HOST-PARASITE FACTORS IN GROUP A STREPTOCOCCAL INFECTIONS*

A Comparative Study of Streptococcal Pyrogenic Toxins and Gram-Negative Bacterial Endotoxin

By NATALIE CREMER,‡ Ph.D., and DENNIS W. WATSON, Ph.D.

(From the Department of Bacteriology, University of Minnesota, Minneapolis)

(Received for publication, July 27, 1960)

There are few reports in the literature (1–4) on the pyrogenicity of streptococci or streptococcal filtrates. The most recent detailed investigations on streptococcal pyrogenic activity were reported by Stetson (5) and Watson (6). Stetson reported on the endotoxic activity of Group A streptococci grown *in vitro*. On the basis of studies with lysates of streptococci disrupted in a Mickle disintegrator, he suggested that streptococci possess an endotoxin similar to that of Gram-negative bacteria. Watson worked with extracellular products of Group A streptococci grown *in vivo*. Sterile extracts, prepared from rabbit lesions produced by intradermal injection of various types of Group A streptococci, contained pyrogenic toxins which were immunologically distinct. These toxins had other manifestations, causing lethality, cardiotoxic and other tissue damage, and enhancement of toxicity. They were identified with the scarlet fever group of toxins.

This study was done to investigate the relationship between streptococcal pyrogenic intracellular and extracellular toxins and between streptococcal pyrogenic toxins and the endotoxin of Gram-negative bacteria. Relationships among these toxins were studied by (*a*) the ability of one toxin upon repeated intravenous injection into rabbits to establish pyrogenic tolerance to a heterologous toxin; (*b*) effectiveness for streptococcal pyrogens of extraction procedures used in isolation of Gram-negative bacterial endotoxins; (*c*) additional comparative studies based on several other toxic manifestations. Using these criteria, the data suggest that Group A streptococci produce two pyrogenic toxins, one primarily intracellular, the other mainly extracellular. The streptococcal pyrogens appear to be chemically different from Gram-negative bacterial endotoxin. However, similar non-specific host factors may be active in the establishment of tolerance to both Gram-negative bacterial endotoxin and

* This investigation was supported by a research grant HTS-5360 from the National Heart Institute, National Institutes of Health, Bethesda.

‡ Present address: Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena.

the streptococcal intracellular toxin. The streptococcal exotoxin appears to be detoxified in a dissimilar manner.

Materials and Methods

Rabbits.—Young American Dutch rabbits weighing on the average 1 kg. were used. They were supplied from a single source and fed a diet of nutrena rabbit pellets. Ten rabbits were used to check each preparation in the tolerance studies; 7 to 8 rabbits were used for each experiment in the studies on extraction procedures.

Bacteria.—Group A streptococci, Types 18, 4, 12, and strain NY-5 were studied. Their history and source are as previously described (6). *Salmonella typhosa*, Ty 2, came from Dr. Maurice Landy, National Institutes of Health, Bethesda, Maryland.

Water.—All water used in this study was freshly prepared, glass-distilled, and shown to be pyrogen-free by testing in rabbits.

Cultivation of Bacteria and Preparation of Extracts.—The streptococci were grown in a completely dialyzable medium (6) with the following modification. After digestion of the beef heart with trypsin, the digest was dialyzed against pyrogen-free, distilled water in a ratio of 1 liter of water for each pound of ground meat. The dialysate was then diluted with an equal amount of distilled water. Five to 6 hour cultures were used in order to minimize lysis of bacteria. They were grown in 12 liter carboys containing approximately 7 liters of medium Every culture was checked for purity at each step in the process by subculture to blood agar plates. The bacteria were harvested by centrifugation in an electrically driven Sharples centrifuge and washed either by resuspension in 6 liters of sterile water and recentrifugation in the Sharples or by suspension three times in sterile water and centrifugation in a refrigerated International centrifuge. Thirty ml. aliquots of a bacterial suspension were exposed to sonic oscillation in a 9 kc. Raytheon for 2½ to 3 hours at a plate voltage of 130. The bacterial suspension was prepared by thoroughly mixing 30 gm., wet weight, of bacteria with 100 ml. of water. The temperature in the cup was maintained at 4°–6°C. by circulation of a water-alcohol mixture kept at approximately −4°C. The extract was freed of cellular debris by centrifugation in a refrigerated Serval at 15,500 *G* for 1 hour. The supernate was dialyzed 24 hours against a balanced salt solution at 2°C., filtered through an 02 Selas, placed in ampules, and kept frozen at −70°C. until immediately prior to use. The *Salmonella* were grown on veal infusion agar. Sterile extracts were prepared in the same manner as for the streptococci.

Production of Extracellular Toxin. The extracellular products of one streptococcal type, 18, were examined. The extracellular products were collected, concentrated, dialyzed, and lyophilized as previously described for NY-5 exotoxin (6). The lyophilized material was dissolved in a balanced salt solution and kept frozen at −70°C.

Determination of Injection Size.—For tolerance studies dosage of both sonic extracts and of T-18 extracellular products was based on total N as determined by the Kjeldahl technique (7).

Recording of Rectal Temperatures.—Two types of thermometers were employed, a TRI-R electronic thermometer and a TE3 thermometer. With the TRI-R thermometer, food and water were not withheld during the experiments; with the TE3 thermometer, the rabbits were kept in a rack restrained only by a loose fitting neck collar. The day prior to the experiment they were placed in the rack for 3 to 5 hours in order to accustom them to the restraint. During the experiment, food and water were withheld. Rabbits with zero hour temperatures greater than 103.5°F. were not used. The average hourly rise above zero hour temperatures of all the rabbits, in each experiment, was determined over a 4 to 5 hour period. All temperature changes were recorded in degrees F.

Controls.—Temperature variation in a total of 120 normal rabbits was followed over periods

of 3 to 5 hours. The average hourly temperature change in uninjected rabbits or in rabbits injected intravenously with diluent, balanced salt solution, or saline, varied from −0.3°–0.2°F. On the basis of these data, a rise in temperature of 1°F. or greater was considered of significance.

Serologic Studies.—Antigenic relationships among the various streptococcal preparations were studied by the Ouchterlony technique.

Balanced Salt Solution.—This solution was prepared by dissolving NaCl 8.0 gm., KCl 0.4 gm., $MgSO_4 \cdot 7H_2O$ 0.2 gm., KH_2PO_4 0.06 gm., Na_2HPO_4 0.05 gm. in 1000 ml. of water.

Extraction Procedures.—Extraction with hot phenol by the method of Westphal *et al.* (8) and with 0.5 N TCA, as described by Webster *et al.* (9), was done. Bacteria were washed three times with distilled water prior to use. The extracted material was dialyzed 36 hours against a total of 18 liters of distilled water at 2°C. and then lyophilized. Pyrogenic dose was based on dry weight.

RESULTS

Relative Potency of Extracts in Producing a Sustained Pyrogenic Response

The size of the intravenous injection necessary to insure a sustained febrile response varied among the preparations. For NY-5 and T-18, in the range of 290 μg. N was satisfactory; for T-12, 330 μg. N; for T-18 extracellular products, 130 μg. N, which represented 2 mg. of lyophilized material. For the group A sonic extracts, 290 μg. N represented 0.1 ml. or less of the undiluted extracts. Four μg. N of the *Salmonella* sonic extracts sufficed. This amount represented 0.1 ml. of a 1–100 dilution of the extract. T-4 extracts were only slightly pyrogenic. For T-4, 3.7 mg. N, or approximately 1.0 ml. of undiluted extracts, was required. Because of the high concentration required for T-4, no further pyrogenic studies were done with this organism. Unless otherwise stated, the micrograms N listed above for each preparation was the injection dose used in all the following experiments.

Establishment of Tolerance

Intravenous injection into rabbits of either streptococcal intracellular products or T-18 extracellular products in sufficient concentration caused a biphasic febrile response resembling that caused by Gram-negative bacterial toxins. After the first injection of streptococcal products the rabbits did not respond with the same high fever when tested the next day. It appeared the higher the temperature on day 1, the more significant the fever still remained on day 2; and the lower the temperature rise on day 1, the less significant the fever on day 2. This reduction in fever on day 2 occurred if either homologous or heterologous streptococcal sonic extract was given. After repeated injections with the same material the rabbits eventually no longer responded with a significant fever at 3, 4, and 5 hours. The 1 hour temperature, however, usually persisted. Tolerance was considered established when the average temperature rise at 3, 4, and 5 hours was less than 1°F. Establishment of tolerance was most successful when approximately 5 to 6 injections were given over a period of

about 7 to 9 days. When animals were rested 1 to 2 weeks after tolerance was established, they responded with significantly elevated temperatures at 3 hours. However, tolerance was quickly reestablished, usually by the next day. If they were rested 2 to 4 days and then rechallenged, the tolerance appeared

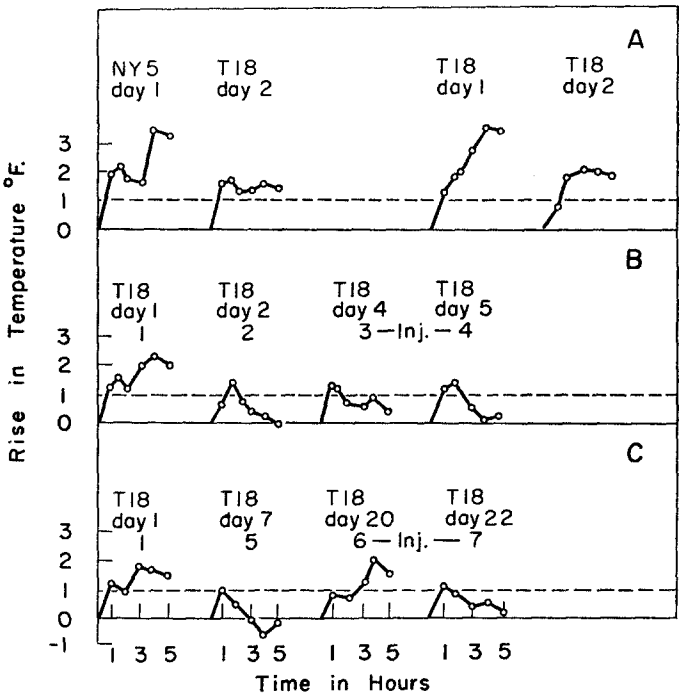

FIG. 1. A. The effect of a second injection on the febrile response of either heterologous or homologous streptococcal sonic extracts. B. The establishment of tolerance by repeated injections of homologous sonic extract over a period of 5 days. C. Loss of tolerance to homologous extract after a rest of 2 weeks and the reestablishment of tolerance upon the next injection. Each fever curve in this experiment and all subsequent experiments represents the average febrile response of 10 rabbits.

to be borderline. These data are given in Fig. 1. In order to establish tolerance to *S. typhosa* extracts, 7 to 9 injections were necessary. These were spaced over a period of about 14 days.

Cross-Tolerance Studies with Streptococcal Products

The day after establishment of tolerance to a homologous preparation, the rabbits were given an intravenous injection of a heterologous extract. The same preparation used to establish tolerance was used in the same concentration in the reciprocal cross. Rabbits tolerant to any one of the three Group A streptococcal sonic extracts studied were tolerant of the others also (Fig. 2).

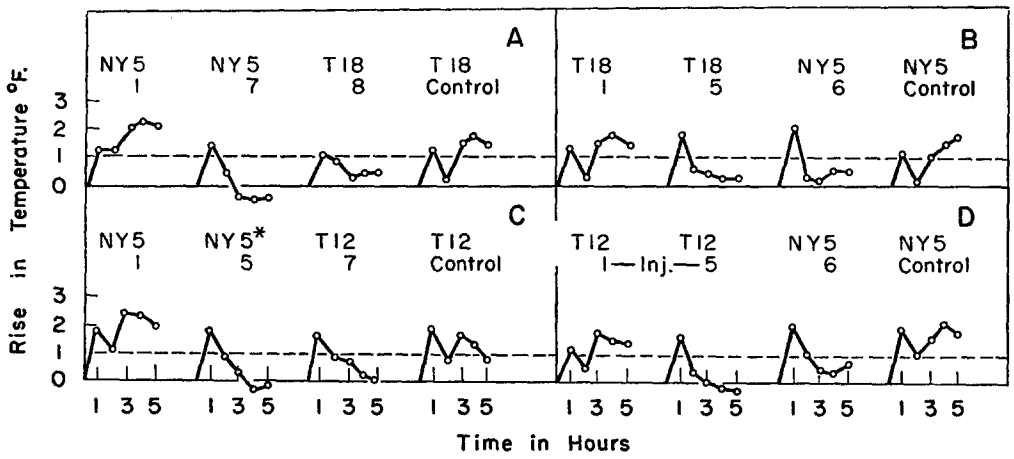

FIG. 2. A and B. Reciprocal cross between Group A streptococci, strain NY-5 and T-18, showing mutual tolerance between them. In this experiment and all the following experiments, control indicates the average hourly temperature response of 10 normal rabbits injected for the first time with the extract specified The controls were done either on the day of the cross or a day or two prior to the cross. C and D: Reciprocal cross between Group A streptococci, strain NY-5 and T-12, showing mutual tolerance between them.

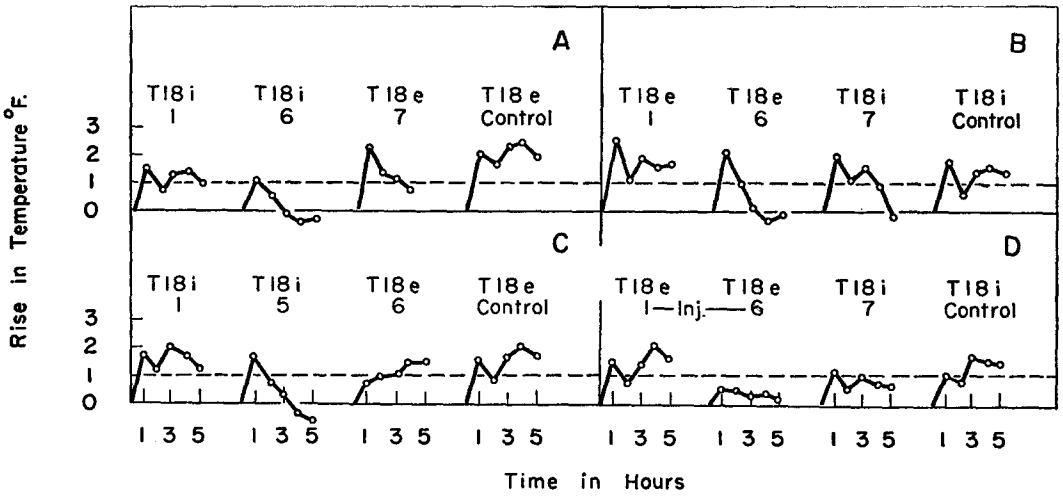

FIG. 3. A and B. Reciprocal cross between T-18 intracellular material, sonic extract (T-18i), and T-18 extracellular products (T-18e). Injection dose for T-18i was 290 μg. N and for T-18e, 130 μg. N. C and D. Reciprocal cross between T-18i and T-18e with the concentration of T-18i the same as in the previous experiment and T-18e at double the former concentration; *e.g.*, 260 μg. N.

* There was an intervening injection of T-18 extracellular products, 260 μg. N.

An absolute mutual tolerance between T-18 sonic extracts (T-18i) and T-18 extracellular products (T-18e) did not occur (Fig. 3 A and B). When a minimal concentration of T-18e was used (130 μg. N) rabbits tolerant of T-18i showed borderline tolerance to T-18e. T-18i in the reciprocal cross caused a 3 hour rise in temperature which was greater than 1°F. By the 4th and 5th hours, however, the temperature rise dropped below 1°F. When the concentration of T-18e was doubled (Fig. 3 C), rabbits tolerant to T-18i were not tolerant to T-18e. They responded with a significant fever at the 3rd, 4th, and 5th hours. In the reciprocal cross at this concentration (Fig. 3 D), rabbits tolerant to T-18e responded with a borderline response to an injection of T-18i.

This inability of T-18 sonic extracts to induce complete tolerance to its extracellular products and the reverse situation, the inability of extracellular products to induce complete tolerance to intracellular material, suggest the presence of two distinct toxins, one primarily intracellular, the other primarily extracellular. It is not illogical to assume the intracellular products, that is the sonic extracts, contain traces of the extracellular pyrogen, and the extracellular products contain traces of intracellular pyrogen. If this be true, a partial but not a complete tolerance to the extracellular pyrogen would occur upon repeated injections of the intracellular pyrogen. The reverse situation would also occur; a partial but not complete tolerance to the intracellular pyrogen would occur upon repeated injections of the extracellular pyrogen.

Serologic studies using the Ouchterlony technique showed that intracellular material did contain traces of extracellular material. Immune serum produced in response to T-18 sonic extracts formed two lines of precipitate with T-18 extracellular material. In order to show the second weaker line, it was necessary to concentrate the globulins from the antiserum. Thus, the intracellular products either shared antigens with the extracellular material or sufficient extracellular antigens adhered to the surface of the bacteria and thus were extracted along with the intracellular products. T-18 produces a very large hyaluronic acid capsule which would serve to hold extracellular products to the cell surface.

Cross-Tolerance Studies between Streptococcal Products and Gram-Negative Bacterial Endotoxin

Development of tolerance between T-18 extracellular products and *S. typhosa* sonic extract was never demonstrated (Fig. 4). This lack of tolerance occurred whether minimal concentrations causing a reproducible febrile response were used (Fig. 4 A and B), or whether increasing concentrations of T-18 extracellular products were employed (Fig. 4 C, D, and E).

Anomalies occurred in tolerance studies between the sonic extracts of the streptococci and the *Salmonella*. All degrees of tolerance were observed from complete lack of tolerance to various combinations of non-reciprocal tolerance. Enough of these data are presented in Fig. 5 to show these discrepancies. In

Fig. 5 (A and B) there is a mutual lack of tolerance between T-18 sonic extract (T-18i) and *S. typhosa* sonic extract (S.T.). The experiment was repeated using new preparations of each extract (Fig. 5 C and D). Now non-reciprocal tolerance occurred. Rabbits tolerant to *S. typhosa* were *not* tolerant to T-18i, but rabbits tolerant to T-18i *were* tolerant to *S. typhosa*. This latter experiment was

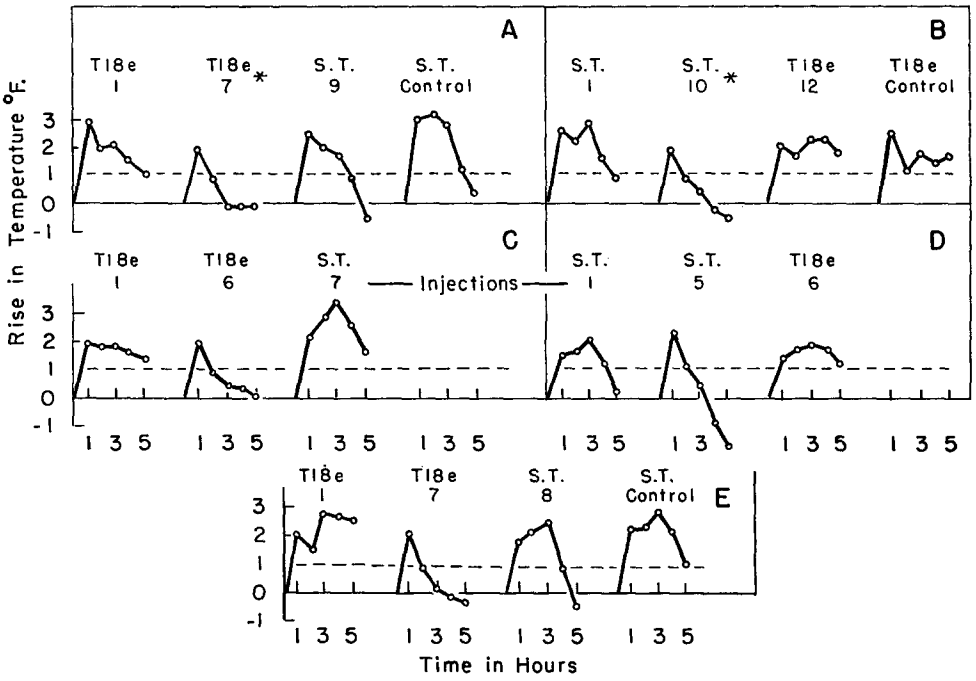

FIG. 4. A and B. Reciprocal cross between T-18 extracellular products (T-18e), injection dose 130 μg. N, and *S. typhosa* (S.T.), injection dose 4 μg N. C and D: Reciprocal cross between T-18e, injection dose 195 μg N, and S.T., injection dose the same as above. E. Cross between T-18e, injection dose 260 μg. N, and S.T., injection dose the same as above. The reciprocal cross was not done.

repeated with the same results. However, in the first experiment (Fig. 5 A and B), there had been an intervening injection of T-18 extracellular products (T-18e). It was possible that this injection had caused reversal in tolerance. Therefore, in the experiment of Fig. 5 (C and D) after the *S. typhosa* challenge injection, an injection of the same preparation and concentration of T-18e as in the previous experiment was given. Upon rechallenge with *S. typhosa*, the animals were still tolerant; no reversal in tolerance had occurred. However, an

* There was an intervening injection of T-18i, 290 μg. N.

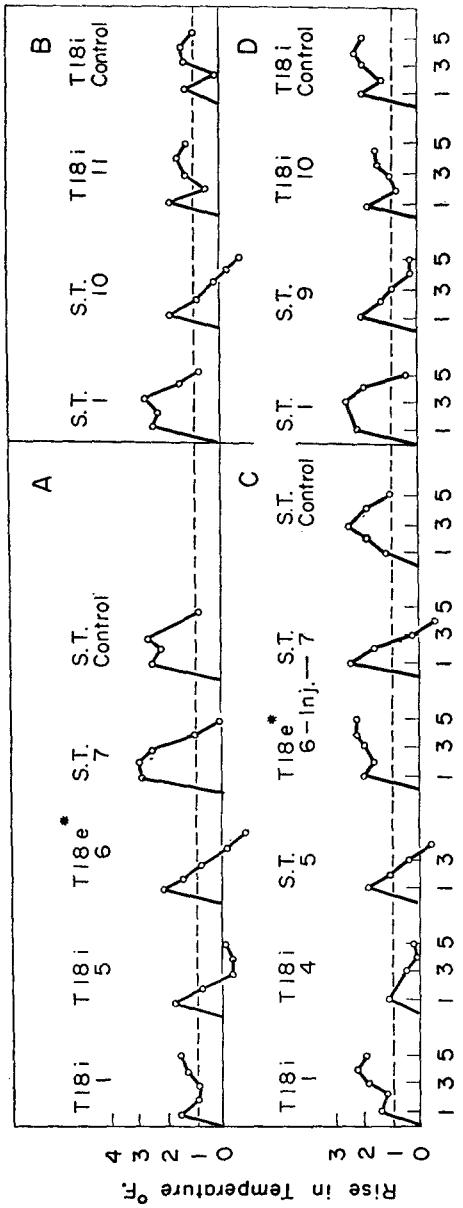

FIG. 5. A and B. Reciprocal cross between the sonic extracts of T-18 and *S. typhosa*, showing a reciprocal lack of tolerance between the two. C and D: Repeat of the above reciprocal cross at the same concentrations but with different preparations of both extracts. The cross now shows a non-reciprocal tolerance. This identical cross was repeated with the same results.

* 130 μg. N T-18e, the same preparation in both experiments.

interesting ancillary observation was the different response of the two groups of rabbits to the *S. typhosa* and T-18e injections. The first group of rabbits responded to T-18e with a barely significant febrile response at 3 hours, which then dropped to normal, while they showed no tolerance to the *S. typhosa* injection. Conversely the second group of rabbits was tolerant of the *S. typhosa* but not of T-18e. Possibly the first T-18 sonic extract contained more of the extracellular products and the second extract less.

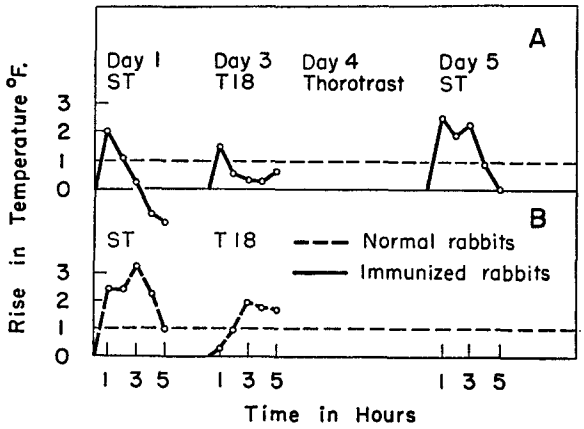

FIG. 6. In Experiment A, 10 rabbits were immunized with equal parts of undiluted *S. typhosa* sonic extract and Freund's complete adjuvant by subcutaneous injections of 1.2 to 1.4 ml. of the mixture at multiple sites. Three weeks later they were given an intradermal injection of 0.3 ml. of the sonic extract to check for Arthus reaction. All reacted positively. Six days later, designated as day 1, they were given an intravenous injection of *S. typhosa* extract, 4 μg. N. They did not respond with a sustained fever. On day 3 an intravenous injection of T-18 sonic extract, 290 μg. N, did not elicit a fever. However, after an intravenous injection of thorotrast, 3 ml. per kg. body weight, the rabbits now responded normally to the *S. typhosa* extract on day 5. In Experiment B, two groups of 10 normal control rabbits were injected with the bacterial extracts on the same day as the immune rabbits.

Because of the ambiguous results obtained in tolerance studies with T-18 intracellular extracts and *S. typhosa*, the effect of active immunization on pyrogenic response was studied. Ten rabbits were immunized to the sonic extract of *S. typhosa* using Freund's adjuvant. Injections were made by the subcutaneous route. After Arthus reactions were strongly positive, the animals were challenged intravenously with the sonic extracts of *S. typhosa* and T-18 (Fig. 6). The same extracts at the same concentration as in the experiment of (Fig. 5 C and D) were used. Neither caused a febrile response in the immunized rabbits at the 3rd, 4th, and 5th hours. The animals were tolerant. If thorotrast, a reticuloendothelial blockading agent, was given intravenously, the tolerance was reversed. The animals now responded with a febrile response to *S. typhosa*.

This result does not rule out the role of humoral factors in tolerance but does emphasize the necessity for a functioning RES in removing toxin or toxin-serum complexes. It is not suggested that specific *S. typhosa* antibody was instrumental in establishing tolerance to streptococcal sonic extract but rather that because of active stimulation of the RES and possible increase in non-specific humoral factors through the immunizing injections, non-specific host resistance was increased. A functioning, actively stimulated RES appears to be as important in tolerance to streptococcal pyrogenic intracellular toxin as it is to Gram-negative bacterial endotoxin (10). Stetson (5) reported some reversal of tolerance to streptococcal lysates by intravenous injection of proferrin, a RES

TABLE I

Partial Reversal of Tolerance by Intravenous Injection of Thorotrast

Intravenous injection	Day	Average* hourly temperature response				
		1	2	3	4	5
NY-5 sonic extract	1	1.8	1.8	2.1	1.9	1.8
	13	1.8	1.5	0.8	0.6	0.8
Thorotrast‡	14	(Not checked)				
NY-5 sonic extract	15	2.2	2.5	1.9	1.0	0.7

* 7 rabbits.

‡ 3 ml. per kg. body weight; 24 to 28 per cent thorium dioxide by volume (Testagar and Co. Inc., Detroit, Michigan).

blockading agent. The finding was repeated in this study using thorotrast as a blockading agent (Table I).

Chemical Extraction of Streptococcal Pyrogen

The endotoxin of Gram-negative bacteria consists of a lipopolysaccharide which is easily extracted by either hot phenol (8) or TCA (9). Therefore, in order to determine whether the streptococcal pyrogenic toxins were chemically the same as the Gram-negative bacterial endotoxin, such extraction procedures were done. Initial studies with hot phenol extraction of streptococci, strain NY-5, showed some activity present in the water phase. Forty-five gm. of bacteria, wet weight, were first extracted two times with 45.6 per cent phenol and a third and fourth time with 90 per cent phenol, at a temperature of 65°-68°C. The water phases were collected and dialyzed against pyrogen-free water for 36 hours at 2°C. They were then lyophilized and tested for pyrogenic activity in rabbits. Results are shown in Table II.

Since there was enough pyrogen present to produce a 1 hour response but insufficient to cause a sustained response, the possibility existed that better extraction would be obtained if the cell wall were damaged prior to phenol extraction or that TCA extraction would be more effective. A new batch of NY-5 was grown and divided into aliquots. One aliquot, 14 gm. wet weight, was alternately frozen in a dry ice–cellosolve bath and thawed in a 55°C. water bath

fifteen times. The bacteria were then extracted with 90 per cent hot phenol. A second aliquot, 14 gm. wet weight, was extracted with TCA. A third aliquot, approximately 18 gm. wet weight,

TABLE II

Pyrogenicity of Streptococcal Extracts Obtained by Hot Phenol Method

Extraction*	Yield	Intravenous injection	Average temperature response in half-hour increments						
			½	1	1½	2	2½	3	3½
	mg.	*mg.*							
45.6 per cent phenol, 2 times	220	0.1	0.0	0.0	0.0	0.3	0.2	0.1	0.3
		1.0	0.5	1.0	1.1	1.0	0.7	0.7	0.5
		6.0	0.4	1.2	1.6	1.4	1.0	0.5	−0.3
90.0 per cent phenol, 2 times	45	2.4	0.2	1.5	2.0	1.7	1.1	0.6	0.0

* Both extractions were done on same batch of bacteria, 45 gm. wet weight.

TABLE III

Studies on Extraction Methods for Streptococcal Pyrogens

Treatment of bacteria prior to extraction	Method of extraction	Yield	Intravenous injection	Average temperature response in half-hour increments								
				½	1	1½	2	2½	3	3½	4	4½
		mg.	*mg.*									
14 gm. frozen-thawed 15 ×	90 per cent phenol 3×	170	1.0	0.1	0.2	0.5	0.4	0.3	0.3	0.3		
14 gm. none	0.5 N TCA at 2–4°C.	*										
0.4 gm. sonic extract	90 per cent phenol‡ 2×	11	0.04	0.0	−0.1	−0.1	−0.1	0.0	0.0	—	−0.1	
			0.44	0.2	0.7	0.5	0.5	0.6	0.7	0.8	0.8	
0.4 gm. sonic extract	0.5 N TCA§ at 2–4°C.	8	0.03	−0.1	−0.1	−0.2	−0.2	−0.1	−0.1	—	−0.4	
			0.32	0.3	0.4	0.5	0.4	0.3	0.7	0.4	0.4	
Sonic extract control prior to treatment			2.0	0.4	1.5	1.8	1.5	1.2	1.7	2.0	2.2	1.9

* Yield was so small it was impossible to measure, therefore pyrogenicity not checked.

‡ Lyophilized material was solubilized in 100 ml. distilled water. Temperature was brought to 65–68°C. and an equal volume of hot 90 per cent phenol added with constant shaking. Extraction was continued for 30 minutes. The water phase was separated, collected, and an additional 100 ml. of water was added to the phenol phase. Extraction was repeated. The water phase was again collected, the two extractions pooled, dialyzed, and lyophilized.

§ Lyophilized material was solubilized in 40 ml. distilled water. An equal volume of 0.5 N TCA was added dropwise with agitation at 2–4°C. Extraction was continued for 3 hours. The fluid harvested after centrifugation was dialyzed 36 hours against distilled water in the cold and then lyophilized.

was exposed to sonic oscillation and an extract prepared as previously described under Materials and Methods. However, rather than dialyzing the extract against balanced salt solution and freezing it, the water extract was immediately lyophilized. The yield of dry material was 1.4 gm. The lyophilized material in 400 mg. aliquots was then treated with 90 per cent phenol and with TCA. Results are shown in Table III. A hot phenol extraction was also done with

T-18 extracellular material (Table IV). Hot phenol extraction and/or extraction with TCA resulted in very small yields, with little or no pyrogenic activity.

Other Comparative Studies with Streptococcal Sonic Extracts

Local Shwartzman Reaction.—The activity of streptococcal sonic extracts in preparing and eliciting the local Shwartzman reaction is shown in Table V.

TABLE IV

Effect of Phenol Extraction on Pyrogenicity of T-18 Extracellular Material*

Starting material	Yield	Intravenous injection	Average temperature response in half-hour increments							
			1	1½	2	2½	3	3½	4	4½
	mg.	*mg.*								
372 mg. lyophilized T-18 extracellular material	193	2.0	0.6	1.0	0.9	—	0.5	0.3	0.3	0.4
Control on material prior to extraction		2.0	1.6	1.6	1.8	1.8	1.8	1.8	1.8	1.8

* See second footnote under Table III.

TABLE V

Activity of Streptococcal Sonic Extracts in Causing the Local Shwartzman Reaction

Intradermal injection of sonic extracts		Intravenous injection 24 hrs. later		Positive/total
	mg. N		*mg. N*	
S. typhosa	0.1	*S. typhosa*	0.2	8/10
T-18, T-4	0.6	*S. typhosa*	0.2	7/10
S. typhosa	0.1	T-18, T-4	1.4	2/10
T-4	0.6	T-4	1.4	0/5
NY-5	1.7	NY-5	4.3	0/8

While streptococcal sonic extract could substitute for either the preparatory or challenging dose in conjunction with *S. typhosa* sonic extract, at the concentrations used, it was incapable of causing the reaction alone.

Heat Inactivation.—Streptococcal sonic extract, concentration 2 mg. lyophilized material/ml., was boiled for 1 hour. The resulting coagulum was removed by centrifugation and the crystal clear supernate inoculated intravenously in 1 ml. amounts into each of 7 rabbits. Such supernates elicited no pyrogenic response.

Enhancement of Toxicity.—Watson (6) reported on the ability of streptococcal pyrogenic exotoxin, strain NY-5, to enhance toxicity of sublethal doses of a *S. typhosa* broth filtrate. Streptococcal sonic extracts were therefore tested for

such activity (Table VI) with essentially negative results. On this basis, the differentiation between NY-5 intracellular and extracellular products was definitive. With T-18, however, the results were not as clear. The large quantities of hyaluronic acid produced by T-18 make it very difficult to wash the bacteria free of extracellular material. Considerable extracellular material would therefore be extracted along with the bacterial cell. Production of hyaluronic acid by NY-5 is considerably less than with T-18.

TABLE VI

Enhancement of Toxicity of Gram-Negative Bacterial Endotoxin by Streptococcal Preparations

First intravenous injection		Second intravenous injection 4 hrs. later	Dead/total
	mg.		
NY-5 sonic extract	2.0*	0.25 ml. *S. typhosa* broth filtrate	0/13
NY-5 extracellular material	1.0‡	" " " " " "	7/8
" " "	0.05	" " " " " "	5/6
" " "	0.02	" " " " " "	3/6
Medium‡		" " " " " "	0/6
T-18 sonic extract	3.0*	" " " " " "	2/8
T-18 extracellular material	2.0	" " " " " "	4/8
Saline, 1 ml.		" " " " " "	0/6

Diluent in all cases was saline. All the streptococcal preparations at these concentrations did not cause death when injected alone.

* Lyophilized sonic extract.

‡ Broth filtrate from a 6 hour culture of NY-5 was precipitated twice according to the method of Pavlov *et al.* (11). The precipitate was then solubilized in distilled water and lyophilized. Two liters of sterile medium were precipitated in the same way; the total resulting material was dissolved in saline and injected into 6 rabbits.

DISCUSSION

The ability of the Group A sonic extracts studied to induce reciprocal pyrogenic tolerance to each other (Fig. 2) suggests that they all produce a similar somatic toxin which is neutralized *in vivo* in the same fashion. This picture is analogous to the Gram-negative bacterial endotoxins (12) where tolerance to one species insures tolerance to other Gram-negative species. Also analogous to the results seen in tolerance to Gram-negative bacterial endotoxins, was the ease with which the second peak of the biphasic febrile response was diminished and the difficulty in repressing the 1 hour peak.

The toxin in the extracellular concentrated products of T-18 appears to differ from intracellular pyrogen in several respects. First, as shown in Fig. 3, an absolute reciprocal tolerance was not observed between the two. Secondly, the response to a challenging injection of *S. typhosa* sonic extracts was markedly different in rabbits tolerant to the intracellular and extracellular material. There was a reproducible, reciprocal lack of tolerance between the extracellular

products of T-18 and *S. typhosa* extracts, while in crosses in rabbits using T-18 intracellular products and *S. typhosa*, there was a great variation in response from reciprocal non-tolerance to non-reciprocal tolerance. Thirdly, extracellular products were very active in enhancing toxicity of Gram-negative bacterial endotoxin while intracellular products in the concentrations used were not.

Two pyrogenic exotoxins for NY-5 and one for T-18 were previously described (6). They were associated with the scarlet fever group of toxins. Following the nomenclature started by Hooker and Follensby (13) for scarlet fever toxins, the two exotoxins of NY-5 were designated A and B, and that of T-18, C. Other types also produced either A or B exotoxin. T-28 and T-12 produced the A toxin, while T-19 produced the B toxin. Tolerance to these toxins followed immunologic specificity. Thus, strains producing the A toxin induced tolerance only to those strains also producing the A toxin but not to T-18, a C toxin producer. The febrile response to the A toxin was prevented by *in vitro* neutralization with potent scarlet fever antitoxin. A fourth difference, therefore, between the extracellular pyrogens and the intracellular pyrogens of this study is the lack of specificity in tolerance among the intracellular pyrogens. If the intracellular pyrogens of NY-5 and T-18 were the same as their respective extracellular pyrogens, no cross-tolerance should develop between them.

The results in this study can be explained if the Group A streptococci produce two distinct pyrogenic toxins: a somatic toxin which shows no type-specificity and an immunologically distinct exotoxin as previously described (6). Further, if the somatic toxin is contaminated with traces of extracellular toxin and *vice versa*, a borderline tolerance but never complete tolerance would occur between the two. Serologic studies using the Ouchterlony technique show that such cross-contamination does exist.

Reduced pyrogenic response to Gram-negative bacterial endotoxin has been associated primarily with increased RES activity (10) and with serum factors other than specific antibody (14–17). However, the pyrogenicity of streptococcal exotoxin produced *in vivo* is neutralized by specific antibody (6). Thus, no tolerance would be expected between these two pyrogens. Such was the finding in this study.

The anomalies in crosses between streptococcal sonic extracts and *Salmonella* sonic extracts are explicable on the following assumptions. First, in different preparations of streptococcal extracts, varying degrees of contamination with exotoxin may occur; this variation may be due to the greater or lesser production of hyaluronic acid capsular material which would tend to hold extracellular products more closely to the bacterial cell surface. Second, tolerance to the somatic toxin of the streptococci and to the Gram-negative bacterial endotoxin is due to similar non-specific factors. Thus, as long as the streptococcal endotoxin contained appreciable quantities of streptococcal exotoxin, rabbits

tolerant to *S. typhosa* would always respond to some degree to streptococcal extracts. However, rabbits tolerant to streptococcal extracts would not necessarily respond to *S. typhosa*, unless the *S. typhosa* endotoxin was present in higher concentration than was the streptococcal endotoxin. Stetson (5) observed that rabbits made tolerant to Group A streptococcal lysates responded with high fever to Gram-negative bacterial endotoxin. His injection dose of Gram-negative bacterial endotoxin was 10 μg. This may have been in excess of the amount present in streptococcal lysates. Rabbits tolerant to Gram-negative bacterial endotoxin always responded with some fever to streptococcal lysates. If the hypothesis of a distinct exotoxin is correct, this response could then be due to traces of streptococcal exotoxin in the lysates.

Gram-negative bacterial endotoxins act as an adjuvant in antibody production to heterologous antigens (18). Stetson and others (5, 19, 20) have shown a similar effect using streptococci or streptococcal lysates as adjuvant. Further, intravenous injection of thorotrast can cause reversal of tolerance to both Gram-negative endotoxins and streptococcal toxins. Cross-tolerance between *S. typhosa* and streptococcal extracts may merely reflect non-specific increased RES capacity and presence or absence of non-specific humoral factors (14–17, 21).

Yields of streptococcal material with phenol and TCA extraction methods were very small and pyrogenicity weak or absent. Although in some cases, 1 hour febrile responses occurred, the fever was not usually sustained. Average hourly temperature changes at 3, 4, and 5 hours always fell below a significant 1°F. response. On the basis of these data, the streptococcal pyrogenic toxins and the Gram-negative bacterial endotoxins appear to be chemically dissimilar. These results do not necessarily rule out the possible importance of lipoid constituents in the toxin. However, rather than a polysaccharide acting as a solubilizing carrier for the toxic lipid moiety, as with Gram-negative bacterial endotoxin (8, 22), a protein may be involved. The fact that the streptococcal products elicit some biologic effects associated with the action of Gram-negative bacterial endotoxins does not necessitate the assumption that the two are chemically identical. Similar biologic effects can result from substances differing chemically. Intracellular streptococcal material could substitute for either the preparatory or provoking injection of typhoid endotoxin in the local Shwartzman reaction but could not substitute for both injections. Stetson (5) found that if the dose were large enough or if the rabbits were made hypersensitive to streptococcal products, the local Shwartzman reaction could be produced by streptococcal lysates alone. Thus, the streptococcal intracellular pyrogenic toxin is considerably less active. This difference in activity was also observed in the present pyrogenic studies in which the streptococcal pyrogenic dose was approximately 100 times greater than the *Salmonella* dose.

SUMMARY

The data presented in this study suggest that Group A streptococci produce two distinct pyrogenic toxins: one primarily an intracellular toxin and the other mainly an extracellular toxin.

Both of these toxins are not extractable by the conventional methods, phenol and TCA, used for isolation of Gram-negative bacterial endotoxin. Their chemical composition, therefore, differs from that of Gram-negative bacterial endotoxin.

On the basis of tolerance studies it appears that similar non-specific host factors may be involved in the detoxification of both the streptococcal intracellular pyrogenic toxin and the Gram-negative bacterial endotoxin. Detoxification of streptococcal exotoxin requires a more immunologically specific mechanism.

Because all the Group A sonic extracts studied elicited reciprocal tolerance to one another, it is suggested that tolerance to the intracellular pyrogenic toxin is not correlated with type-specificity.

BIBLIOGRAPHY

1. Centanni, E., Ueber infektious Fieber. Das Fiebergift der Bakterien, *Deutsch. med. Woch.*, 1894, **20,** 148, 176.
2. Tui, Co, and Schrift, M. H., Production of pyrogen by some bacteria, *J. Lab. and Clin. Med.*, 1942, **27,** 569.
3. Duval, C., and Hibbard, R. J., Experimental glomerulonephritis induced in rabbits with the endotoxic principle of *Streptococcus scarlatinae*, *J. Exp. Med.*, 1926, **44,** 567.
4. Probey, T. F., and Pittman, M., The pyrogenicity of bacterial contaminants found in biologic products, *J. Bact.*, 1945, **50,** 397.
5. Stetson, C., The endotoxic properties of lysates of Group A hemolytic streptococci, *J. Exp. Med.*, 1956, **104,** 921.
6. Watson, D. W., Host-parasite factors in Group A streptococcal infection. Pyrogenic and other effects of immunologic distinct exotoxins related to scarlet fever toxins, *J. Exp. Med.*, 1960, **111,** 255.
7. Kabat, E. A., Mayer, M. M., and Heidelberger, M., Experimental Immunochemistry, Springfield, Illinois, Charles C. Thomas, 1948.
8. Westphal, O., Lüderitz, O., and Bister, F., Ueber die Extraktion von Bakterien mit Phenol/Wasser, *Z. Naturforsch.*, 1952, **7b,** 148.
9. Webster, M. E., Sagin, J. F., Landy, M., and Johnson, A. G., Studies on the O antigen of *Salmonella typhosa*. I. Purification of the antigen, *J. Immunol.*, 1955, **74,** 455.
10. Beeson, P. B., Tolerance to bacterial pyrogens. II. Role of the reticulo-endothelial system, *J. Exp. Med.*, 1947, **86,** 39.
11. Pavlov, P. B., Akimova, V. V., and Pomiankevich, A. N., Purified adsorbed scarlet fever toxin. I. The production of high titre scarlet fever toxin, *J. Microbiol. Epidem., and Immunol.*, 1957, **28,** 1636 (English translation, New York, Pergamon Press).

12. Beeson, P. B., Tolerance to bacterial pyrogens. I. Factors influencing its development, *J. Exp. Med.*, 1947, **86,** 29.
13. Hooker, S. B., and Follensby, E. M., Studies on scarlet fever. II. Different toxins produced by hemolytic streptococci of scarlatinal origin, *J. Immunol.*, 1934, **27,** 177.
14. Grant, R., Refractoriness to pyrogens. Effects of incubation of pyrogen with plasma from normal and refractory donors on the response of refractory recipients, *Am. J. Physiol.*, 1953, **173,** 246.
15. Farr, R. S., Clark, S., Proffitt, J., and Campbell, D. H., Some humoral aspects of the development of tolerance to bacterial pyrogens in rabbits, *Am. J. Physiol.*, 1954, **177,** 269.
16. Yoshioka, M., and Johnson, A. G., Alteration of bacterial endotoxin by human alpha globulin, *Bact. Proc.*, 1960, 116.
17. Freedman, H. H., Passive transfer of tolerance to pyrogenicity of bacterial endotoxin, *J. Exp. Med.*, 1960, **111,** 453.
18. Johnson, A. G., Gaines, S., and Landy, M., Studies on the O antigens of *Salmonella typhosa*. V. Enhancement of antibody response to protein antigens by the purified lipopolysaccharide, *J. Exp. Med.*, 1956, **103,** 225.
19. Lewis, P., and Loomis, D., Allergic irritability. III. The influence of chronic infections and of trypan blue on the formation of specific antibody, *J. Exp. Med.*, 1926, **43,** 263.
20. Schultz, M. P., and Swift, H. F., The effect of antecedent infection and immunization with streptococci upon the reactivity of rabbits to horse serum, *J. Exp. Med.*, 1934, **60,** 337.
21. Landy, M., Skarnes, R. C., Rosen, F. S., Trapani, R. J., and Shear, M. J., Inactivation of biologically active ("endotoxic") polysaccharides by fresh human serum, *Proc. Soc. Exp. Biol. and Med.*, 1957, **96,** 744.
22. Neter, E., Anzai, H., Gorzynski, E. A., Nowotny, A., and Westphal, O., Effects of lipoid A component of *Escherichia coli* endotoxin in dermal reactivity of rabbits to epinephrine, *Proc. Soc. Exp. Biol. and Med.*, 1960, **103,** 783.