Table 2) confirms that the absence of GTA in the Similac was responsible, at least in part, for the high PFC numbers as compared with animals reared on Laboratory Chow which contained GTA.

The GTA-mediated immunosuppression observed in this investigation could be explained as a form of sequential antigenic competition (25). In this type of competition a normal response is produced to the first antigen administered while the response to the second is suppressed, with maximum suppression observed at the peak PFC response of the first. Gershon and Kondo (17) showed that such competition is mediated by T cells. Since immunoglobulin G

immune complexes have been shown to activate suppressor T cells (23) which are capable of suppressing B-cell activation, GTA-mediated immunosuppression could be explained as antigenic competition mediated by immune complex stimulation of suppressor T cells. All rats used in the present investigation which were fed the Laboratory Chow diet did produce anti-GTA immunoglobulin G (data not shown; 5) and did show GTA-mediated immunosuppression. Rats producing no anti-GTA, but injected with GTA-anti-GTA immunoglobulin G complexes, also showed suppression of anti-SRBC responses. Both situations could be explained by activation of suppressor T cells by immune complexes. A constant low-level T-cell-mediated immunosuppression (stimulated by naturally occurring GTA-anti-GTA complexes) could also explain the generally lower PFC numbers observed in the conventionally raised rats (Table 1) compared with those raised on the GTA-free diet (Table 2).

A criticism of the above hypothesis is that no antigenic competition or suppression was observed when GTA was injected into the rats fed the GTA-free diet (Table 2). Several explanations can be offered for this lack of immuno-

suppression. Since lipid-free GTA was used, it probably could not bind directly to lymphocytes, thus eliminating the possibility of mitogenic stimulation of suppressor T cells (4). The immunogenicity of lipid-free GTA is also questionable since there are conflicting reports in the literature (9, 20). Injection of lipid-free GTA may not stimulate antibody formation; thus no immune complex stimulation of suppressor T cells could occur. The low dosage used in the present investigation did not stimulate a detectable serum antibody response. The lipid requirement for GTA-mediated immunosuppression in mice as reported by Miller et al. (22) could also be explained by the above arguments. Mice reportedly do not produce naturally occurring anti-GTA (14). The lipid-free GTA of Miller et al. (22), being poorly immunogenic (15), was unable to stimulate production of anti-GTA antibodies (within the time frame of their experiment) and probably was unable to attach directly to lymphocyte membranes (4). Therefore, no immune complex- or mitogen-stimulated suppressor T cells were generated, and thus no immunosuppression occurred in mice with lipid-free GTA. The results of the present studies and those of Miller et al. (22) should not be compared with the GTA-mediated suppression and enhancement reported by Chorpenning et al. (11). These authors used C3H/HEJ mice, which possess a genetic inability to respond to lipopolysaccharide, with the defect possibly occurring at the macrophage level (27). It is possible that such mice would have an altered response to GTA; however, no GTA response data have been reported.

The above hypothesis of immune complex-mediated immunosuppression is supported by other investigations of immunoregulation. Immunosuppression by such complexes has been shown to operate at the T-cell (23) and B-cell levels (1). Immune complex-mediated immunosuppression of a heterologous antibody response has also been previously reported (24); therefore, the results of the present investigation are not entirely unique. Further in vitro studies are being planned to clarify the mechanism of TA-mediated immunosuppression which hopefully will identify the function of immune complexes and suppressor T cells in this phenomenon.

LITERATURE CITED

- Askonas, B. A., A. J. McMichael, and M. E. Roux. 1976. Clonal dominance and the preservation of clonal memory cells mediated by antigen-antibody. *Immunology* 31:541-551.
- Battisto, J., and F. Pappas. 1973. Regulation of immunoglobulin synthesis by dextran. *J. Exp. Med.* 138:176-193.
- Beachey, E. H., T. M. Chiang, I. Ofek, and A. H. Kang. 1977. Interaction of lipoteichoic acid of group A streptococci with human platelets. *Infect. Immun.* 16:649-654.
- Beachey, E. H., J. B. Dale, S. Grebe, A. Ahmed, W. A. Simpson, and I. Ofek. 1979. Lymphocyte binding and T cell mitogenic properties of group A streptococcal lipoteichoic acid. *J. Immunol.* 122:189-195.
- Bolton, R. W., and F. W. Chorpenning. 1974. Naturally occurring cellular and humoral immunity to teichoic acid in rats. *Immunology* 27:517-523.
- Bolton, R. W., H. Rozmiarek, and F. W. Chorpenning. 1977. Cyclic antibody formation to polyglycerophosphate in normal and injected rats. *J. Immunol.* 118:1154-1158.
- Chiang, T. M., M. L. Alkan, and E. H. Beachey. 1979. Binding of lipoteichoic acid of group A streptococci to isolated human erythrocyte membranes. *Infect. Immun.* 26:316-321.
- Chisari, F. V., R. S. Northrup, and L. C. Chen. 1974. The modulating effect of cholera enterotoxin on the immune response. *J. Immunol.* 113:729-739.
- Chorpenning, F. W., H. R. Cooper, J. W. Oldfather, and J. J. Lynch. 1979. Immunogenicity of soluble versus cellular glycerol teichoic acid. *Infect. Immun.* 26:211-216.
- Chorpenning, F. W., H. R. Cooper, and S. Rosen. 1975. Cross-reactions of *Streptococcus mutans* due to cell wall teichoic acid. *Infect. Immun.* 12:586-591.
- Chorpenning, F. W., J. J. Lynch, H. R. Cooper, and J. W. Oldfather. 1979. Modulation of the immune response to sheep erythrocytes by lipid-free glycerol teichoic acid. *Infect. Immun.* 26:262-269.
- Chorpenning, F. W., and H. B. Stamper. 1973. Spontaneous adsorption of teichoic acid to erythrocytes. *Immunochemistry* 10:15-20.
- Cooper, H. R., F. W. Chorpenning, and S. Rosen. 1978. Lipid-free glycerol teichoic acids with potent membrane binding activity. *Infect. Immun.* 19:462-469.
- Decker, G. P., F. W. Chorpenning, and G. T. Frederick. 1972. Naturally-occurring antibodies to bacillary teichoic acid. *J. Immunol.* 108:214-222.
- Fiedel, B. A., and R. W. Jackson. 1976. Immunogenicity of a purified and carrier-complexed streptococcal lipoteichoic acid. *Infect. Immun.* 13:1585-1590.
- Franzl, R. W., and P. D. McMaster. 1968. The primary immune response in mice. I. The enhancement and suppression of hemolysin production by bacterial endotoxin. *J. Exp. Med.* 127:1087-1107.
- Gershon, R. K., and K. Kondo. 1971. Antigenic competition between heterologous erythrocytes. *J. Immunol.* 106:1524-1531.
- Hanna, E. E., and D. W. Watson. 1973. Enhanced immune response after immunosuppression by streptococcal pyrogenic exotoxin. *Infect. Immun.* 7:1009-1011.
- Jerne, N., and A. Nordin. 1963. Plaque formation in agar by single antibody-forming cells. *Science* 140:405.
- Knox, K. W., M. J. Hewitt, and A. J. Wicken. 1970. Studies on the group F antigen of lactobacilli: antigenicity and serological specificity of teichoic acid preparations. *J. Gen. Microbiol.* 60:303-313.
- Miller, G. A., and R. W. Jackson. 1973. The effect of a *Streptococcus pyogenes* teichoic acid on the immune response of mice. *J. Immunol.* 110:148-156.
- Miller, G. A., J. Urban, and R. W. Jackson. 1976. Effects of streptococcal lipoteichoic acid on host responses in mice. *Infect. Immun.* 13:1408-1417.
- Moretta, L., S. R. Webb, C. E. Grossi, P. M. Lydyard, and M. D. Cooper. 1977. Functional analysis of two human T-cell subpopulations: help and suppression of B-cell responses by T cells bearing receptors for IgM and IgG. *J. Exp. Med.* 146:184-200.

24. **Morgan, E. L., and C. H. Tempelis.** 1978. The requirement for the Fc portion of antibody in antigen-antibody complex-mediated suppression. *J. Immunol.* **120**:1669-1671.
25. **Radovich, J., and D. W. Talmage.** 1967. Antigenic competition: cellular or humoral. *Science* **158**:512-514.
26. **Rozmiarek, H., R. W. Bolton, and F. W. Chorpensing.** 1977. Environmental origin of natural antibodies to teichoic acid. *Infect. Immun.* **16**:505-509.
27. **Ruco, L. P., M. S. Meltzer, and D. L. Rosenstreich.** 1978. Macrophage activation for tumor cytotoxicity: control of macrophage tumoricidal capacity by the LPS gene. *J. Immunol.* **21**:543-548.
28. **Schwab, J. H.** 1975. Suppression of the immune response by microorganisms. *Bacteriol. Rev.* **39**:121-143.
29. **Winkelstein, J. A., and A. Tomasz.** 1978. Activation of the alternative complement pathway by pneumococcal cell wall teichoic acid. *J. Immunol.* **120**:174-178.