MODIFICATION OF HOST RESPONSES TO BACTERIAL ENDOTOXINS*

II. Passive Transfer of Immunity to Bacterial Endotoxin with Fractions Containing 19S Antibodies

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The first paper in this series (1) presented evidence for an immunologic mechanism of pyrogenic tolerance; this was based on the induction of specific tolerance and an observed anamnestic response to endotoxins comparable to classical immune systems. It was suggested that antibodies other than those specific for the O antigen are produced in animals made tolerant to endotoxin; these antibodies protect hyperreactive cells from the primary and secondary toxic activities of the endotoxin and assist the reticuloendothelial system (RES) to destroy the toxin (1, 2).

This paper extends these observations by characterizing an antibody capable of conferring pyrogenic and lethal tolerance to Gram-negative bacterial endotoxins.

Materials and Methods

Animals.—Young adult American Dutch rabbits (1.0 to 1.2 kg) were used under the same conditions as previously described (1).

Toxins.—Purified *Escherichia coli* 08 endotoxin lot $CO8_{2158}S_5$ was generously supplied by Dr. Otto Westphal, Max Planck Institute for Immunobiology, Freiburg, West Germany. The methods of preparation of stock solutions, working solutions, and determination of febrile response (minimal pyrogenic dose–3 hours, MPD-3) were given previously (1, 2). A dose of 100 MPD-3 (0.8 μg/kg) given intravenously was used as a standard for pyrogenicity testing.

Preparation of Serum from Rabbits Tolerant to Endotoxin (TRS).—Four groups, each group comprising 10 to 15 rabbits, were rendered tolerant to endotoxin by the schedule given in the previous paper (1). Tolerance was confirmed by testing their pyrogenic response to 100 MPD-3/kg of endotoxin. Animals were bled 48 hours after the last injection of toxin; this serum collected at 4°C was pooled and stored at −20°C.

Normal Rabbit Serum (NRS).—Serum from normal rabbits was prepared and processed as described above.

Hyperimmune Rabbit Serum (HRS).—Rabbits were hyperimmunized with heat-killed washed cells of *E. coli* 08 at a concentration of 10^9 cells/ml. Animals received a series of intravenous injections at 2-day intervals for 9 days. The final injections were given subcutaneously

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on the 16th and 23rd day. Each animal received 12 ml of antigen. On the 30th day the animals were bled and the serum processed as described above. The O agglutination titer was 2560 expressed as the reciprocal of the highest dilution of serum giving agglutination.

All sera used in these experiments were heated at 56°C for 30 minutes.

Diethylaminoethyl (DEAE) Cellulose Chromatography.—Chromatography of TRS was done in water-jacketed columns (5 by 150 cm) at 4°C. The method was the same as recently described for the fractionation of mouse serum (3) using selectacel standard DEAE cellulose. Fractions were used for titration of anti-O antibodies, immunoelectrophoretic analyses, passive transfer studies and additional purifications.

Exclusion Chromatography.—Sephadex G-200 was used to isolate 19S γ_1-immunoglobulin from fraction IV obtained by DEAE chromatography of TRS. 40 gm sephadex G-200 was

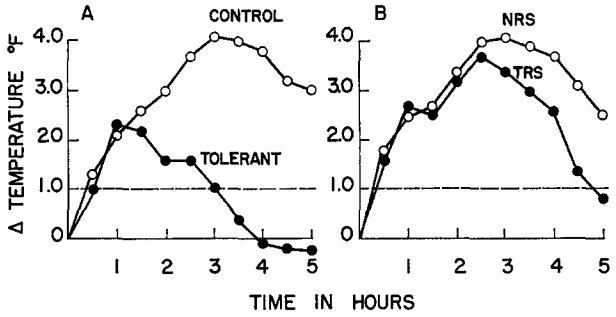

FIG. 1. Active and passive tolerance to COO8 endotoxin. A. Each curve represents the mean febrile response of 15 rabbits to 100 MPD-3/kg of endotoxin; *control*, normal rabbits; *tolerant*, the above rabbits after 6 daily injections of endotoxin. B. Each curve represents the mean febrile response of 5 rabbits to 100 MPD-3/kg of endotoxin injected intravenously 24 hours after the passive transfer of serum; *NRS*, animals received intravenously 5 ml/kg of normal rabbit serum; *TRS*, animals received intravenously 5 ml/kg of serum from tolerant rabbits.

washed with buffered-saline and packed in a 5 by 100 cm water-jacketed column maintained at 4°C. 20 ml (1000 mg protein) of DEAE fraction IV from 200 ml of TRS, equilibrated with buffered saline, was passed through the column. Eluates collected in a fraction collector at a rate of 25/ml/tube/hour were assayed for protein concentrations in a Beckman DB spectrophotometer at 280 mμ. Samples within the peaks were pooled, concentrated by pervaporation, and finally dialyzed against 0.15 M NaCl solution; these were used for passive transfer studies.

Density Gradient Zone Ultracentrifugation.—A slight modification of the method of Stanworth *et al.* (4) was used as recently described (3).

Agar Gel Double Diffusion and Immunoelectrophoresis.—Immunoelectrophoresis was done on glass plates (8.2 x 10.1 cm or 12.7 x 17.8 cm) with veronal buffer pH 8.6, 0.05 M and 0.75 per cent Noble agar. A current of 3 ma/cm width and 4 v./cm length was applied for 2.5 or 3.5 hours. Ouchterlony analyses were made on the same type of plates used for immunoelecphoresis. After the lines of precipitate developed, plates were washed free of soluble protein with 0.15 M NaCl solution, dried at 45°C, and stained with azocarmine. Goat anti-rabbit serum was used in the analyses.

Procedure for Passive Transfer of Tolerance with Serum or Fractions.—Rabbits were conditioned in racks for 5 to 6 hours, 1 to 2 days prior to use. These animals were randomly di-

vided into groups of 5 to 10 rabbits for each serum or fraction tested. Various amounts of serum or fractions were injected intravenously; 24 hours later the animals were given 100 MPD-3/kg intravenously of endotoxin and the pyrogenic response recorded over a 5 hour period.

RESULTS

Passive Transfer of Pyrogenic Tolerance with Serum from Tolerant Animals.— Because of the many reports on the presence of endotoxin-modifying substances

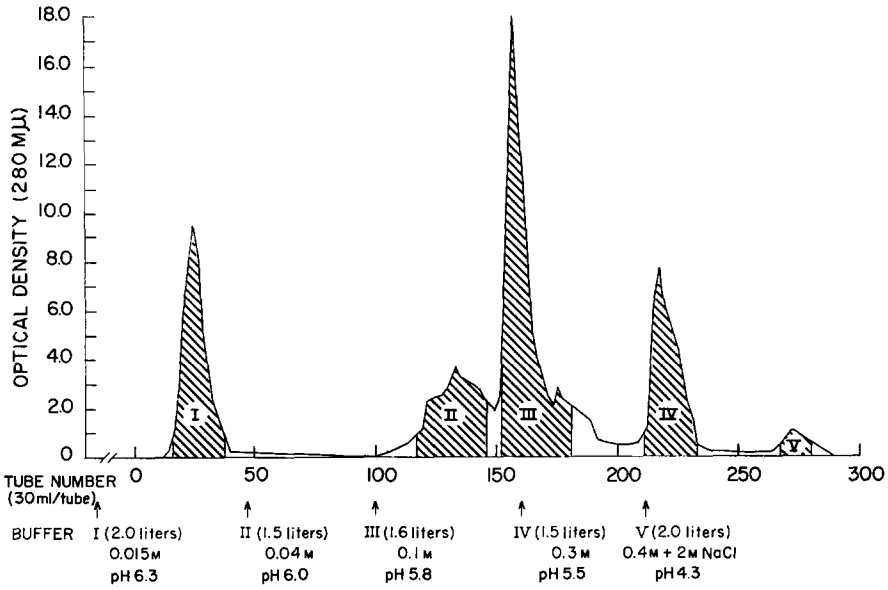

FIG. 2. DEAE chromatography of TRS. The 5 fractions represented by the shaded areas under each curve were obtained from 250 ml of TRS. The fractions were concentrated by pervaporation and dialyzed against 0.15 M NaCl solution.

present in normal serum (5–7), it was necessary to use a passive transfer technique in which the serum is given 24 hours prior to injection with endotoxin. Serum for these passive transfer studies was obtained from animals made tolerant by repeated injections of endotoxin. Fig. 1 A gives the pyrogenic response to 100 MPD-3/kg of endotoxin in these animals before and after the development of tolerance. Since 100 MPD-3/kg is the standard dose used to test for pyrogenic tolerance in the following experiments, these curves also serve as references for pyrogenic response in normal and tolerant animals.

Rabbits receiving 5 ml/kg of serum from tolerant rabbits (TRS) (Fig. 1 A) were partially tolerant as shown in Fig. 1 B when compared with those receiving normal rabbit serum (NRS).

Passive Transfer of Tolerance with DEAE Fractions of TRS.—The slight degree of passive tolerance obtained with serum from tolerant donors suggested that the failure to obtain a greater degree of tolerance could be attributed to the relatively small amount of antibody in the TRS. In an effort to concentrate larger amounts of antibody into a small volume, a large volume of TRS was fractionated by DEAE chromatography. As shown in Fig. 2, the serum was fractionated into 5 groups of proteins designated I, II, III, IV, and residual V

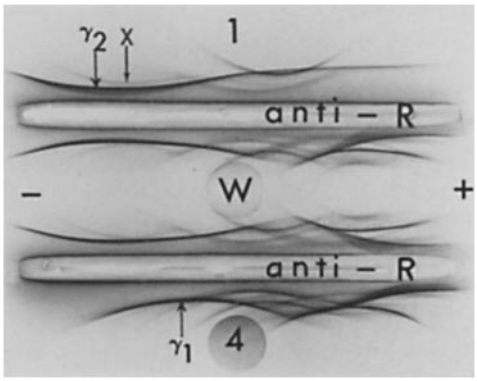

FIG. 3. Immunoelectrophoretic analysis of DEAE fractions I and IV. *1* is fraction I (0.2 ml 10 mg/ml); *W* is whole TRS (0.2 ml 1:4 dilution); *4* is fraction IV (0.2 ml 10 mg/ml). Each trough contained 1 ml of goat anti-rabbit serum (anti-R). 7S γ_2-, 19S γ_1-immunoglobulins and *X* are indicated by arrows. X has an electrophoretic mobility comparable to 7S γ_2 but is a distinct component similar to that observed in the mouse (3).

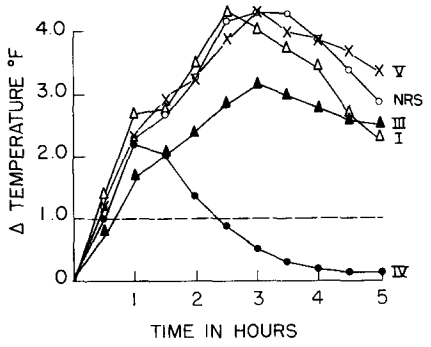

FIG. 4. Passive transfer of pyrogenic tolerance with DEAE fractions of TRS. Each curve represents the mean febrile response of 5 or 10 rabbits to 100 MPD-3/kg of endotoxin injected intravenously 24 hours after the passive transfer of serum or fractions; group *NRS* received 5 ml/kg of normal rabbit serum; group *I*, 5 ml (275 mg)/kg of fraction I; group *III*, 5 ml (430 mg)/kg of fraction III; group *IV*, 4 ml (172 mg)/kg of fraction IV; and group *V*, 4 ml (44 mg)/kg of fraction V.

From the immunoelectrophoretic patterns given in Fig. 3, fraction 1 contained the 7S γ_2-immunoglobulin, and fraction 4, the 19S γ_1-immunoglobulin.

These fractions isolated by DEAE chromatography were tested for their ability to transfer pyrogenic tolerance to normal rabbits. Results given in Fig. 4 show that fraction IV containing 19S γ_1-immunoglobulin gave complete pyrogenic tolerance in rabbits tested with 100 MPD-3/kg of endotoxin. The protein transferred in this fraction would be equivalent to that in 30 ml of TRS. Other fractions including fraction I containing most of the γ_2-immunoglobulin failed to confer pyrogenic tolerance. Fraction III gave a partial tolerance which might indicate contamination with 19S γ_1-immunoglobulin.

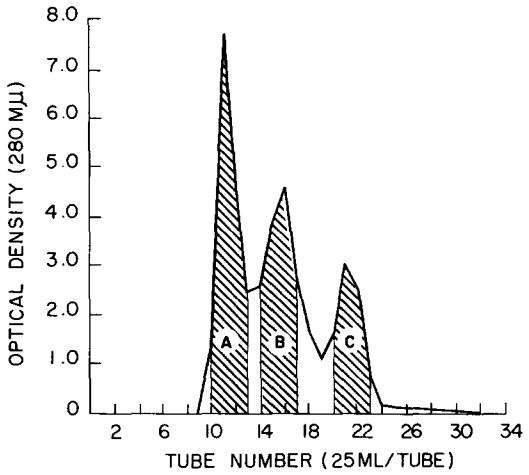

FIG. 5. Sephadex G-200 fractionation of DEAE fraction IV of TRS. The contents of the tubes in the shaded area of each curve were pooled to give fractions A, B, and C. Each fraction was concentrated by pervaporation and dialyzed against 0.15 M NaCl solution.

Passive Transfer of Pyrogenic and Lethal Tolerance with Sephadex G-200 Fractions of DEAE Fraction IV.—As shown in Fig. 4, complete pyrogenic tolerance was transferred with DEAE fraction IV. Although this fraction contained a high concentration of 19S γ_1-immunoglobulin, the immunoelectrophoretic analysis (Fig. 3) showed a high degree of contamination with other serum proteins. In an attempt to purify further the 19S γ_1-immunoglobulin, fraction IV was fractionated by exclusion chromatography on sephadex G-200. Data in Fig. 5 show three main fractions designated A, B, and C. The macromolecule, 19S γ_1-immunoglobulin appeared exclusively in fraction A as determined by immunoelectrophoretic and sucrose density gradient ultracentrifugation analyses.

Fractions A, B, and C were tested for their ability to transfer pyrogenic tolerance and to protect against the lethal activity of endotoxin. Results given

in Fig. 6 show that of the three fractions tested, only A at a concentration of 24 mg/kg gave complete pyrogenic tolerance and protected against the lethal activity of endotoxin. Since fraction A contained primarily 19S γ_1-immunoglobulin, the protective activity appears to be associated with 19S antibody.

Non-Relation between O Specific Antibodies and Passive Transfer of Tolerance. —It is known that the specific O antibodies are associated with 19S γ_1-immuno-

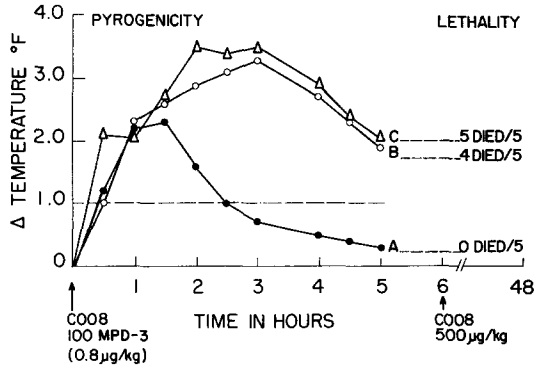

FIG. 6. Passive transfer of pyrogenic tolerance and immunity to the lethal effect of endotoxin with sephadex G-200 fractions. Each curve represents the mean febrile response of 5 rabbits to 100 MPD-3/kg of endotoxin given intravenously 24 hours after the injection of fractions; group *A* received 1 ml (24 mg)/kg of fraction A; group *B*, 2 ml (30 mg)/kg of fraction B; and group *C*, 1.5 ml (30 mg)/kg of fraction C. Six hours after the injection of 100 MPD-3/kg of endotoxin all animals received intravenously 500 μg/kg of endotoxin. Deaths were recorded within 48 hours.

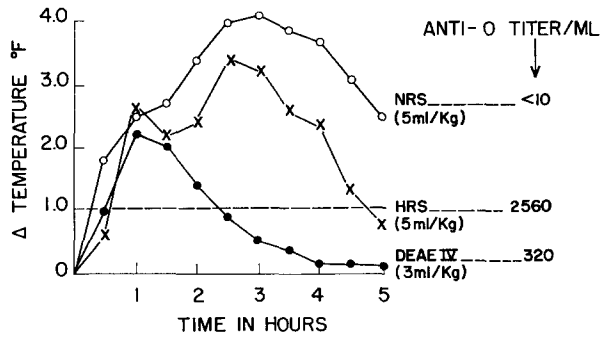

FIG. 7. Non-relation between O-specific antibodies and passive transfer of tolerance. Each curve represents the mean febrile response of 5 rabbits to 100 MPD-3/kg of endotoxin given intravenously 24 hours after the injection of serum or fraction; group *NRS* received intravenously 5 ml/kg of normal rabbit serum (anti-O titer < 10/ml); group *HRS*, 5 ml/kg of hyperimmune rabbit serum (anti-O titer, 2560/ml); group *DEAE IV*, 3 ml/kg of DEAE IV of TRS (anti-O titer 320/ml).

globulins (8). Therefore, we have compared the anti-O titers of serum and fractions with their ability to transfer pyrogenic tolerance. Data in Fig. 7 show no correlation between the anti-O titer and the capacity to transfer pyrogenic tolerance. DEAE fraction IV, with a low titer of anti-O antibody, gave complete pyrogenic tolerance to 100 MPD-3/kg of endotoxin while a larger quantity of high titered anti-O serum (HRS) gave only partial tolerance to the same dose of endotoxin; this partial tolerance can be accounted for by the presence of some antiendotoxin antibody. This confirms our previous observations that the mechanism of pyrogenic tolerance is due to an antibody not directed toward the O-specific determinant of the polysaccharide.

DISCUSSION

Beeson (9) established the importance of the reticuloendothelial system (RES) in the mechanism of pyrogenic tolerance; this, however, does not preclude the role of classical immunoglobulins as an important accessory to the normal functioning RES. As in many other classical immune mechanisms, antibody without a functioning RES would not give immunity. The apparent absence of specificity and the failure to transfer tolerance passively as shown by Beeson (10) resulted in the belief that pyrogenic tolerance is a non-specific refractory state comparable to a pharmacologic tolerance.

More recently investigators have attempted to implicate classical antibodies not only in pyrogenic tolerance but also in the mechanism of toxicity; these investigations have recently been reviewed (2, 11–13). There is evidence (14), however, that endotoxins have a true primary toxicity not dependent on an antigen-antibody reaction as suggested by Stetson (13). In addition to a primary toxicity, there may be a secondary toxicity associated with acquired hypersensitivity; these appear to be interdependent activities (2). In the previous paper (1) in this series, some degree of specificity between two endotoxins was demonstrated by means of cross-tolerance tests. In addition, an excellent anamnestic response was observed comparable to classical immunologic mechanisms. It is quite possible, therefore, that the apparent non-specficity observed by Beeson (10) is due to a common determinant in many endotoxins prepared from diverse sources. In the present paper, an immunologic mechanism of tolerance was confirmed by successful transfer of pyrogenic and lethal tolerance by 19S γ_1-immunoglobulin fraction isolated from tolerant rabbit serum. These results might indicate that Beeson's (10) failure to transfer tolerance may be related to the low quantity of antibody in the tolerant serum used for passive transfer. The belief had also predominated that the only antibody directed toward the endotoxin is the one specific for the O determinant of the polysaccharide. Morgan (15) presented convincing evidence that this antibody plays no major role in the mechanism of pyrogenic tolerance; we have confirmed this observation in the present investigation. It follows, therefore,

that the O determinant is not the toxophore group. It appears that the anti-endotoxin described in this report is a γ_1-immunoglobulin of the 19S class specific for a toxophore group common to many endotoxins and assists the RES in the uptake and destruction of the toxin.

Because the term tolerance was adopted (16) to describe the absence of the normal immunologic response to an antigen, a concept completely unrelated to pyrogenic tolerance, a certain amount of confusion exists in the use of this term. In view of the evidence that acquired resistance to the various toxic reactions of endotoxins, including pyrogenicity, results from a classical immunological mechanism, it seems appropriate to use the term *immunity* in this context rather than *tolerance.*

SUMMARY

Serum from rabbits rendered tolerant or immune to 100 MPD-3/kg of endotoxin when passively transferred to normal rabbits gave partial tolerance to the standard dose of endotoxin. The same serum was fractionated by DEAE chromatography into 4 major fractions. Immunoelectrophoretic analysis indicated that the 7S γ_2- and the 19S γ_1-immunoglobulins were separated into two distinct fractions. Of the four fractions tested, only fraction IV containing 19S γ_1-immunoglobulin conferred complete pyrogenic tolerance to 100 MPD-3/kg of endotoxin.

Additional fractionation of DEAE fraction IV by exclusion chromatography on sephadex G-200 gave 3 fractions. Of these only the first, containing 19S γ_1-immunoglobulin, conferred complete pyrogenic and lethal tolerance to normal rabbits. There was no correlation between the quantity of O-specific antibodies and the ability to transfer tolerance.

It is concluded that endotoxin tolerance involves a classical immune mechanism which includes both 19S γ_1-immunoglobulin specific for toxophore groups common to many endotoxins and a normally functioning RES.

To avoid confusion with immunologic tolerance, it is suggested that the term endotoxin immunity be substituted for endotoxin tolerance.

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