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Antigenic Competition Between an Endotoxic Adjuvant and a Protein Antigen

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Antigenic competition between bovine gamma globulin (BGG) and endotoxin from a smooth strain (S-ET) and a rough (R-ET) heptoseless mutant strain of *Salmonella minnesota* was studied in mice. Both endotoxins acted as adjuvants for enhancing the antibody response to BGG. However, other work showed that the R-ET had minimal antigenicity, and it was used as a control for the competition studies. Antigenic competition between BGG and endotoxin as expressed by a suppression of the antibody response to BGG could not be demonstrated when varying adjuvant doses of S-ET or R-ET were injected simultaneously with a small constant dose of BGG into normal mice. However, mice presensitized with S-ET several weeks before immunization with the S-ET and BGG combination produced anti-BGG levels which were four to eightfold lower than in normal mice. Nearly complete suppression of the anti-BGG response could be obtained in presensitized mice by reducing the BGG dose 10-fold or by increasing the adjuvant dose of endotoxin. Mice pretreated with R-ET and challenged with BGG plus S-ET or R-ET showed no depression of the anti-BGG response. These and other experiments confirmed the immunological basis of the competitive effect.

Under certain conditions, the immunological response elicited by an antigen may be impaired by the simultaneous or previous injection of a second antigen. This phenomenon, generally referred to as antigenic competition [*see* reviews by Adler (3, 4)], has been observed between synthetic antigens (8), haptenic groups on separate or common protein carriers (6, 9, 26), particulate antigens (11), particulate and soluble antigens (2), and soluble antigens (1).

As adjuvants, bacterial endotoxins enhance antibody responses of rodents to a number of protein antigens (15, 16, 22). In many instances, those endotoxins derived from smooth strains of bacteria are potent immunogens themselves (18, 19). Since antigenic competition between endotoxic adjuvants and protein antigens has not yet been studied, the present investigations were undertaken to define conditions under which a competitive effect could be demonstrated on the humoral antibody response of mice to bovine gamma globulin (BGG) by endotoxins derived from two strains of *Salmonella minnesota*. In this report, antigenic competition is defined to have been effected when the antibody response to BGG, in the experimental case, is less than that stimulated by BGG and adjuvant doses of endotoxin.

The rationale for the succeeding experimental designs is as follows. It has been established that endotoxins from smooth strains of *Salmonella* are antigenic (18, 19). However, endotoxins from heptoseless mutant strains of *Salmonella* which lack the normal polysaccharide determinant sugars (20) are poor antigens (24; Leong, Milner, and Rudbach, *unpublished data*); in some cases no detectable antibody response can be elicited in animals repeatedly inoculated with them. These polysaccharide-deficient endotoxins are toxic and were found to still possess adjuvanticity; they were employed as nonimmunogenic controls in these studies on antigenic competition.

MATERIALS AND METHODS

Mice. Male albino mice of the Rocky Mountain Laboratory stock weighing 20 to 22 g were used in all experiments.

Protein antigen. Bovine gamma globulin (lot no. A30702, fraction II from bovine plasma) was purchased from Armour Pharmaceutical Co., Kankakee, Ill.

Endotoxins. Cultures of *S. minnesota* wild type S and its heptoseless (Re) mutant strain R595 were provided by O. Lüderitz (20). The bacteria were

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grown at 37 C for 24 hr in shake flasks containing Trypticase Soy Broth (BBL). *E. coli* 0111:B4 (Difco) was grown at 37 C for 24 hr in M-9 medium (25) containing 0.5% glucose. Cells were harvested by centrifugation and washed repeatedly with cold saline. Cell walls were prepared from *E. coli* by cell disruption at 20,000 psi with a Sorvall refrigerated fractionator (13). Endotoxins were extracted from cell walls of *E. coli* and from whole cells of *S. minnesota* wild type and of its rough heptoseless mutant by the hot phenol-water method of Westphal, Lüderitz, and Bister (29).

Immunizations. In general, groups of mice were pretreated with varying doses of endotoxins from either *S. minnesota* wild type (S-ET) or from its rough heptoseless mutant (R-ET). At 7, 14, and 21 days after pretreatment, these mice and groups of normal mice were immunized with mixtures of BGG and S-ET or R-ET. On days 3, 6, 9, 12, 15, and 18 postimmunization, six mice from each group were sacrificed and the sera pooled. All solutions of endotoxins, BGG, and combinations of the two were made with pyrogen-free saline immediately preceding each experiment and were used within 1 hr after preparation; all injections were given intravenously.

Antiserum. Anti-*S. minnesota* serum was prepared from mice given six weekly intravenous injections of 20 μg of *S. minnesota* wild type endotoxin. Control sera were obtained from normal mice. All sera were collected, pooled, sterilized by filtration, and stored at −20 C.

Passive hemagglutination. Bovine gamma globulin was attached to tannic acid-treated sheep red blood cells (SRBC) by the procedure of Stavitsky (28), and SRBC were sensitized with boiled endotoxin from *S. minnesota* (wild type) by the method described by Neter et al. (23). Phosphate buffered saline (PBS), *p*H 7.2 (0.01 M PO_4; 0.15 M NaCl), containing 1.5% normal rabbit serum (heated for 30 min at 56 C and twice adsorbed with SRBC) was used as diluent. Serial twofold dilutions of serum samples were made beginning with 1:10. The volume of each dilution was 0.5 ml to which 0.05 ml of a 2% suspension of sensitized SRBC was added. The tubes were incubated at room temperature for 4 hr and placed in the refrigerator overnight, and the titers were read. The resulting titers were expressed as values of x derived from the equation $x = \log_2 (HD/10)$, where HD is the reciprocal of the highest dilution of serum giving a positive hemagglutination pattern.

RESULTS

Adjuvant activity of S. minnesota endotoxins. The first set of experiments was designed to establish a reproducible system for detecting, in a quantitative fashion, the adjuvant activity of *S. minnesota* endotoxins on anti-BGG responses of mice. Data in Fig. 1 show the adjuvant effect of S-ET and R-ET on the antibody responses of mice to BGG. Incorporation of endotoxin with various doses of BGG resulted in shorter induction periods and increased anti-BGG levels as compared to the responses of mice receiving BGG alone. With the S-ET adjuvant dose, the anti-BGG responses increased as the amount of BGG injected was increased. Average peak titers in sera of mice immunized with BGG and S-ET were 2 to 3 logs higher than the titers elicited with comparable doses of BGG and R-ET. Even 100 μg of the poorly immunogenic R-ET injected with 12 μg of BGG did not increase the anti-BGG titer above that stimulated by 10 μg of S-ET plus 12 μg of BGG (cf. Fig. 4). These findings indicate a lack of antigenic competition between the simultane-

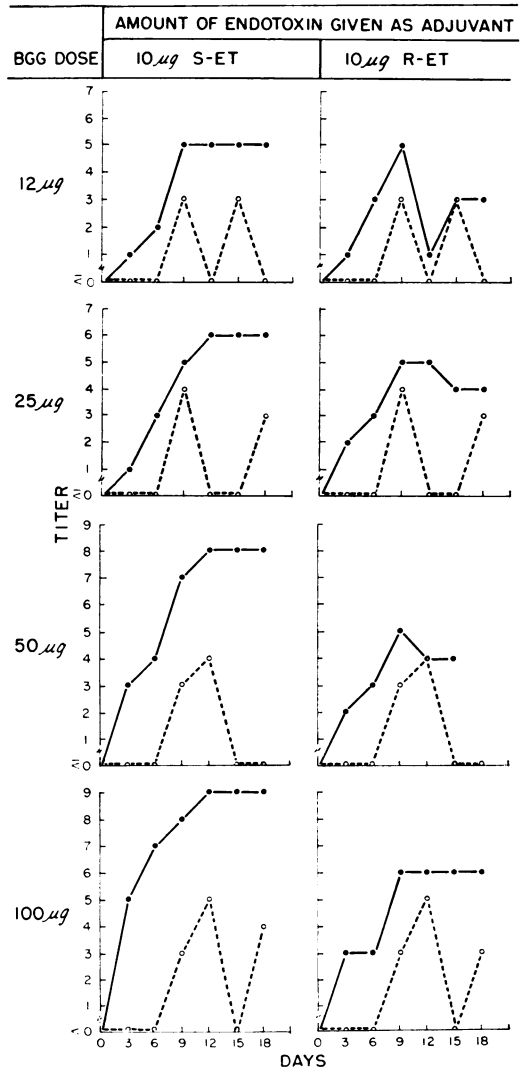

FIG. 1. *Adjuvant effect of endotoxins derived from Salmonella minnesota wild type (S-ET) and its rough mutant strain R_{595} (R-ET) on the primary antibody response of normal mice to bovine gamma globulin (BGG). Symbols: ●, BGG plus endotoxin; ○, control, BGG alone.*

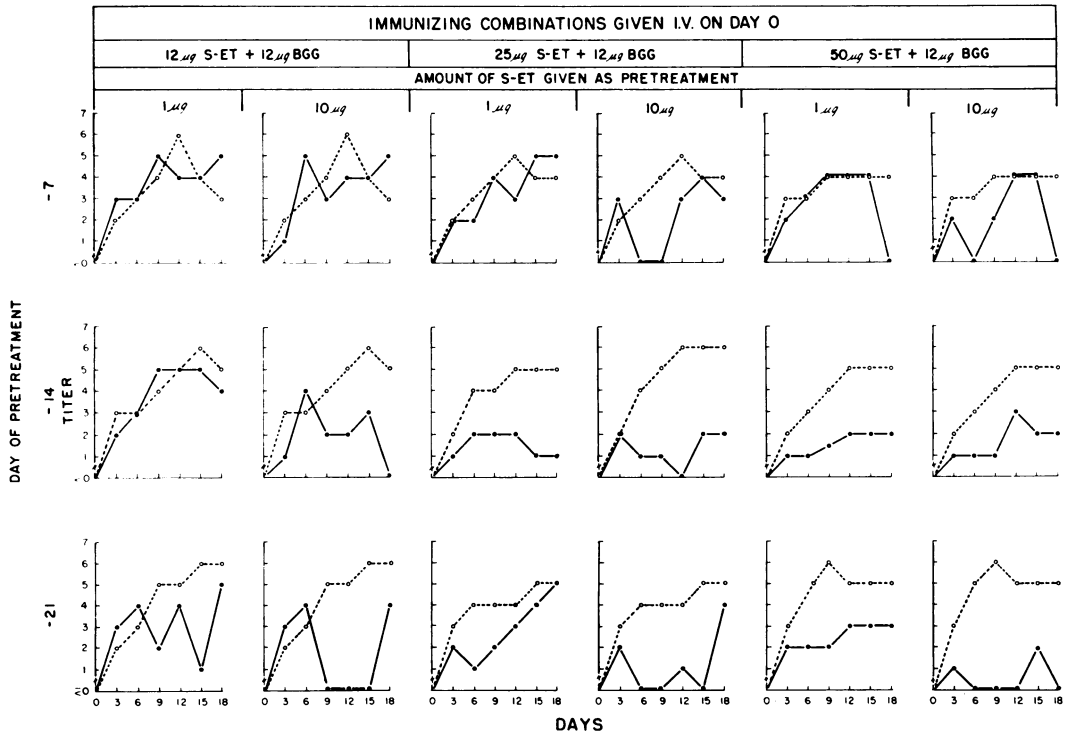

FIG. 2. *The effect of pretreatment with endotoxin from Salmonella minnesota wild type (S-ET) on the anti-BGG response of mice immunized with combinations of BGG and S-ET. Symbols:* ●, *S-ET pretreated and BGG plus S-ET immunized;* ○, *controls immunized with BGG plus S-ET only.*

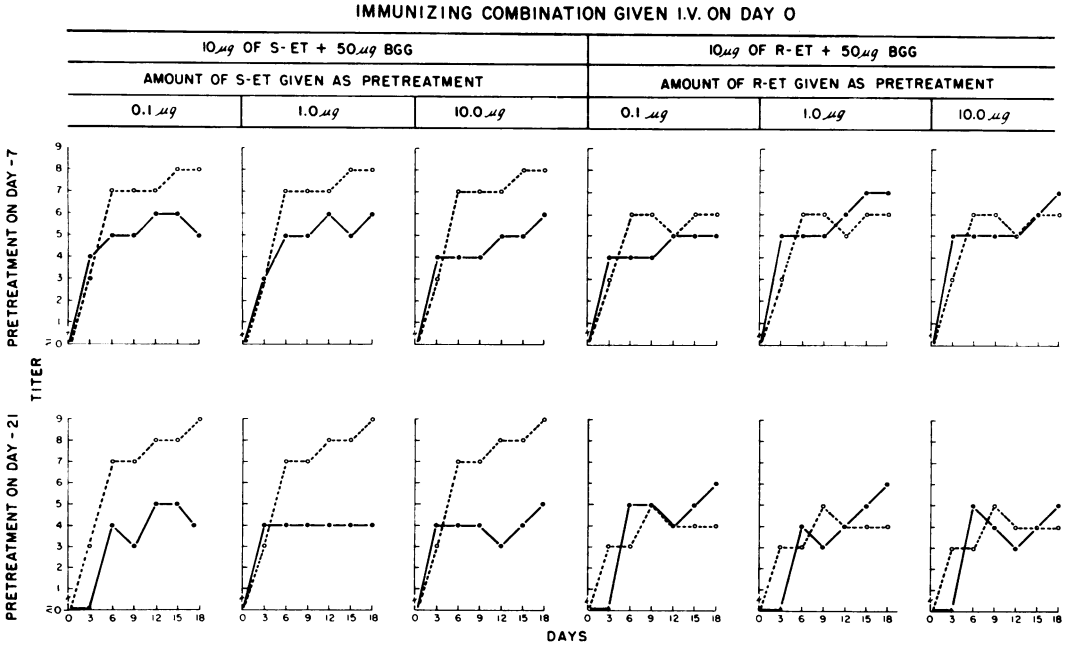

FIG. 3. *The anti-BGG responses of mice pretreated with various doses of endotoxins from Salmonella minnesota wild type (S-ET) or from its rough mutant strain* R_{595} *(R-ET) before immunization with combinations of BGG and S-ET (R-ET). Symbols:* ●, *endotoxin pretreated and BGG plus endotoxin immunized;* ○, *controls immunized with the BGG plus endotoxin combinations only.*

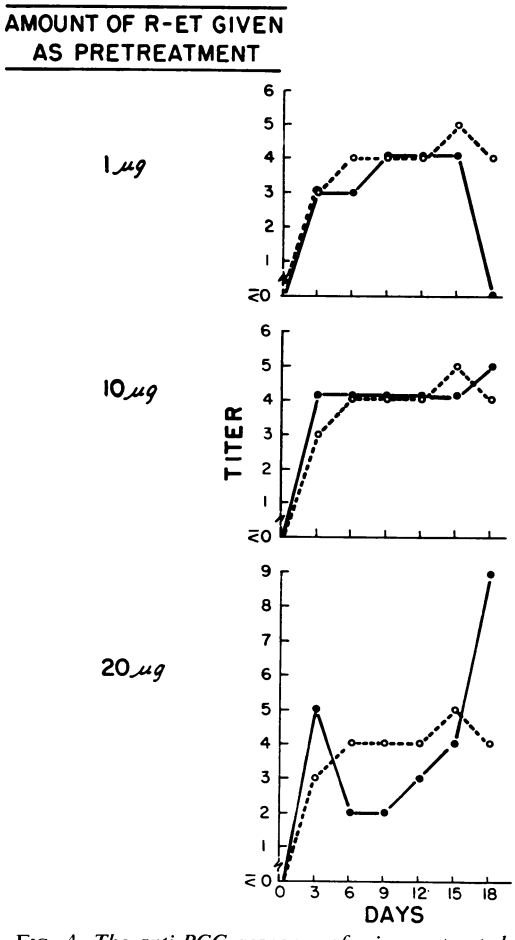

FIG. 4. *The anti-BGG response of mice pretreated with endotoxin from Salmonella minnesota heptoseless mutant R_{595} (R-ET) 21 days before immunization with 12 μg of BGG + 100 μg of R-ET given intravenously on day zero. Symbols: ●, R-ET pretreated and BGG plus 100 μg of R-ET immunized; ○, control, immunized with BGG plus R-ET only.*

ous injection of an adjuvant dose of S-ET and varying doses of BGG in normal mice.

Effect of pretreatment of mice with S-ET on antigenic competition between BGG and S-ET. Next attempted was a demonstration of antigenic competition between adjuvant doses of S-ET and BGG in mice presensitized to S-ET (Fig. 2). Antigenic competition to the S-ET and BGG immunization, characterized by a decreased anti-BGG response, was observed in mice treated with 10 μg of S-ET 14 or 21 days prior to immunization. It was also observed in groups treated with 1 μg at day 14 with the higher adjuvant doses of endotoxin and at day 21 in all cases. Furthermore, both sensitizing doses of S-ET tended to increase the depression of the responses when longer intervals between injections and when larger adjuvant doses of endotoxin were employed. This is suggestive of an immunological basis for the competition. Control mice responded as expected demonstrating that the increased quantities of S-ET employed as adjuvant in the immunization combinations were not responsible, per se, for the depressed immune responses to BGG in presensitized mice.

Comparison of effect of pretreatment with S-ET and R-ET on antigenic competition. Figure 3 shows the anti-BGG responses of mice pretreated with three dose levels of S-ET or R-ET 7 and 21 days before immunization with 50 μg of BGG and 10 μg of the homologous endotoxin. Antigenic competition was clearly demonstrated in mice treated 21 days previously with S-ET and immunized with S-ET and BGG; some competitive effect was also observed in the groups pretreated at day 7. In contrast, it was not possible to demonstrate a competitive effect with R-ET given as the pretreating and adjuvant dose with BGG (Fig. 3). Furthermore, an attempt to elicit competition between BGG and R-ET by pretreating with R-ET, increasing the adjuvant dose of R-ET to 100 μg, and decreasing the amount of BGG to 12 μg in the immunizing injection was unsuccessful (Fig. 4). A 21-day interval between pretreatment and immunization was chosen because this time was found to be optimal for the competitive effect with S-ET (Fig. 2).

Requirement for similar antigenic specificities in the pretreatment and adjuvant doses of endotoxin to elicit antigenic competition. The data presented above indicated that S-ET had to be used as either the pretreating dose or as the adjuvant to elicit a competitive effect on the antibody response to BGG. The data in Fig. 5 show that, in fact, S-ET was needed in both doses. Antigenic competition to BGG could not be established in mice pretreated with S-ET 21 days prior to immunization with R-ET and BGG (group I), nor could the competitive effect be demonstrated if the sequence was reversed with R-ET presensitization followed by S-ET as the adjuvant (group II). The antibody responses of the experimental groups were similar to those of the controls.

Another experiment was designed to confirm the requirements for both pretreatment and adjuvant doses to contain immunogenic endotoxin of the same specificity in order to establish a competitive situation. Figure 6 shows that pretreatment of mice with a highly immunogenic endotoxin from *Escherichia coli* 0111:B4 would not sensitize for competition between BGG and adjuvant doses of the *Salmonella* endotoxins. Anti-BGG responses to the immunizing mixtures were similar in control and experimental groups.

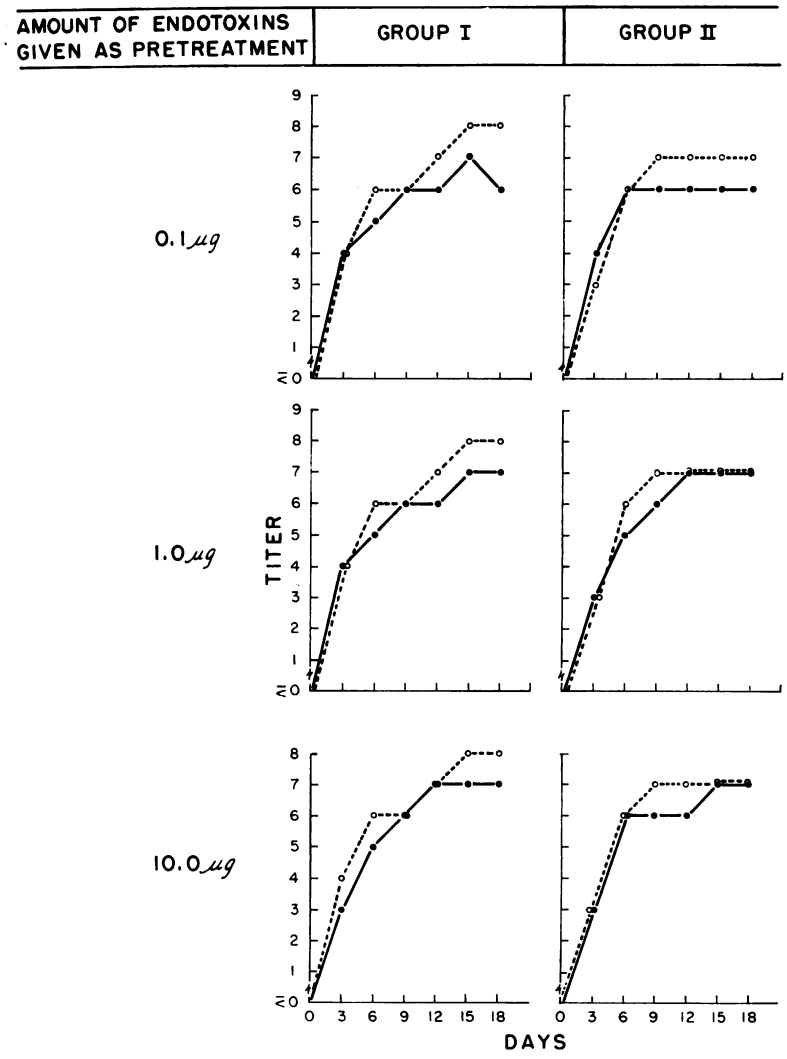

FIG. 5. *The anti-BGG responses of mice pretreated 21 days previously with endotoxin from S. minnesota wild type (S-ET) and endotoxin from its rough mutant R_{595} (R-ET). Group I, mice pretreated with S-ET 21 days before immunization with 50 μg of BGG plus 10 μg of R-ET; group II, mice pretreated with R-ET 21 days before immunization with 50 μg of BGG plus 10 μg of S-ET. Symbols: ●, endotoxin pretreated and BGG plus ET immunized; ○, control, immunized with BGG plus endotoxin combination only.*

Influence of antibodies to S-ET on antigenic competition. Since elicitation of a state of antigenic competition required presensitization of mice with an immunogenic endotoxin of the same specificity as the adjuvant dose, the possibility existed that humoral antibodies stimulated by the first pretreating injection of endotoxin might be neutralizing the adjuvanticity of the second dose of endotoxin. As a control for this possibility, three groups of normal mice (42 mice per group) were given 0.5 ml of mouse anti-S-ET serum intraperitoneally (agglutinin titer of serum 1:640). At 6, 12, and 24 hr after injecting the serum, six mice were sacrificed, and their sera were assayed individually for antibody to S-ET. The mice remaining in each pretreatment period were immunized with 12 μg of S-ET and 12 μg of BGG. Similarly, an equal number of mice were inoculated with 0.5 ml of normal mouse serum and immunized with the BGG and S-ET combination at the same time intervals. A third group of normal mice received only the immunizing combination. The results (Fig. 7) indicate that competition between BGG and S-ET was not the result of a

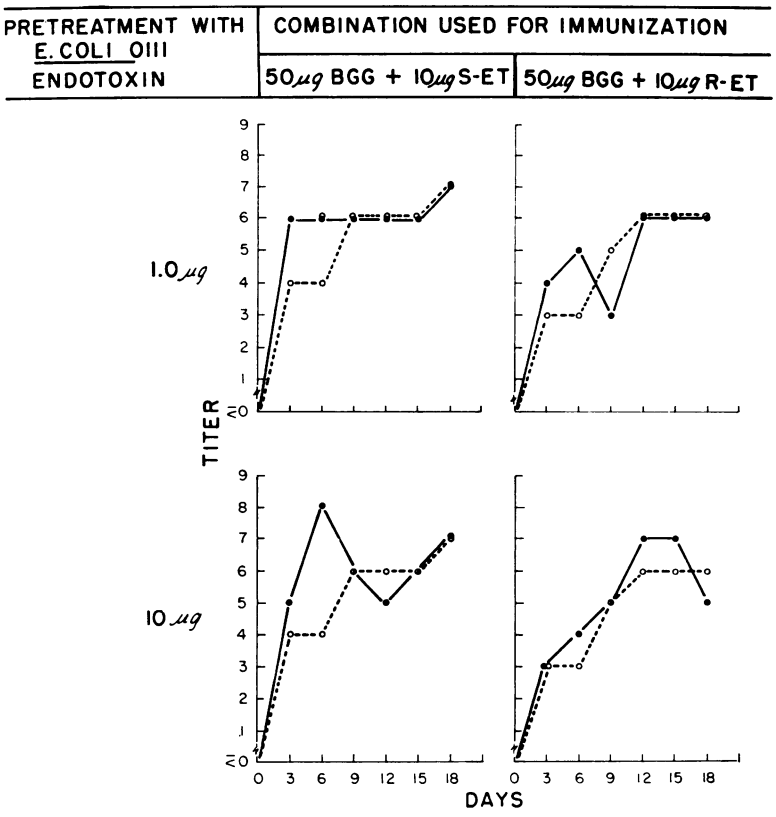

FIG. 6. *Specificity studies: the anti-BGG responses of mice pretreated 21 days previously with various doses of Escherichia coli 0111 endotoxin before immunization with combinations of 50 μg of BGG and endotoxins from Salmonella minnesota wild type (S-ET) or from its rough mutant R_{595} (R-ET) on day zero. Symbols: ●, E. coli 0111 endotoxin pretreated and BGG plus S. minnesota endotoxin immunized; ○, control, immunized with BGG plus S. minnesota endotoxin combination only.*

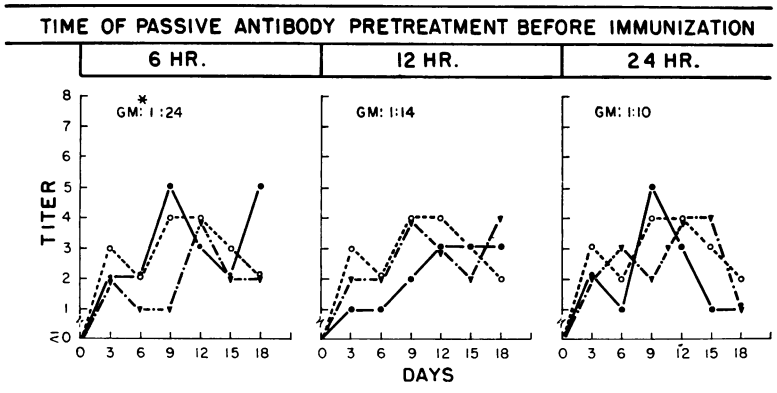

FIG. 7. *The effect of passive anti-S. minnesota antibody on the immune response of mice to BGG. Normal mice were given 0.5 ml of anti-Salmonella minnesota serum intraperitoneally (ip) 6, 12, and 24 hr before immunization with 12 μg of BGG plus 12 μg of S. minnesota wild type endotoxin (S-ET) given intravenously (iv). Control mice received 0.5 ml of normal mouse serum ip on the same time intervals before iv immunization with the antigen combination. The anti-S. minnesota serum was prepared in mice given multiple iv injections of S-ET, hemagglutinin titer 1:640. Symbols: ●, anti-BGG responses of mice given anti-S. minnesota serum; ▼, anti-BGG responses of mice given normal mouse serum; ○, control, normal mice immunized with the antigen combination only; ✳ geometric mean titer of residual anti-S-ET antibody in passively immunized mice before injection with the antigen combination.*

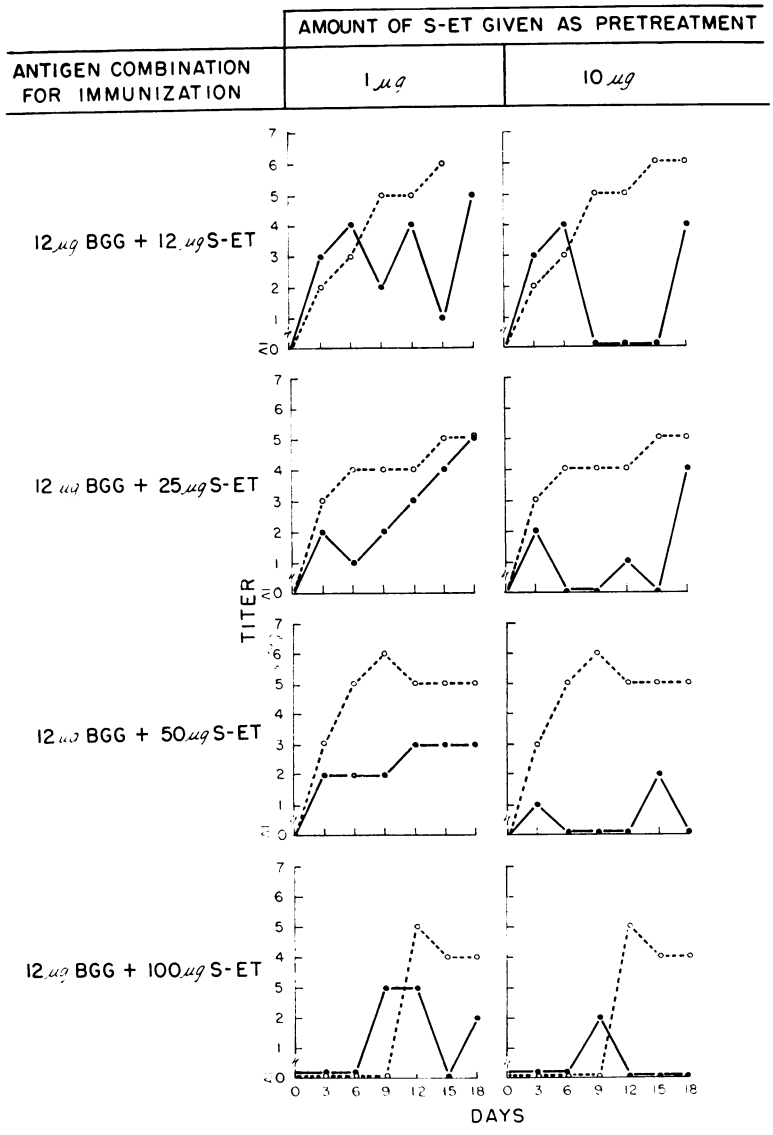

FIG. 8. *The anti-BGG response of mice pretreated with endotoxin from S. minnesota wild type (S-ET) 21 days before immunization with a constant dose of 12 μg of BGG and increasing amounts of S-ET intravenously on day zero. Symbols: ●, S-ET pretreated and BGG plus S-ET immunized; ○, control, immunized with BGG plus S-ET only.*

neutralization of the adjuvanticity of S-ET by specific antibody. No significant differences were observed between the anti-BGG titers of passively immunized mice and those of the controls given normal mouse serum or immunized only with the BGG and S-ET combination. Mice passively immunized with anti-S-ET serum possessed residual antibodies to S-ET at the time of immunization with the BGG-S-ET combination. The geometric mean titers of the 6-, 12-, and 24-hr pretreatment groups were 1:24, 1:14, and 1:10, respectively.

A second control experiment was designed to determine the possible neutralization of the adjuvant dose of S-ET by residual antibodies from the presensitizing injection. The rationale for this test was as follows. If antibodies to S-ET were neutralizing the adjuvanticity of the second dose of S-ET, then by increasing the size of the adjuvant dose of S-ET, the neutralizing effect of specific antibody could be overcome by the excess of S-ET. However, if the first dose of S-ET sensitized mice for an active competitive action by the sec-

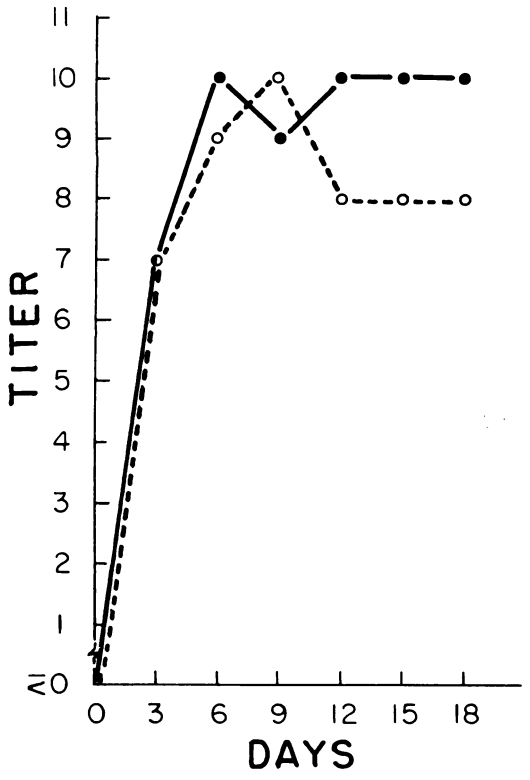

FIG. 9. *The anti-BGG response of mice immunized intravenously (iv) with two injections of BGG and endotoxin. Two groups of mice were injected iv with 12 μg of BGG plus 12 μg of endotoxin from S. minnesota wild type (S-ET) or 12 μg of BGG plus 12 μg of endotoxin from its mutant R_{595} (R-ET), respectively. Twenty-one days later, each group was boosted iv with the same BGG-endotoxin combination. Symbols: ●, 12 μg of BGG plus 12 μg of S-ET; ○, 12 μg of BGG + 12 μg of R-ET.*

ond injection of S-ET, increasing the size of the second or adjuvant dose of S-ET should enhance competition. As shown (Fig. 8), the latter was the case. Competition was greatest in sensitized mice receiving 50 μg of adjuvant doses of S-ET. The adjuvant dose of 100 μg of S-ET was overtly toxic and it delayed and depressed the anti-BGG response even in normal mice, but it still elicited antigenic competition in sensitized animals.

Effect of two doses of endotoxin and BGG on the anti-BGG response. Two groups of mice were pretreated with BGG plus S-ET or R-ET and were immunized 21 days later with the same respective antigen combination (Fig. 9). The results of this experiment show no antigenic competition to the anti-BGG response when both doses contained BGG. Apparently, competition could only be manifested to a primary response to BGG by a secondary anti-S-ET response.

DISCUSSION

Bacterial endotoxins have been shown to act as adjuvants in enhancing antibody production to a number of protein antigens (15, 16, 22). Similarly, in the present study S-ET and R-ET were found to augment the antibody responses of mice to BGG. A shorter inductive period with subsequently increased and sustained levels of antibody to BGG were observed when the endotoxins were injected with BGG, as compared to the response to BGG alone.

One of the problems which has prevented the incorporation of purified endotoxin adjuvants with prophylactic agents for use in human and veterinary medicine is the toxicity of such preparations. Reports have appeared which point out methods for circumventing the toxicity by chemical detoxification of endotoxins derived from smooth strains of gram-negative bacteria; however, these modified endotoxins are still antigenic (17). From results presented above, it would appear that the use of whole gram-negative bacteria (7), endotoxins, or modified endotoxins from smooth strains of gram-negative bacteria as universal adjuvants would be limited because of potential sensitization of the host by the first injection of an adjuvant-antigen mixture for competition with the response to a second antigen injected with the same lipopolysaccharide adjuvant. Our investigation suggests a method for avoiding this problem. The heptoseless endotoxin still possessed adjuvanticity for enhancing the humoral antibody response to BGG. However, the endotoxin had low immunopotency and was unable to initiate antigenic competition in mice primed with either R-ET or S-ET. Therefore, chemical detoxification of this material, or one like it, might yield an adjuvant suitable for various prophylactic vaccines.

It would appear that true antigenic competition between an adjuvant dose of endotoxin and the immunological response to a second antigen occurs only in the presence of a secondary response to the endotoxin. Franzl and McMaster (12) stated that antigenic competition did not explain the inhibited immunological response in mice which received erythrocyte antigens and a single injection of *Salmonella typhosa* endotoxin; apparently, the inhibition observed in their system was more closely related to toxicity than to the antigenicity of the endotoxin. We also observed inhibition of the response to BGG by the single injection of an overtly toxic dose of endotoxin (Fig. 8).

Another possible explanation for the reduced anti-BGG response in mice, which were first injected with S-ET and then immunized with BGG and S-ET, is that tolerance to the adjuvant effect

of endotoxin was produced by the first injection (10). However, tolerance to biological effects of endotoxin, produced by a single injection of endotoxin and exhibiting no interendotoxin specificity, wanes within 48 hr (14). Our data show that both the presensitizing and adjuvant doses had to contain immunogenic endotoxin of the same immunological specificity in order to establish competition with the anti-BGG response. Furthermore, the competition was more pronounced when the BGG and S-ET were given 3 weeks after the first dose of S-ET than at shorter intervals. A later phase of tolerance to endotoxin does require interendotoxin specificity between the two doses and is related to circulating O-antibody (14). The data presented (Fig. 7 and 8) apparently ruled out this type of effect in our system.

The experimental system chosen for the present work employed amounts of endotoxin which would be used as adjuvant doses and limited the amounts of an experimental antigen, the response to which would need enhancement. Therefore, we would not wish to imply from the results obtained with the present restricted system that a specific immune response to the competing antigen can be used as a measure of the magnitude of competing pressures exerted on all test antigens. For example, Abramoff and Wolfe (1) showed that bovine hemoglobin effectively suppressed the primary antibody response to bovine serum albumin without inducing detectable antibodies to itself. Other studies employing synthetic polypeptides have shown that a nonimmunogenic material given in Freund adjuvant could completely inhibit the delayed cutaneous response to a fully immunogenic copolymer (8).

Recently, it has been shown that the cellular events in the immune response require the interaction of at least two cell types of different origins. Briefly, antigen reactive cells of thymic origin first recognize and react with antigen. These cells then interact with and subsequently trigger the differentiation of bone marrow derived precursor cells into specific antibody producing cells (21, 27). On the basis of this two cell model, other investigators have suggested that competition of antigens takes place at the cellular level, that is, antigens uncouple the reaction between antigen reactive cells and precursor cells (5). As indicated by this information, the results of the present work could be interpreted as the utilization of the antigen reacting cells by the priming stimulus, S-ET, which would produce a deficiency of cells available for processing the second antigen, BGG, administered in sequence. However, the competitive effect exerted on BGG was observed only after the BGG-S-ET combination was injected into S-ET primed mice. In this case, then, the depression of the primary response to BGG could be the result of an insufficient number of antigen reactive cells available to BGG caused by the enhanced efficiency in processing S-ET after a second exposure to the antigen. In contrast to S-ET, endotoxin from the rough heptoseless mutant R_{595} was unable to effect antigenic competition. The weak immunogenicity of this endotoxin is probably attributable to its deficiency in polysaccharide and consequent lack of major O-antigenic determinants. Therefore, S-ET with its full complement of O-determinants could be expected to exert greater immunological pressure than R-ET and to compete more effectively for antigen-processing cells.

ACKNOWLEDGMENT

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LITERATURE CITED

1. Abramoff, P., and H. R. Wolfe. 1956. Precipitin production in chickens. XIII. A quantitative study of the effect of simultaneous injection of two antigens. J. Immunol. 77:94–101.
2. Adler, F. L. 1956. Antibody formation after injection of heterologous immune globulin. J. Immunol. 76:217–227.
3. Adler, F. L. 1957. Antibody formation after injection of heterologous immune globulin. II. Competition of antigens. J. Immunol. 78:201–210.
4. Adler, F. L. 1964. Competition of antigens. Progr. Allergy 8:41–57.
5. Albright, J. F., T. F. Omer, and J. W. Deitchman. 1970. Antigen competition: antigens compete for a cell occurring with limited frequency. Science 167:196–198.
6. Amkraut, A. A., J. S. Garvey, and D. H. Campbell. 1966. Competition of haptens. J. Exp. Med. 124:293–306.
7. Barr, M., and M. Llewellyn-Jones. 1953. Interference with antitoxic responses in immunisation with combined prophylactics. Brit. J. Exp. Pathol. 34:233–240.
8. Ben-Efraim, S., and P. Liacopoulos. 1967. Inhibition, no-effect or enhancement of immune responses following injection of mixtures of immunogenic and non-immunogenic synthetic polypeptides. Immunology 12:517–524.
9. Brody, N. I., and G. W. Siskind. 1969. Studies on antigenic competition. J. Exp. Med. 130:821–832.
10. Condie, R. M., and R. A. Good. 1956. Inhibition of immunological enhancement by endotoxin in refractory rabbits. Immunochemical study. Proc. Soc. Exp. Biol. Med. 91:414–418.
11. Eidinger, D., S. A. Khan, and K. G. Millar. 1968. The effect of antigenic competition on various manifestations of humoral antibody formation and cellular immunity. J. Exp. Med. 128:1183–1200.
12. Franzl, R. E., and P. D. McMaster. 1968. The primary immune response in mice. I. The enhancement and suppression of hemolysin production by a bacterial endotoxin. J. Exp. Med. 127:1087–1107.
13. Fukushi, K., R. L. Anacker, W. T. Haskins, M. Landy, K. C. Milner, and E. Ribi. 1964. Extraction and purification of endotoxin from *Enterobacteriaceae*: a comparison of selected methods and sources. J. Bacteriol. 87:391–400.
14. Greisman, S. E., E. J. Young, and F. A. Carozza, Jr. 1969. Mechanisms of endotoxin tolerance. V. Specificity of the early and late phases of pyrogenic tolerance. J. Immunol. 103:1223–1236.
15. Johnson, A. G. 1964. Adjuvant action of bacterial endotoxins

on the primary antibody response, p. 252–262. *In* M. Landy and W. Braun (ed.), Bacterial endotoxins. Rutgers Univ. Press, New Brunswick, N.J.

16. Johnson, A. G., S. Gaines, and M. Landy. 1956. Studies on the O antigen of *Salmonella typhosa*. V. Enhancement of antibody response to protein antigens by the purified lipopolysaccharide. J. Exp. Med. **103**:225–246.
17. Johnson, A. G., and A. Nowotny. 1964. Relationship of structure to function in bacterial O antigens. III. Biological properties of endotoxoids. J. Bacteriol. **87**:809–814.
18. Landy, M., A. G. Johnson, M. E. Webster, and J. F. Sagin. 1955. Studies on the O antigen of *Salmonella typhosa*. II. Immunological properties of the purified antigen. J. Immunol. **74**:466–478.
19. Landy, M., R. P. Sanderson, and A. L. Jackson. 1965. Humoral and cellular aspects of the immune response to the somatic antigen of *Salmonella enteritidis*. J. Exp. Med. **122**:483–504.
20. Lüderitz, O., A. M. Staub, and O. Westphal. 1966. Immunochemistry of O and R antigens of *Salmonella* and related *Enterobacteriaceae*. Bacteriol. Rev. **30**:192–255.
21. Mitchell, G. F., and J. F. A. P. Miller. 1968. Immunological activity of thymus and thoracic-duct lymphocytes. Proc. Nat. Acad. Sci. U.S.A. **59**:296–303.
22. Munoz, J. 1964. Effect of bacteria and bacterial products on antibody response. Advan. Immunol. **4**:397–440.
23. Neter, E., O. Westphal, O. Lüderitz, E. A. Gorzynski, and E. Eichenberger. 1956. Studies of enterobacterial lipopolysaccharides. Effects of heat and chemicals on erythrocyte-modifying, antigenic, toxic and pyrogenic properties. J. Immunol. **76**:377–385.
24. Raynaud, M., M. Digeon, and C. Nauciel. 1964. Studies on the endotoxin and antigens of a rough strain of *Salmonella typhi*, p. 326–344. *In* M. Landy and W. Braun (ed.), Bacterial endotoxins. Rutgers Univ. Press, New Brunswick, N.J.
25. Roberts, R. B., P. H. Abelson, D. B. Cowie, E. T. Bolton, and J. Britten. 1955. Studies of biosynthesis in *Escherichia coli*, p. 5. Carnegie Inst. Wash. Publ. 607.
26. Schechter, I. 1968. Antigenic competition between polypeptidyl determinants in normal and tolerant rabbits. J. Exp. Med. **127**:237–250.
27. Shearer, G. M., and G. Cudkowicz. 1969. Distinct events in the immune response elicited by transferred marrow and thymus cells. I. Antigen requirements and proliferation of thymic antigen-reactive cells. J. Exp. Med. **130**: 1243–1261.
28. Stavitsky, A. B. 1954. Micromethods for the study of proteins and antibodies. I. Procedure and general applications of hemagglutination and hemagglutination-inhibition reactions with tannic acid and protein-treated red blood cells. J. Immunol. **72**:360–367.
29. Westphal, O., O. Lüderitz, and F. Bister. 1952. Über die Extraktion von Bakterien mit Phenol/Wasser. Z. Naturforsch. **7b**:148–155.