A PURIFIED GROUP A STREPTOCOCCAL PYROGENIC EXOTOXIN

Physicochemical and Biological Properties Including the Enhancement of Susceptibility to Endotoxin Lethal Shock*

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(Received for publication 25 August 1969)

After Dick and Dick (1, 2) discovered scarlet fever toxin in culture filtrates of hemolytic streptococci, many investigators attempted to purify and characterize the toxin (3–10). Stock and his associates (7–10) did the most significant work on its physicochemical properties; they also believed that the erythrogenic property was a manifestation of its primary toxicity. Also, because scarlet fever toxins or erythrogenic toxins were thought to be produced only by specific scarlet fever strains of hemolytic streptococci, and because of the relatively low incidence of scarlet fever cases after the antibiotic era, the toxins were not considered to be of great significance in the pathogenesis of Group A streptococcal infections and their sequelae.

More recently Schwab et al. (11, 12) showed that when Group A streptococci grow in the tissue of animals, such as the skin of rabbits, they produce an extracellular toxin. Although this toxin was not highly lethal, it could modify the host so that the tissues, including the heart, became vulnerable to damage by a wide variety of microbial and other toxins. Later this enhancement activity was found to be associated with the so-called erythrogenic toxins (13). These toxins, whether produced in vivo or in vitro, induced fever in rabbits and were called streptococcal pyrogenic exotoxins (13–15). Three distinct pyrogenic exotoxins were demonstrated by inducing specific pyrogenic immunity in rabbits (13).

The present work extends these observations by describing the physicochemical properties and the various biological activities of a highly purified toxin prepared from culture filtrates of Group A streptococci, type 10, strain NY-5.

Because all Group A streptococci tested produce one or more of these pyrogenic exotoxins, and because of their potent biological activity including enhanced susceptibility to injury and lethal endotoxin shock, blockage of the

* Supported by United States Public Health Service Grants AI-06487 and HE-5360.

‡ Dr. Kim is the recipient of Research Career Development Award AI37, 388.

reticuloendothelial system (RES)[1] (16) and immunosuppression (17), it is suggested that this group of toxins might play a significant role in the pathogenesis of Group A streptococcal infections and their sequelae; in combination with Gram-negative bacterial infections they could prepare the host for fatal shock.

Materials and Methods

Strains of Streptococci.—The organisms used to produce the exotoxins were Group A hemolytic streptococcus (*Streptococcus pyogenes*) type 10, strains NY-5 and T-18 from Naval Medical Research Unit-4, Great Lakes, Ill. The organisms were maintained as lyophilized cultures after growth in Todd-Hewitt broth and suspension in 1% whole defibrinated fresh rabbit blood.

Production of Exotoxin.—Pyrogen-free freshly prepared modified Stock's medium (8, 13) inoculated with a 5% exponentially growing culture in the same medium, was incubated at 37°C for 5–6 hr. To minimize contamination by intracellular products derived from cell autolysis, the media was preheated to 37°C before inoculation and the cultures were shaken gently at 10–15 min intervals. Under these conditions the logarithmic phase was maintained for 5–6 hr, at which time the cultures were cooled rapidly in an ice bath, and the cell-free culture filtrates obtained by continuous flow centrifugation by the Sharples or Sorval centrifuges; finally the supernatant fluids were passed through Seitz sterilizing filters. At each step, the purity of the culture and sterility of culture filtrates were checked.

Purification of Exotoxin.—A modified method of Ando et al. (18) was used. Briefly, one part of the crude cell-free culture filtrate was cooled to 1–2°C and three parts of cold absolute ethyl alcohol (ETOH) (precooled to −20°C) were added and thoroughly mixed. The toxin settled out as a precipitate when the solution was kept at 1–2°C for 48 hr. The clear supernatant fluid was siphoned off and the precipitated toxin collected by centrifugation at 1500 *g* for 20 min. The sediments were dissolved in cold acetate-buffered saline (ABS) (acetate buffer 0.005 M, pH 4.0 with 0.15 M NaCl) at a ratio of 1 part ABS to 15 parts original culture filtrate. The mixture was kept in the refrigerator (1–2°C) for 24 hr. In this step the exotoxin was dissolved and the precipitate remaining represented inert material; this was discarded after centrifugation at 1500 *g* for 10 min. The toxin was reprecipitated from the clear supernatant fluid by the addition of 4 parts of cold −20°C ETOH. This procedure was repeated until all of the precipitate dissolved completely in ABS. Usually five or more repeated precipitations gave highly purified toxin preparations. Finally the dissolved toxin was dialyzed against pyrogen-free distilled water at 1–2°C and lyophilized. About 1200 mg of purified NY-5 exotoxin were obtained from 100 liters of culture filtrate.

Physicochemical Analysis.—Sedimentation velocity and sedimentation equilibrium experiments for sedimentation coefficient and molecular weight determinations were performed as described elsewhere (19). The heat stability was tested by incubating the toxin at 65°C for 30 min or in a boiling water bath for 2 min. Activity of the heated exotoxin was determined by pyrogenic assays in rabbits.

Proteins were determined by the micro-Kjeldahl nitrogen method (20), and optical density measurements at 280 mμ in a DB spectrophotometer with bovine albumin as a protein standard; lipids were analyzed by gas chromatography, and hyaluronic acid by the method of

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[1] *Abbreviations used in this paper:* ABS, acetate-buffered saline; DNases, deoxyribonucleases; ETOH, ethyl alcohol; MPD-3, minimal pyrogenic dose–3 hr; NADases, nicotinamide adenine dinucleotidases; PBS, phosphate-buffered saline; RES, reticuloendothelial system; STD, skin test doses.

Dische (21). Amino acid composition of the toxin was determined in a Beckman 120B automatic analyzer after hydrolyzing the toxin with 6 N and 9 N hydrochloric acid at 105°C in vacuum-sealed vials. In addition, the purified toxin preparations were tested for streptolysins O and S (22), nicotinamide adenine dinucleotidases (NADases) (23, 24), deoxyribonucleases (DNases) (25), and endotoxin (26).

Biological Assays.—Young American Dutch rabbits, 3 months old, 1.0–1.2 kg were used to determine the various biological activities of the toxin, including pyrogenicity, lethality within 48 hr, skin sensitivity as described previously for endotoxin (26, 27), cytotoxicity (28), and

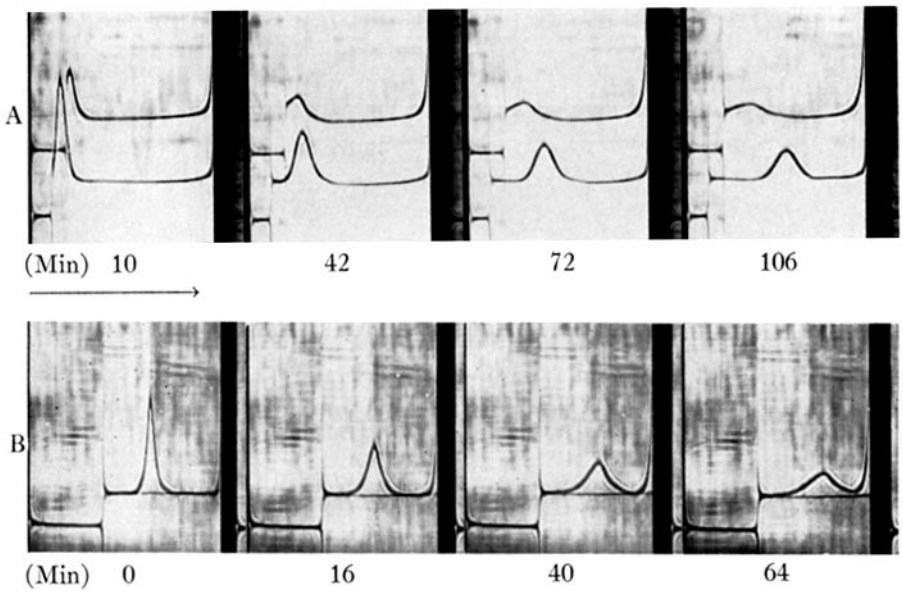

FIG. 1. Sedimentation patterns of streptococcal pyrogenic exotoxin (NY-5). A. Top patterns (+1° wedge window), toxin (NY-5) at 5 mg/ml. Bottom patterns, bovine serum albumin at 5 mg/ml for comparison. B. Synthetic boundary run (double sector, capillary-type cell), toxin (NY-5) at 5 mg/ml. Solvent, phosphate-buffered saline (0.005 M Na_2HPO_4-NaH_2PO_4 buffer + 0.15 M NaCl, pH 7.0) (PBS). Speed, 56, 100 rpm at 20°C. Arrow, indicates direction of sedimentation. Photographs were taken at indicated time after reaching the indicated rotor speed; phase plate angle was 60°C. The calculated sedimentation coefficient ($s_{20,w}$) of toxin (NY-5) was 1.8S.

enhanced susceptibility to endotoxin shock (15). In addition, guinea pigs and human volunteers were used for the skin reactivity of the toxin and 3-wk old baby rabbits, 2-month old mice, and adult cynomolgus monkeys were used to test the enhancement effect of the toxin.

Endotoxin.—Endotoxin from *Salmonella typhosa* 0901 prepared by the hot-phenol water extraction method of Westphal and Lüderitz (29) was used for cross-immunity testing and enhancement to endotoxin shock experiments.

Immunization against Toxins.—The following injection schedule was used to develop pyrogenic immunity to streptococcal pyrogenic exotoxin. After the control test with 10 minimal pyrogenic doses–3 hr (MPD-3)/kg intravenously on day 1, the rabbits were injected with 10 MPD-3/kg of the toxin intravenously every other day for 2 wk. Finally, 2 days after the last injection, the rabbits were tested for pyrogenic immunity to the homologous toxin with

10 MPD-3/kg intravenously. The rabbits were then cross-tested with other toxins to determine cross-reactivity or specificity. Pyrogenic immunity to endotoxins is described elsewhere (26, 27, 30).

The *toxin solutions* were prepared in sterile pyrogen-free phosphate-buffered saline (PBS) (0.005 M phosphate buffer, pH 7.0, plus 0.15 M NaCl) for all biological assays. All the *glassware* and *reagents* used for purification and assays of the toxin were maintained pyrogen-free.

TABLE I

Chemical Composition of Purified Streptococcal Pyrogenic Exotoxin (NY-5)

Amino acids	Amino acids	
	6 N HCl (20 hr)	9 N HCl (26 hr)
	moles/100 moles	*moles/100 moles*
Aspartic	14.7	15.1
Threonine	5.5	5.2
Serine	7.3	6.9
Glutamic acid	14.4	15.1
Proline	4.2	4.4
Glycine	9.2	8.9
Alanine	8.6	8.3
Half cystine	Trace	Trace
Valine	5.1	5.2
Methionine	0.8	0.8
Isoleucine	3.9	3.9
Leucine	6.3	6.0
Tyrosine	3.4	3.5
Phenylalanine	3.1	3.1
Lysine	8.5	8.6
Histidine	1.9	1.9
Arginine	3.1	3.1
(Total amino acids)	(100.0)	(100.0)
Glucosamine	31.6	19.5
Galactosamine	None	None
Muramic acid	None	None
Diaminopimelic acid	None	None
Ammonia	21.4	30.0

RESULTS

Physicochemical Properties of the Toxin

A high degree of purification was achieved by combined differential solubility in alcohol and acidic buffer, centrifugation, and dialysis. Ion exchange or exclusion chromatography were not used because of the danger of pyrogen contamination. The toxin was primarily protein (80%) complexed with hyaluronic acid (20%) with a trace of lipid. Fatty acids were detectable by gas chromatography but not in significant amounts for quantitation. Biological activity of the toxin

was destroyed by heating at 65°C for 30 min or boiling for 2 min. The purified toxin preparations were free of streptolysins O and S, NADases, DNases, mucopeptides, and endotoxin. Although strain NY-5 is known to produce both types A and B toxins (31), the purified toxin is type A.

Sedimentation velocity studies were performed in a Spinco Model E analytical ultracentrifuge (19). As shown in Fig. 1A and B, a single boundary throughout the migration indicated relative homogeneity. The sedimentation coefficient was 1.8S ($s_{20,w}$). The molecular weight of the exotoxin, at a concentration of 0.1–2.0 mg/ml in PBS, was determined by high speed sedimentation equilibrium, meniscus depletion method of Yphantis (32). Calculations were

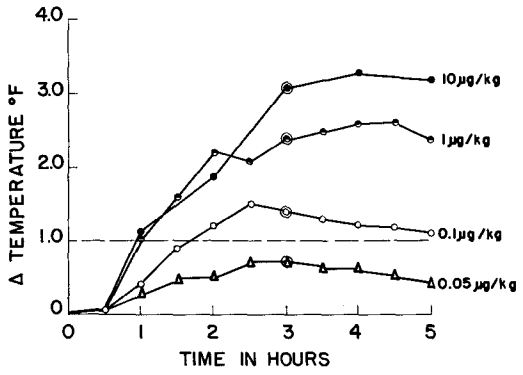

FIG. 2. Titration of streptococcal pyrogenic exotoxin (NY-5) for minimal pyrogenic dose–3 hr (MPD-3) in young adult rabbits. Each curve represents the mean febrile response of five rabbits injected intravenously with indicated concentrations of the toxin.

made with a Control Data 3300 computer using a Fortran program developed by Small et al. (33). The whole cell average molecular weight was 29,400.

The amino acid composition of the toxin is listed in Table I and is typical of an acidic protein. The high value of glucosamine is due to the presence of hyaluronic acid. The absence of muramic acid and diaminopimelic acid indicates that the purified toxin is devoid of cell-wall products.

Biological Properties of the Toxin

A. Pyrogenicity.—Pyrogenic activity of the toxin was tested in young 3-month old American Dutch rabbits. Representative febrile response curves for the titration of pyrogenic activity are given in Fig. 2. The minimal pyrogenic dose–3 hr (MPD-3) (27) is presented in Fig. 3. The mean febrile response at 3 hr is plotted against the log of the concentration; the resulting regression line is drawn to 1°F and the quantity of the toxin at the intercept represents the MPD-3. The MPD-3 value of the toxin was 0.07 μg/kg. For controls, rabbits

injected intravenously with 1 ml/kg 100 *X* concentrated modified stocks dialyzable medium or PBS, as well as 100 μg/kg heat inactivated toxin gave no febrile responses.

B. Lethality.—Lethal activity of the toxin was tested in 3-month old rabbits as shown in Table V. The LD_{50} was 3500 μg/kg.

C. Skin Reactivity.—All eight volunteers among laboratory personnel tested gave positive skin tests with 10^{-6} μg of exotoxin intradermally as summarized in Table II. These results show that $>10^9$ skin test doses (STD) per mg of toxin is higher than reported earlier for these exotoxins (1–3, 7–9).

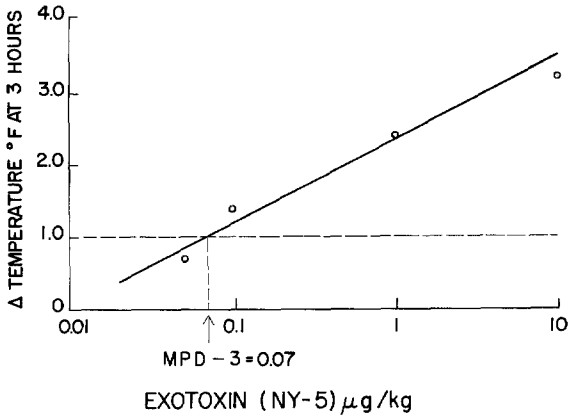

FIG. 3. Determination of minimal pyrogenic dose–3 hr (MPD-3) of streptococcal pyrogenic exotoxin (NY-5). Each point represents the mean febrile response of five rabbits 3 hr after the intravenous injection with indicated concentrations of NY-5. The linear regression line (least square fits of the data) is drawn to 1°F and the quantity of the toxin (NY-5) at the intercept is the MPD-3. The MPD-3 of the toxin was 0.07 μg/kg intravenously.

D. Cytotoxicity.—The tissue culture method of Heilman et al. (28) was used for measuring the cytotoxicity of these toxins. As reported in Table III, macrophages from young rabbit spleens showed 50% inhibition of migration at 96 hr with 10 μg/ml of the toxin giving a cytotoxic index of less than 0.5. These results are consistent with the in vivo test of RES blockage and the immunosuppression by the toxin (16, 17).

E. Enhancement of Susceptibility to Endotoxin Shock.—

Young adult rabbits: To determine the most effective combination to enhance susceptibility to lethal shock, various combinations of endotoxin and exotoxin were tested as shown in Table IV. Rabbits pretreated with endotoxin or exotoxin and 3 hr later tested with homologous endotoxin or exotoxin showed little enhancement effect with the doses used (groups A and B). Animals pretreated

with endotoxin and 3 hr later tested with exotoxin showed some enhancement (group C). When rabbits were pretreated with the exotoxin, however, and subsequently (3 hr later) tested with endotoxin, a marked enhancement was observed (group D). When the two toxins were mixed in vitro just prior to the

TABLE II

Skin Reactivity to Streptococcal Pyrogenic Exotoxin (NY-5) on Human Volunteers

Volunteers	Skin reaction to exotoxin (NY-5)	
	10^{-5} μg/intradermally	10^{-6} μg/intradermally
D. W.	45 × 45*	20 × 20*
Y. K.	25 × 25	20 × 20
E. H.	15 × 15	13 × 13
J. B.	15 × 15	15 × 15
R. H.	N.D.‡	5 × 5
J. K.	N.D.	10 × 10
R. S.	N.D.	5 × 5
J. H.	16 × 21	14 × 16

* mm at 24 hr.

‡ N.D., not done.

TABLE III

Cytotoxic Effect of Streptococcal Pyrogenic Exotoxin (NY-5) on Rabbit Spleen Macrophages

Experiment No.	Macrophage migration*		
	Control cultures	Cultures with exotoxin (10 μg/ml)	Cytotoxic index‡
	mean ± SE	*mean ± SE*	
1	284 ± 4.5	127 ± 8.1	0.45
2	307 ± 7.4	141 ± 5.1	0.46
3	324 ± 11.4	140 ± 8.2	0.43

* Migration values are the mean ocular micrometer units ± standard error of the mean measured at 96 hr.

‡ Cytotoxic index $= \frac{\text{Average migration in cultures with toxin.}}{\text{Average migration in controls}}$

injection (group E), the enhancement effect was markedly reduced; therefore, the enhancement was not a simple additive effect.

To analyze the degree of enhancement, a two-factor factorial experiment in complete random design was done. Young adult rabbits, 3 months old, were randomly allocated into 16 groups. 4 different levels of the exotoxin—0.1, 1, 5, and 10 μg/kg—were injected intravenously, and 3 hr later the animals were tested by the intravenous injection of endotoxin at 4 levels—0.1, 1, 5, and 10 μg/kg. Deaths were recorded over a 48 hr period. Additional rabbits of the same age and source were used for titration of LD_{50} of the exotoxin and endotoxin when injected separately.

TABLE IV

Enhanced Susceptibility of Adult Rabbits to Endotoxin Shock by Streptococcal Pyrogenic Exotoxin (NY-5) with Various Combinations

Experimental group	First injection		Interval between injections	Second injection		Results	
	Toxin	Intravenous dose		Toxin	Intravenous dose	Dead/total	Mortality
		μg/kg	hr		μg/kg		%
A	Endotoxin	10	3	Endotoxin	10	0/10	0
B	Exotoxin	10	3	Exotoxin	10	0/10	0
C	Endotoxin	1	3	Exotoxin	10	0/5	0
	"	10	3	"	10	1/5	20
D	Exotoxin	1	3	Endotoxin	5	8/10	80
	"	10	3	"	10	10/10	100
E	Exotoxin	5	0 (mixed in vitro before	Endotoxin	5	1/5	20
	"	5	injection)	"	10	2/5	40
	"	10		"	5	0/5	0
	"	10		"	10	4/10	40

TABLE V

Enhanced Susceptibility of Adult Rabbits to Endotoxin Shock by Streptococcal Pyrogenic Exotoxin

Pretreatment with exotoxin (NY-5) given intravenously	Results (dead/total) Endotoxin (ST 0901) given intravenously 3 hr after exotoxin injection								LD_{50} given intravenously
	0	0.1	1	5	μg/kg 10	250	500	1,000	
μg/kg									μg/kg
0					0/5	0/5	5/9	6/7	500
0.1		0/5	0/5	2/5	3/5				6.9
1		0/5	3/10	8/10	8/10				2.5
5		0/5	5/10	9/10	10/10				1.1
10	0/5	1/10	7/10	9/10	10/10				0.47
100	0/6								
500	2/10								
1000	2/10								
2000	4/10								
5000	6/10								
LD_{50}, μg/kg	3500	>10	5.0	0.38	<0.1				

As shown in Table V, the LD_{50} of the endotoxin alone was 500 μg/kg and of the exotoxin alone 3500 μg/kg. When animals were pretreated with exotoxin 3 hr prior to the injection of endotoxin, susceptibility to lethal shock was markedly increased; with 0.1, 1, 5, and 10 μg of the exotoxin per kg body weight, the LD_{50} of the endotoxins were 6.9, 2.5, 6.1, and 0.47 μg/kg, respectively. The quantitative relationship of this enhancement effect is given in Fig. 4. When

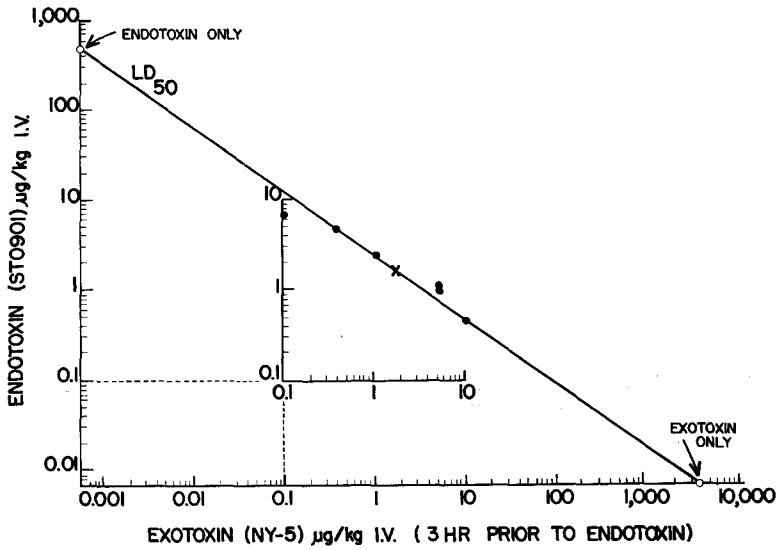

FIG. 4. Quantitative relationship between streptococcal pyrogenic exotoxin (NY-5) and the endotoxin (ST0901) in the enhancement effect of susceptibility to lethal shock in young adult rabbits. The LD_{50} of the data shown in Table V is plotted on a log-log scale (inner) and the linear regression line (least square fits of the data) is extended to the LD_{50} of exotoxin and endotoxin, and then the outer ordinate and abscissa were plotted.

TABLE VI

An Experimental Test of the Quantitative Relationship between Exotoxin and Endotoxin in the Enhancement Effect of Susceptibility to Lethal Shock in Adult Rabbits

Group	Exotoxin (NY-5) given intravenously	Endotoxin (ST0901) 3 hr after exotoxin injection given intravenously	K* Fig. 4	Results	
	X	N	$K = NX$	Dead/ total	Mortality
	µg/kg	µg/kg			%
I	1.5	1.5	2.25	7/10	70
II	50.0	0.045	2.25	5/10	50
III	0.045	50.0	2.25	7/10	70

* $K = NX$ = exotoxin (µg/kg, intravenously) × endotoxin (µg/kg, intravenously, 3 hr after the exotoxin injection).

the LD_{50} is plotted on a log-log scale, a linear relationship pertains. As observed, there is mutual supplementation between the exotoxin and the endotoxin. By the graphic determination it is possible to estimate that the enhancement effect for endotoxin and exotoxin are greater than 100,000 and 1,000,000-fold, respectively. This is a highly quantitative and reproducible observation. If N is the endotoxin in µg/kg and X is the exotoxin in µg/kg injected intravenously

3 hr prior to the endotoxin, $NX = K$, where K is the constant for the LD_{50} dose of the enhancement effect. From the data in Fig. 4 where $N = 1.5$ and $X = 1.5$, K is equal to 2.25. The validity of the relationship ($K = 2.25$) was tested in the designed experiment shown in Table VI. The results are consistent with those predicted from the constant K.

Baby rabbits: Animals highly resistant to endotoxin shock, such as baby rabbits (3 wk of age) were also made susceptible to endotoxin shock after pre-

TABLE VII

Enhanced Susceptibility of Adult Cynomolgus Monkeys to Endotoxin Shock by Streptococcal Pyrogenic Exotoxin

Exotoxin (NY-5) given intravenously	Endotoxin (ST0901) 3 hr after exotoxin injection given intravenously	Results	
		Dead/total	Mortality
μg/monkey	*μg/monkey*		%
2000	None	0/3	0
None	8000	0/5	0
500	2000	7/7*	100

* All died within 24 hr.

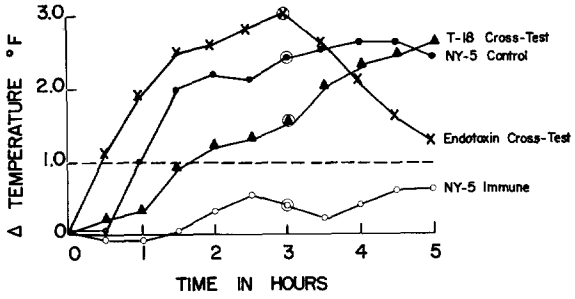

FIG. 5. Streptococcal pyrogenic exotoxin (NY-5) immune rabbits tested with endotoxin (*Salmonella typhosa* 0901) and streptococcal pyrogenic exotoxin (T-18). Each curve represents the mean febrile response of 5–10 rabbits injected intravenously with 10 MPD-3/kg of the toxins.

treatment with exotoxin. An experiment using the two-factor factorial design was performed with baby rabbits. The LD_{50} dose of endotoxin and exotoxin were 4500 μg/kg and >4000 μg/kg, respectively. Intravenous injection of baby rabbits with 10 μg/kg of exotoxin, which is less than 1/400th the LD_{50} dose, increased the susceptibility to endotoxin from an LD_{50} of 4500 μg/kg to 16 μg/kg.

Adult mice: The enhancement effect could also be demonstrated in the mouse but to a lesser extent than in adult rabbits. The LD_{50} dose of endotoxin and exotoxin in the mouse is 310 μg per mouse and 3600 μg per mouse, respectively.

Pretreatment of the mouse with a sublethal dose of exotoxin increased susceptibility to endotoxin shock from an LD_{50} of 310 μg to 50 μg per mouse.

Adult monkey. Adult cynomolgus monkeys were surprisingly resistant to endotoxin lethal shock; animals were refractory to 2000 μg of the exotoxin and 8000 μg of endotoxin when the toxins were injected individually. When the monkeys, however, were pretreated with 500 μg of exotoxin and 3 hr later given

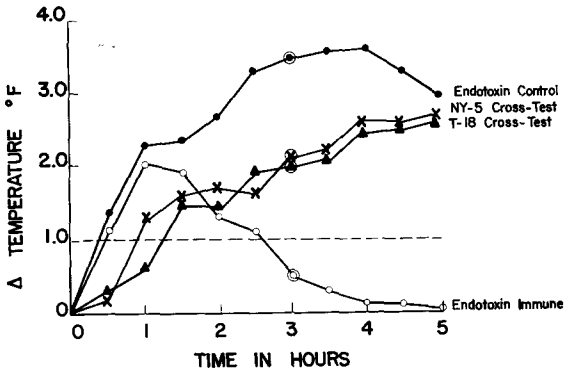

FIG. 6. Endotoxin (*Salmonella typhosa* 0901) immune rabbits tested with streptococcal pyrogenic exotoxins from NY-5 and T-18. Each curve represents the mean febrile response of 5–10 rabbits injected intravenously with 100 MPD-3/kg of endotoxin or 10 MPD-3/kg of exotoxin.

TABLE VIII

Actively Acquired Immunity against the Enhancement Effect of Streptococcal Pyrogenic Exotoxin (NY-5) and Endotoxin (ST0901)

Exotoxin (NY-5) given intravenously	Endotoxin (ST0901) 3 hr after exotoxin injection given intravenously	Results (Dead/total)		
		Normal rabbits	NY-5 immune rabbits	ST0901 immune rabbits
μg/kg	*μg/kg*			
1.5	1.5	7/10	0/10	0/10
10.0	10.0	10/10	3/7	6/8

2000 μg of endotoxin, all of the animals became extremely ill and died within 24 hr (Table VII).

F. Immunogenicity.—Rabbits injected intravenously with small amounts of exotoxin (10 MPD-3/kg) every other day for 2 wk became immune to the toxin. Rabbits made immune to the type A exotoxin (NY-5) and then tested with the type C(T-18) exotoxin and endotoxin (ST0901) showed no cross-immunity, indicating that the exotoxin (NY-5) was not contaminated with endotoxin, and confirms the immunologic specificity of the exotoxins (Fig. 5).

Conversely, rabbits immunized with endotoxin and later tested with exo-

toxins also showed no cross-immunity, again indicating the specificity of the immunity to the exotoxin (Fig. 6). Furthermore, animals immunized against either exotoxin or endotoxin were refractory to the enhancement effect as manifest by their resistance to endotoxin lethal shock (Table VIII).

DISCUSSION

Previously we postulated that Gram-negative bacterial endotoxin and streptococcal pyrogenic exotoxin have two interdependent activities (26, 27, 34, 35). Both toxins have a primary or intrinsic toxicity and, in addition, a secondary toxicity dependent on the immunologic state of the host. We believe that the secondary toxicity depends upon the acquisition of hypersensitivity after infection or exposure to "natural" flora; this could be directed at some common determinant within the toxins or to cross-reacting antigens.

The mechanism of the biological activity of the exotoxin is not known. Originally, Dick and Dick (1) described the Dick test as a method for the detection of human beings susceptible to scarlet fever; they assumed that the skin reactivity was the primary toxicity of the toxin, a concept most frequently accepted by contemporary reviewers. In contrast, Dochez and Stevens (36) emphasized the role of hypersensitivity in the pathogenesis of the Dick test. We believe that these two extreme concepts can be combined into a single mechanism comparable to that proposed for the Gram-negative bacterial endotoxins (27, 35): The exotoxin is produced within the host or in vitro in a complex form closely associated with hyaluronic acid. The hyaluronic acid is not necessary for the primary toxicity but acts only as a carrier. The primary toxicity which can act either alone or interdependently with an acquired hypersensitivity is associated with the heat-labile portion of the low molecular weight protein. This portion of the molecule exists in more than one antigenic form and explains the immunologic specificity that distinguishes at least three toxins A, B, and C (13). The primary toxicity of the exotoxins can be measured by several activities such as pyrogenicity, lethality, cytotoxicity, RES suppression (16), immunosuppression (17), and the most significant of all, the enhancement of susceptibility of experimental animals to other injuries, especially the enhancement of susceptibility to endotoxin shock. The secondary toxicity is an allergic reaction to a heat-stable portion which is antigenically common to all the pyrogenic exotoxins. The host may acquire delayed type hypersensitivity to this component as a result of clinical and subclinical infections and oral and intestinal flora with cross-reacting antigens.

In the absence of specific neutralizing antibodies and hypersensitivity, only the primary toxicity will be manifest and the host will be more resistant to the toxin (26, 34). If the host, however, has acquired hypersensitivity without the formation of neutralizing antibody (antitoxin), the primary toxicity induces within the host hypersusceptibility to hypersensitivity reactions. Here the

primary and secondary toxicities are mutually interdependent and the host becomes highly susceptible to the toxin (26, 27, 35). Either blocking the primary toxicity by neutralization of the toxin with antitoxin, actively or passively, or destroying the primary toxic portion by heat inactivation, reduces or eliminates the hyperreactivity resulting in reduced skin reaction in the host, even in the presence of delayed type hypersensitivity to the toxin. We believe, therefore, that the rash in scarlet fever or the erythema in the Dick test results from the combination of the primary or intrinsic toxicity and the secondary toxicity which is a delayed type hypersensitivity to a heat-stable portion of the toxin. Recently, Hribalova and Schuh (37) have added evidence for a hypersensitivity component in the dermal activity of these exotoxins; development of a positive Dick reaction was inhibited by cortisone comparable to that observed in a local reaction of delayed hypersensitivity. Schuh (38) has also confirmed our observations on the pyrogenic activity of these exotoxins. Therefore, in defining the biological activities of these exotoxins one must consider not only the properties of the toxins, but also the immunologic state of the host—namely, the relative immunity vs. hypersensitivity. For example, two persons could have negative Dick tests but for entirely different reasons. The very young child has acquired neither hypersensitivity nor immunity to the toxin. Here only the primary toxicity would be acting and the absence of hypersensitivity would result in a negative skin reaction; this is the situation prevalent in our young American Dutch rabbits. The absence of erythrogenic activity in the skin of these animals contributed to our failure to associate the pyrogenic exotoxins with erythrogenic toxins in our earlier investigations (11, 12). On the other hand, an adult who has had repeated streptococcal infections and acquired an allergy of infection may also give a negative Dick test if concomitantly he acquired high levels of neutralizing antibody (antitoxin). Here the primary toxicity which results in hypersusceptibility is blocked and the individual will give a negative test to the small amounts of toxin used in the Dick test, even in the presence of hypersensitivity.

The great variability of the immunologic state of the host, which can exist in a population of different ages subjected to diverse environmental conditions, should be apparent; this, of course, was the basis for the concept of streptococcosis as defined by Powers and Boisvert (39) which is so helpful in understanding the pathogenesis of streptococcal infections and their sequelae. If one, therefore, considers the number of variables within the host, as described above, and in addition, recognizes the presence of at least three immunologically distinct toxins (and there are probably more), then one can understand some of the discrepancies and the difficulty in establishing a unified concept for the pathogenesis of the Dick test. Neither is it difficult to understand how the rash, perhaps the least important activity of these exotoxins, obscured for many years the importance of these exotoxins in the pathogenesis of Group A

streptococcal infections and their sequelae. Since the erythema is a secondary toxicity, and fever is a better measure of primary toxicity, we prefer to designate these toxins as streptococcal pyrogenic exotoxins. Furthermore, these toxins are rapidly produced in vivo or in vitro by all Group A streptococcal types tested. It follows, therefore, that the rash is dependent on the immunological state of the host, not on the strains or types of Group A streptococci from which the toxins were derived.

One of the most striking and perhaps significant biological activities of these exotoxins is their ability to enhance the susceptibility of animals to various injuries, especially to endotoxin lethal shock (11–15). The mechanism of the enhancement is not known. The ability of these exotoxins, however, to block the RES (16) and to act as immunosuppressants (17) could account for some of the enhancement effect because it is known that the RES and antibodies play a major role in the elimination of endotoxin (30). Complete suppression of the RES, however, cannot account for the great increase in susceptibility to endotoxin shock brought about by these exotoxins. We have preliminary evidence that the primary mechanism may involve membrane permeability, thereby altering the blood-brain barrier. Because the enhancement effect is evident in baby rabbits and adult mice, where delayed hypersensitivity plays a minor role, it is possible that the enhancement effect of the exotoxins is caused by the primary or intrinsic toxicity alone. The degree of enhancement, however, increases with the acquisition of delayed type hypersensitivity; this implies that the primary and secondary toxicities can be mutually interdependent. Since animals and man have ample opportunity to become exposed and thereby sensitized to both Gram-negative endotoxins and streptococcal exotoxins, it is possible that this mechanism contributes to unknown causes of lethal shock. Because of our observations in rabbits, mice, and monkeys, the clinical implications of combined Group A streptococcal and Gram-negative bacterial infections is apparent. The ubiquity of Gram-negative bacterial endotoxins and their potential for contaminating such products as antilymphocyte immunoglobulins and many other materials purified by column chromatography could result in fatal accidents when administered to patients with clinical or subclinical Group A streptococcal infections.

This potent enhancement effect of streptococcal exotoxins can be blocked in experimental animals by active immunization with specific exotoxin, A, B, or C. Immunization against the toxic component of bacterial endotoxin is also possible (30, 40). Neither is a good antigen, and methods of detoxification and increasing their antigenicity should be resolved. Also, it is necessary to continue the search for new, distinct immunologic types of the streptococcal exotoxins.

Antitoxin immunity may not prevent infection, but it might protect against the immediate as well as the late sequelae of Group A streptococcal infections. Indeed, the acute tissue damage produced by these exotoxins may be necessary

to evoke the postulated autoimmune mechanisms (41) or lesions resulting from cell-associated toxins of the Group A streptococci (14, 42–44).

SUMMARY

Purified pyrogenic exotoxin from Group A streptococcal filtrates (*Streptococcus pyogenes*, type 10, strain NY-5) has been characterized primarily as a protein complexed with hyaluronic acid. Amino acid composition and analysis revealed a typical acidic protein with an average molecular weight of 29,000. The purified exotoxin was free of streptolysins O and S, nicotinamide adenine dinucleotidases (NADases), deoxyribonucleases (DNases), mucopeptide, and endotoxins. The biological activity was destroyed when the exotoxin was heated at 65°C for 30 min or boiled for 2 min.

The biological activities investigated were pyrogenicity in rabbits (minimal pyrogenic dose–3 hr, 0.07 μg/kg), lethality in rabbits (LD_{50}, 3500 μg/kg), skin test dose in human skin (> 10^9 skin test doses, per mg toxin), cytotoxicity of rabbit spleen macrophage (Cytotoxic Index 0.5–10 μg/ml), enhancement of susceptibility to endotoxin shock (in rabbits >100,000-fold), and antigenic analysis (A-type toxin).

The exotoxin was immunogenic and it was possible, therefore, to immunize animals against the various toxic activities. The immunity was specific for the A-type toxin.

The clinical implications of the highly significant enhancement effect of these exotoxins are discussed. It is suggested that clinical or subclinical infection with Group A streptococci could prepare the host for fatal shock from Gram-negative infections or the inadvertent injection of small amounts of Gram-negative bacterial endotoxins.

We are grateful for the assistance of Mr. David Peterson for the analyses given in Table I

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