Murine Typhus Toxin: Studies on Identification of the Neutralizing Factor Present in Normal Human Serum

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Studies were made on the isolation and identification of the Rickettsia typhi toxin-neutralizing factor (TNF) previously demonstrated in normal human serum. By means of various methods of separating serum proteins, such as filtration on Sephadex G-200, dextran precipitation, hydroxyapatite chromatography, and ultracentrifugation, TNF was found to be closely associated with purified serum β-lipoprotein, although no serological relationship with this protein was demonstrated. Lipase as well as trypsin digestion of purified preparations of β-lipoprotein destroyed the TN activity. No evidence was obtained for an association of TNF with the immunoglobulins or with any serum protein other than β-lipoprotein. Further studies revealed that (i) serum specimens with TN titers of 1:1024 and others with titers of 1:8 or less contained the same concentration of β-lipoprotein; (ii) purified preparations of β-lipoprotein isolated from TNF positive and negative sera, and which had the same protein concentration, differed as much as 250-fold in TN titer; and (iii) the TN activity of a serum could be removed by absorption with antiserum to β-lipoprotein from a positive donor, but not with antisemum to β-lipoprotein from a negative donor.

A toxic reaction causing death within a few hours occurs in mice injected with yolk sacs of embryonated hens' eggs heavily infected with Rickettsia typhi or Rickettsia prowazekii (3, 11). Although the toxicity is intimately associated with the living organisms, for purposes of discussion it has been referred to as the rickettsial "toxin." This lethal effect can be neutralized by the serum of man and lower animals convalescent from infection with these agents (11, 12). Recently it was reported (1) that the toxin of the rickettsia of murine typhus, R. typhi, was neutralized by the serum of some normal human beings and monkeys. Thus, by means of toxin-neutralization (TN) tests in mice, 50% of 207 normal persons were found to have serum TN titers of 1:32 or greater against R. typhi toxin. That this TN factor (TNF) was nonspecific, and not formed from exposure to R. typhi or R. prowazekii, was suggested by the prevalence of TNF in the serum of persons of all ages from widely separated localities and, particularly, by its presence in the serum of persons under 20 years of age who resided in a typhus-free area and had not received typhus vaccine. The nonspecific nature of the murine TNF was also indicated by its failure to react in complement-fixation or agglutination tests with antigens of R. typhi (1).

The present paper reports the results of some attempts to identify and isolate this TNF from normal human serum.

MATERIALS AND METHODS

Toxin-neutralization tests. The Wilmington strain of R. typhi was employed in neutralization tests conducted by a method previously described (2, 12). In brief, serial twofold dilutions of serum or other material to be tested were made with Snyder I solution (14), a phosphate-buffered sucrose-glutamate solution, pH 7.2, and mixed with an equal volume of toxic suspension diluted so that each 0.5 ml of the resulting mixture contained two LD₅₀ toxic doses. A 0.5-ml amount of each mixture was injected intravenously into each of four mice. The neutralization titer of a serum was the highest dilution that protected two or more mice. Neutralization titers are expressed as the final dilution of a serum or other material after it was mixed with a toxic suspension.

Gel filtration. A 5-ml amount of serum was applied to a Sephadex G-200 column (3 by 100 cm) which previously had been equilibrated with M/15 phosphate buffer, pH 7.35, in 0.85% saline (PBS). It was eluted with PBS at a constant flow rate of 0.35 ml/min, and the optical density at 280 nm was monitored.
Samples of 6-ml volume were collected by using a drop counter and automatic fraction collector for dilution of β-lipoprotein. Two methods were used for isolation of β-lipoprotein. First, β-lipoprotein was obtained from serum by precipitation with dextran sulfate (Sodium Dextran Sulfate 500, molecular weight of 5 × 10^6, Pharmacia, Uppsala, Sweden) by the procedure outlined by Cornwell and Kruger (7). To 10 ml of serum was added 1.0 ml of 1 M calcium chloride solution and 0.2 ml of a 10% solution of dextran sulfate. After 24 hr at 4°C, the mixture was centrifuged at 5,000 × g for 10 min, and the supernatant fluid was removed. To remove certain serum proteins adhering to the sediment, the latter was dissolved in 1.0 ml of 5% sodium chloride solution and precipitated by dilution with 10 ml of 0.1 M calcium chloride. This precipitate was collected by centrifugation, and the supernatant fluid (wash 1) was saved for testing. After the precipitate was purified once more by the same procedure (wash 2), it was dissolved in 0.5 ml of 0.1 M sodium oxalate, and then 0.2 M of sodium chloride and 0.25 ml of a 2% solution of protamine sulfate was added. Finally, this solution of β-lipoprotein was centrifuged to remove precipitated calcium oxalate and the dextran sulfate-protamine complex.

In the second method, β-lipoprotein was isolated by chromatography on hydroxyapatite columns. The hydroxyapatite was prepared as described by Tiselius et al. (17), and chromatography was performed as described by Hjertén (13) and Berg (4). Chromatography was done at room temperature in a column (0.9 by 20 cm) equilibrated with 0.01 M phosphate buffer, pH 6.8. Elution of various substances was accomplished with 0.1 M, 0.25 M, and 0.65 M phosphate buffers, pH 6.8. Flow rate was maintained at 1.6 ml/min, 5-ml samples of effluent were collected, and absorbance at 280 nm was monitored.

Antiserum. Antiserum to human serum β-lipoprotein isolated by the methods just described was produced in rabbits. Portions of β-lipoprotein preparations, diluted to have an optical density (280 nm) of about 0.6, were mixed with an equal volume of complete Freund's adjuvant. Rabbits were immunized by injecting 0.5 ml of this emulsion into the footpad of each hind leg. Rabbits were bled 4 weeks later, and the sera were removed, pooled, and stored at −10°C. In addition, monospecific antisera used for gel diffusion analyses were purchased from Behring Diagnostics, Inc., 400 Crossways Park Drive, Woodbury, N.Y. 11797. These included antisera to β-lipoprotein, α1-lipoprotein, α2-macroglobulin, haptoglobin, immunoglobulins M, G, and A (IgM, IgG, and IgA) and to whole serum; all were to antigens of human origin.

Assay of rabbit antisera to β-lipoprotein for antibody against TNF. Antibody to TNF was detected by determining the capacity of an antisera to absorb the TNF from increasing concentrations of a selected serum which was known to have a high TN titer (test serum). Absorption of TNF resulted in loss of neutralizing or protective activity of the serum. Serial twofold dilutions of the test serum were made up to, and including, its neutralization end point. To each of these dilutions was added an equal volume of undiluted antiserum to be assayed. This method assured antibody excess in at least some of the serum dilutions. The mixtures were incubated at 37°C for 2 hr and stored overnight at 5°C, and the precipitates were then removed by centrifugation at 2,000 × g for 30 min. The supernatant fluid from each dilution was mixed with toxin and tested in mice as described for TN tests. As a control, this same procedure was followed except that Snyder I diluent was used instead of antiserum.

Immunodiffusion analysis. Gel double-diffusion tests were conducted with 0.75% agarose (Sea Kem brand, Marine Colloids, Inc., Springfield, N.J.) dissolved in 0.9% sodium chloride. Microscope slides (75 by 25 mm) were overlaid with 2.5 ml of the agarose solution. Wells, 2 mm in diameter with edges 4 mm apart, were filled with antigen and antibody and incubated in a humid chamber at room temperature for 24 to 48 hr.

Enzyme treatments. Preparations of β-lipoprotein isolated on hydroxyapatite columns and whole serum were subjected to enzymatic digestion with lipase and trypsin. Lipase (lot 448, Nutritional Biochemicals Corp., Cleveland, Ohio) solutions in 0.2 M phosphate buffer, pH 6.8, were added to the test sample to give a lipase concentration of 0.3 mg/ml of the mixture. Controls included in each test consisted of (i) test sample plus buffer, (ii) test sample plus boiled lipase, and (iii) lipase plus buffer. In addition, activity of the lipase was measured by digestion of an olive oil emulsion. The mixtures were incubated at 37°C for 4 hr, stored overnight at 5°C, and then tested for TN activity.

Trypsin (lot 4268, Nutritional Biochemicals Corp.) dissolved in M/15 PBS, pH 8.0, was added to test samples to give a trypsin concentration of 0.3 mg/ml of the mixture. Controls and test procedures were similar to those for lipase digestion except that activity of the trypsin was measured by digestion of the gelatin of a developed photographic film strip.

Ultracentrifugation. Ultracentrifugation was done in a Beckman L2-65B ultracentrifuge with an SW 50.1 rotor with swinging buckets of 5 ml capacity each. The solvent density of the serum was raised to 1.063 by mixing 2.5 ml of serum with 2.5 ml of a solution containing 181.7 grams of sodium chloride per liter (8). These samples were centrifuged at 80,000 × g for 48 hr at 12 to 18°C. The layers were removed from the bottom with a hypodermic syringe and needle. Various layers were analyzed for TN titer and for certain serum proteins by gel diffusion analysis.

Microagglutination (MA) and complement-fixation (CF) tests. MA and micro CF tests were conducted as described by Fiset et al. (9, 10). The particulate antigens for both tests consisted of a highly purified suspension of rickettsiae prepared from chicken embryo yolk sacs infected with the Wilmington strain of R. typhi (10).

RESULTS

Sephadex G-200 gel filtration. Normal human serum with a high TN titer was separated into three major peaks (Fig. 1) by filtration on a Sephadex G-200 column. TN activity was found only in fractions of the first peak and, although all
such fractions were active, the highest TN titers were usually found in fractions on the ascending side of this peak. The second and third peaks represented, respectively, the bulk of the IgG and albumin eluted from the column. Because serum proteins of a molecular weight greater than 200,000 are the major ones found in the first peak, these results suggested that the TN factor was either a large-molecular-weight serum protein or a substance closely associated with one. Hence, further studies to identify the TNF of normal human serum were concerned chiefly with attempts to find an association of this factor with certain of the known large-molecular-weight serum proteins, such as IgM, $\alpha_2$-macroglobulin, $\beta$-lipoprotein, or other large lipoproteins. Because preliminary tests involving preparative zone electrophoresis and diethylaminoethyl-cellulose chromatography seemed to show a lack of association between TNF and the first two of these proteins, further studies were centered on the $\beta$-lipoproteins.

**Precipitation of TNF by dextran sulfate.** The use of dextran sulfate for isolation of $\beta$-lipoprotein directly from human serum has been described (7, 15, 16). Sera with high TN titers were treated with dextran sulfate, and the various fractions were tested for TN activity and, by gel diffusion tests, for presence of IgM, IgG, $\alpha_2$-macroglobulin, $\alpha_1$-lipoprotein and $\beta$-lipoprotein. The results are summarized in Table 1. At least 90% of the TN activity of the original serum was precipitated with the $\beta$-lipoprotein fraction by dextran sulfate.

Serum IgM, IgG, $\alpha_2$-macroglobulin, and $\alpha_1$-lipoprotein, but not $\beta$-lipoprotein, were demonstrated in the supernatant fluid after removal of the precipitated complex. Traces of some of these proteins often adhered to the precipitate but, except for $\alpha_1$-lipoprotein, were usually removed from it by washing. There appeared to be no association of TNF with IgM or $\alpha_2$-macroglobulin because most of these two proteins remained in the supernatant fluid which had little neutralizing activity. Furthermore, they could not be demonstrated by gel diffusion analysis in the final prepa-

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**Table 1.** Toxin-neutralization (TN) tests and gel diffusion analysis on various fractions after treatment of serum with dextran sulfate

<table>
<thead>
<tr>
<th>Expt</th>
<th>Fractiona</th>
<th>Reciprocal of TN titer</th>
<th>Reaction with designated antiserum in gel diffusion analysisa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>$\beta$-Lipoprotein</td>
</tr>
<tr>
<td>51-2</td>
<td>Untreated serum</td>
<td>256</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>Supernatant</td>
<td>&lt;8</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Wash 1</td>
<td>&lt;8</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>$\beta$-Lipoprotein</td>
<td>256</td>
<td>+</td>
</tr>
<tr>
<td>51-4</td>
<td>Untreated serum</td>
<td>256</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>Supernatant</td>
<td>16</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Wash 1</td>
<td>&lt;8</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>$\beta$-Lipoprotein</td>
<td>512</td>
<td>+</td>
</tr>
<tr>
<td>53-1</td>
<td>Untreated serum</td>
<td>512</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>Supernatant</td>
<td>16</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Wash 1</td>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Wash 2</td>
<td>&lt;8</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>$\beta$-Lipoprotein</td>
<td>1024</td>
<td>+</td>
</tr>
</tbody>
</table>

a Presence (+) or absence (−) of precipitin reaction between fraction and specific antiserum. IgM, Immunoglobulin M; IgG, immunoglobulin G; NT, not tested.

b Supernatant, supernatant fraction after removal of $\beta$-lipoprotein-dextran sulfate complex; Wash, supernatant fraction remaining after solution and reprecipitation of this complex; $\beta$-lipoprotein, final preparation after removal of calcium ions and dextran sulfate.
Isolation of TNF on hydroxyapatite columns. Results of experiments with dextran sulfate suggested that the TNF of normal human serum was associated with the \( \beta \)-lipoproteins. To confirm this observation, \( \beta \)-lipoprotein was isolated from serum having a high TN titer by chromatography on hydroxyapatite columns. The results of one such experiment are shown in Fig. 2. Fractions representing each of the four elution peaks of the chromatogram were tested for TN activity and, by gel diffusion, for the presence of \( \beta \)-lipoprotein, IgM, IgG, \( \alpha_2 \)-macroglobulin, \( \alpha_1 \)-lipoprotein, and haptoglobin. Peaks I, II, and III together contained all other serum proteins, but no demonstrable \( \beta \)-lipoprotein. TN activity and \( \beta \)-lipoprotein were found only in peak IV, and this peak did not contain demonstrable amounts of the other proteins tested for. The \( \beta \)-lipoprotein fraction (fraction 20) had a TN titer of 1:256, whereas the other fractions had little, if any, TN activity.

Variation in the purity of the \( \beta \)-lipoprotein fraction appeared to be related to the use of different preparations of hydroxyapatite. Thus, in repeated runs this fraction, eluted with 0.65 M buffer, sometimes contained traces of other serum proteins, principally IgG and \( \alpha_1 \)-lipoprotein. In such cases, the \( \beta \)-lipoprotein could be purified by recycling this fraction on the hydroxyapatite column (Fig. 3).

Quantitative tests of purified preparations of \( \beta \)-lipoprotein. TN titers ranging from less than 1:8 up to 1:2048 were previously observed (1). These findings demonstrated that persons differ in the concentration of TNF in their sera. The following quantitative tests were performed to determine whether the concentration of TNF and that of \( \beta \)-lipoprotein were related. First, a comparison was made of the TN activity of \( \beta \)-lipoprotein from a positive donor (one with high serum TN titer) and a negative donor (one whose serum has little, if any, TN activity) after the preparations had been adjusted to the same optical density at 280 nm. The TN titer of the preparation from the positive donor was 1:256 and that of the preparation from the negative donor was less than 1:2.

![Fig. 2. Chromatography of human serum on hydroxyapatite column for isolation of \( \beta \)-lipoprotein. Distribution of selected serum proteins and of R. typhi toxin-neutralizing activity. Used for elution were 0.01, 0.10, 0.25, and 0.65 M phosphate buffer, pH 6.8. Lower part of figure indicates presence (+) or absence (−) of designated serum proteins in peak fractions as determined by gel diffusion analysis. TN titer is expressed as reciprocal.](image)

![Fig. 3. Gel diffusion test showing the purity of \( \beta \)-lipoprotein preparations obtained by hydroxyapatite chromatography. \( \beta \)-Lipoprotein antigen (well A) yielded only a single precipitin line with rabbit antiserum to human serum (well I), and this line reacted in a pattern of identity with that produced with specific antiserum to \( \beta \)-lipoprotein (well 2). Antiserum to human serum (well 3) yielded several precipitin lines with a 1:10 dilution of whole human serum (well B).](image)
Secondly, the concentration of \( \beta \)-lipoprotein in specimens of whole serum from five positive (TN titers of 1:1024) and five negative (TN titers of 1:8 or less) donors was compared by radial immunodiffusion (Behring Diagnostics, Inc.). As determined by this method, the concentration of \( \beta \)-lipoprotein in the sera of the two groups was essentially the same. In addition, no antigenic differences could be demonstrated between the \( \beta \)-lipoproteins of positive and negative donors by gel diffusion tests (Fig. 4).

**Absorption of TNF with antiserum to \( \beta \)-lipoprotein.** Rabbits were immunized with \( \beta \)-lipoprotein obtained by hydroxyapatite chromatography or by precipitation with dextran sulfate. Each of the resulting antisera was tested for its capacity to absorb TNF from selected test sera. Antisera were prepared against \( \beta \)-lipoprotein from positive and negative donors. Results of representative experiments are summarized in Table 2. Antisera prepared against \( \beta \)-lipoprotein isolated from positive donors reduced the TN titer of the test serum at least 32-fold, from 1:256 before to 1:8 after absorption. In contrast, no more than a twofold reduction in titer of the test serum was effected by absorption with antisera against \( \beta \)-lipoprotein from a negative donor. This difference was not due to concentration of anti-\( \beta \)-lipoprotein in the antisera employed because, by quantitative precipitin tests, it was found that the antiserum to \( \beta \)-lipoprotein from a negative donor contained more antibody than did the antiserum to \( \beta \)-lipoprotein from a positive donor.

![Image](http://iai.asm.org/)

**Fig. 4.** Gel diffusion patterns showing the antigenic relationship of \( \beta \)-lipoproteins from toxin-neutralizing factor positive (well P) and negative (well N) donors. The corresponding antisera to these \( \beta \)-lipoproteins are in wells p and n.

<table>
<thead>
<tr>
<th>Determination on 02</th>
<th>Antiserum to ( \beta )-lipoprotein (reciprocal of TN titer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test serum not absorbed</td>
<td>HAa 256  DEXb 256  HAa 128  DEXb 256</td>
</tr>
<tr>
<td>Test serum absorbed</td>
<td>8  8  128  128</td>
</tr>
<tr>
<td>Antiserum used for absorption</td>
<td>&lt;2  &lt;2  &lt;2  &lt;2</td>
</tr>
</tbody>
</table>

* HA, \( \beta \)-Lipoprotein for rabbit immunization prepared by hydroxyapatite chromatography.  
* DEX, \( \beta \)-Lipoprotein for rabbit immunization prepared by precipitation with dextran sulfate.

The method of preparing \( \beta \)-lipoprotein for rabbit immunization had no effect on the quality of the absorbing antisera. Thus, \( \beta \)-lipoprotein isolated by hydroxyapatite chromatography or by precipitation with dextran sulfate produced antisera which behaved alike in absorption tests (Table 2).

Absorption of test sera with an antiserum to the \( \beta \)-lipoprotein of a negative donor removed most of the \( \beta \)-lipoprotein detectable by the gel diffusion test (Fig. 5). Nevertheless, the absorbed serum retained its original TN property.

**Effect of enzymes on the TNF.** \( \beta \)-Lipoprotein isolated from sera of TNF-positive donors by hydroxyapatite chromatography was digested with lipase and with trypsin. The effect of these enzymes on the neutralizing activity of the lipoprotein preparations is shown in Table 3. The TN titer of the \( \beta \)-lipoprotein sample was reduced from 1:128 before to 1:8 or less after digestion with lipase. Essentially the same result was obtained by digestion of the \( \beta \)-lipoprotein sample with trypsin.

Whole serum was similarly treated with lipase and with trypsin at the same time that tests on \( \beta \)-lipoprotein were conducted. In contrast to their effect on TNF of \( \beta \)-lipoprotein preparations, neither enzyme had any effect on the TNF present in a serum specimen which had a TN titer of 1:512.

Digested and undigested samples of \( \beta \)-lipoprotein were examined by gel diffusion with specific antiserum to \( \beta \)-lipoprotein. This antiserum did not produce a precipitin line with a
Table 3. Effect of lipase and trypsin on the toxin-neutralizing (TN) activity of β-lipoprotein isolated from serum containing the TN factor

<table>
<thead>
<tr>
<th>Determination on</th>
<th>TN titer (reciprocal)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lipase</td>
</tr>
<tr>
<td>β-Lipoprotein and buffer</td>
<td>128</td>
</tr>
<tr>
<td>β-Lipoprotein and enzyme</td>
<td>8</td>
</tr>
<tr>
<td>β-Lipoprotein and boiled enzyme</td>
<td>128</td>
</tr>
<tr>
<td>Enzyme and buffer</td>
<td>&lt;8</td>
</tr>
</tbody>
</table>

lipase digest of β-lipoprotein, but did produce distinct lines with the control mixtures, i.e., mixtures of β-lipoprotein with buffer or with boiled lipase (Fig. 6). However, although digestion with trypsin reduced the neutralizing activity of the β-lipoprotein sample, the digested sample still gave a precipitin line with antiserum to β-lipoprotein.

Ultracentrifugation. Serum from TNF-positive donors was ultracentrifuged at a serum density of 1.063, and fractions of 1.0 ml each were collected. The thin, yellow layer which floated on top of the sample contained most of the β-lipoprotein demonstrable by gel diffusion and consistently possessed a higher TN titer (1:1024) than any of the other fractions. Moreover, the sediment which contained most of the IgM and α2-macroglobulin of the original sample had a TN titer of 1:16 or less.

MA and CF tests. A β-lipoprotein preparation with a high TN titer was tested in MA and CF tests. No agglutination or CF reaction with R. typhi organisms was produced by this prepara-

![Image](http://iai.asm.org/Downloadedfrom/http://iai.asm.org/)

**Fig. 5.** Gel diffusion test of serum, with high titer of toxin-neutralizing factor (TNF), before (control) and after absorption with antiserum to β-lipoprotein isolated from serum of a TNF positive or negative donor. Outside wells were dilutions of the test serum to which an equal volume of either diluent (control) or undiluted antiserum was added. Center wells (A) contained antiserum to β-lipoprotein from a positive donor. Both absorbing antisera removed all demonstrable β-lipoprotein from all but the 1:2 dilution of the test serum.

**Fig. 6.** Gel diffusion tests of purified preparation of β-lipoprotein following digestion with lipase and with trypsin. β-Lipoprotein mixed with active enzyme (well 1), with boiled enzyme (well 2), and with buffer (well 3). Antiserum to β-lipoprotein (well B). Only lipase destroyed the capacity of the β-lipoprotein to form a precipitin line with its specific antiserum.
tion. Furthermore, it failed also to inhibit fixation of complement by R. typhi and its specific antiserum. The CF titers of the R. typhi antiserum in the presence, as well as in the absence, of the β-lipoprotein preparation were identical.

**DISCUSSION**

In a previous study (1), evidence was presented which indicated that the factor in normal human serum which neutralized the toxin of murine typhus rickettsiae was of non-specific origin, and not the result of exposure to R. typhi or R. prowazeki. Results of the present study confirm this observation. Indeed, the evidence obtained strongly suggests that the neutralizing factor is not an immunoglobulin. Instead, the TNF present in normal human sera was found to be closely associated with serum β-lipoprotein. When serum was treated by methods commonly employed for isolation of β-lipoprotein, such as filtration on Sephadex G-200, dextran sulfate precipitation, hydroxyapatite chromatography, and ultracentrifugation at 1.063 serum density, most or all of the neutralizing activity was found in fractions containing this protein.

The destruction of the neutralizing activity of β-lipoprotein preparations by the enzyme lipase provides further evidence of a relationship between this serum component and the TNF. Because trypsin also destroys this activity, it seems that both the lipid and protein moieties are essential for neutralization by the TN factor. When whole sera rather than β-lipoprotein preparations were treated with lipase or trypsin, no effect on the TN activity was demonstrable. However, it is possible that a different result might be obtained by use of higher concentrations of enzyme and longer periods of incubation than employed in our tests, or both.

Other results of this study strengthen the conclusion of an association of the TNF with β-lipoprotein. Thus, within the limits of the sensitivities of the tests employed, no evidence was obtained for an association of TNF with the immunoglobulins or with any serum protein other than β-lipoprotein. However, sera with TN titers ranging from less than 1:8 to 1:2048 showed no difference in their β-lipoprotein concentrations by radial immunodiffusion tests with commercial antisera. Furthermore, purified preparations of β-lipoprotein isolated from serum specimens with a high TN titer and others with a low TN titer and adjusted to the same optical density at 280 nm differed as much as 250-fold in TN titer. In addition, it was established that the single precipitin band given by purified β-lipoprotein preparations from positive and negative sera was not associated with TNF. The purification and enzyme treatment studies, however, strongly suggested that TNF had properties similar to β-lipoproteins. If TNF is indeed a lipoprotein, it is antigenically different from the β-lipoprotein demonstrable by gel diffusion with the antisera employed. The gel diffusion studies also suggested that TNF or its antibody, or both, were found in concentrations too low to be detected by immunodiffusion or that they formed a nonprecipitating complex.

Results of serum absorption experiments support the above conclusion. When a test serum with neutralizing activity was absorbed with antiserum against β-lipoprotein from a positive donor, the TN activity was removed. However, little, if any, effect on the TN activity of the test serum was produced when absorption was conducted with antiserum to β-lipoprotein from a negative donor. Despite differences in TN activity of the absorbed sera, all demonstrable β-lipoprotein was removed by the negative as well as the positive β-lipoprotein antisera. Nevertheless, both antisera were known to have similar precipitating strengths.

Although serological studies established that TNF was antigenically different from the β-lipoprotein measured by the antisera employed, they did not rule out the possibility that TNF may be a type of β-lipoprotein with its own antigenic specificity. Antigenic differences in human serum β-lipoprotein are well known (5, 6).

The mechanism by which TNF acts is clearly different from that involved in antibody neutralization of toxins or infectious agents. TNF did not agglutinate R. typhi and did not fix complement or inhibit complement fixation. Furthermore, TNF did not combine with R. typhi cells (E. J. Bell, unpublished data). TNF must, then, protect mice from rickettsial toxin by a mechanism which does not involve an antigen-antibody reaction. Further investigation on the nature of TNF may provide information on the problem of the primary site of action of the rickettsial toxin and of the mechanism by which it induces the vascular changes which result in the death of this animal.

**ACKNOWLEDGMENT**

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**LITERATURE CITED**

