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Interactions of Human Neutrophils with Leukotoxic Streptococci

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Most strains of *Streptococcus pyogenes* contain a toxin which can kill neutrophils. Previous workers failed to show any correlation between leukotoxin content and virulence for animals or humans. We examined the in vitro interactions of a leukotoxic streptococcus and a nonleukotoxic variant with human neutrophils. At ratios of 200 streptococcal colony-forming units per neutrophil, the toxic strain killed 92.8 ± 2.0% of neutrophils, and the nontoxic strain killed only 9.0 ± 1.2%. Despite this, ingestion of the two strains was equal. Postphagocytic oxidative metabolism was equivalent with low numbers of either toxic or nontoxic streptococci but depressed with high numbers of leukotoxic streptococci. At 120 min, neutrophils were able to kill leukotoxic (99.6 ± 0.3% killed) and nonleukotoxic streptococci (99.5 ± 0.2% killed) equally efficiently ($P = 0.42$). Thus, leukotoxicity does not interfere with the ability of neutrophils to destroy streptococci. This may explain why leukotoxicity does not appear to be an important factor in streptococcal virulence.

Most strains of *Streptococcus pyogenes* (group A, beta-hemolytic streptococci) contain a leukotoxic factor. When organisms containing large quantities of this factor are ingested by or come in contact with human polymorphonuclear neutrophils (PMN), the neutrophils are destroyed (11, 23). Death of the PMN is associated with intracytoplasmic rupture of PMN granules; this is thought to be the leukocidal mechanism (8). The leukotoxic factor has been identified by previous workers as cell-bound streptolysin S (SLS) (14).

Despite the potent and dramatic action of the toxin, a number of studies have shown no correlation between leukotoxic activity of strains of streptococci and their virulence in humans or animals (10, 15, 17, 19, 20).

To understand this paradox, we carefully examined the interaction of human PMN with two closely related streptococcal strains which differed principally in their content of SLS and hence their capacity to kill PMN. We examined PMN bactericidal activity and those PMN functions associated with bactericidal capacity, including phagocytosis, oxygen consumption, hexose monophosphate (HMP) shunt activity, protein iodination, and degranulation.

MATERIALS AND METHODS

Bacteria. *S. pyogenes* strains C203S and C203U were obtained from the American Type Culture Collection, Rockville, Md. The parent strain (C203) of the test organisms was originally isolated from a patient with scarlet fever. Strain C203S is an M protein-poor (13), streptolysin S-rich organism (14). Its derivative C203U is also M protein poor, but lacks streptolysin S and is therefore not leukotoxic (14).

On standard sheep blood agar plates only strain C203S was beta hemolytic on the plate surface, indicating the presence of SLS. Both strains were beta hemolytic when inoculated under the agar surface, confirming the presence of the oxygen-inhibitable streptolysin O.

Although strain C203U does contain streptolysin O this strain was not leukotoxic in these aerobic studies. The leukotoxicity of strain C203S was inhibited by trypan blue but not by cholesterol. In addition, there was no release of a leukotoxic factor into the supernatant fluid of bacterial cultures, indicating that the leukotoxicity in these assays was due to cell-bound SLS rather than to streptolysin O (14). Hemolytic activity was quantitated aerobically by the method of Ginsberg et al. (7). There was one hemolytic unit in 2 × 10^5 colony-forming units (CFU) of C203S and in 4 × 10^8 CFU of C203U.

Early stationary-phase (5-h) brain heart infusion cultures of both strains were used. For the quantitation of phagocytosis, ^{14}C-labeled bacteria were prepared by adding 40 μCi of ^{14}C-labeled L-amino acids (New England Nuclear Corp., Boston, Mass.) to 15 ml of the bacterial culture for the final 3 h of incubation.

Bacteria were washed in phosphate-buffered saline and resuspended in phosphate-buffered saline containing activation mixture yielding the following final concentrations: 9 mM glucose, 6 mM $MgSO_4$, and 2 mM cysteine. This mixture enhances the activity of the toxin (14). The leukotoxic factor could be inactivated by heating washed suspensions of *S. pyogenes* to 56°C for 20 min. Heating decreased bacterial viability by 99%.

Opsonized bacteria were prepared by tumbling washed suspensions of the bacteria with 20% fresh normal human serum in phosphate-buffered saline at 12 rotations per min for 30 min at 37°C followed by

centrifugation at 27,000 × g for 15 min and suspension in phosphate-buffered saline with activation mixture.

Bacterial suspensions were adjusted to desired concentrations by using a Gilford 2400 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) at 580 μm. Bacterial counts were performed in a Petroff-Hauser chamber and verified with serial dilution and plate counts on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) containing 0.4% defibrinated sheep blood. Microscopic examination showed that there were 5.5 ± 0.5 (mean ± standard error of the mean) leukotoxic streptococci per chain and 7.0 ± 0.4 nonleukotoxic streptococci per chain. One CFU was equivalent to one chain.

Neutrophils. Human PMN were obtained from normal whole venous blood by sedimentation in an equal volume of heparinized (10 U/ml; Connaught Laboratories, Willowdale, Ontario, Canada) 3% dextran 70 in normal saline (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) for 1 h at 22°C at a 60° angle. The leukocyte-rich supernatant fluid was removed and centrifuged at 200 × g for 10 min at 22°C. For assays of oxygen consumption, leukotoxicity, HMP activity, and iodination, erythrocytes were lysed with 3 ml of iced 0.22% heparinized (10 U/ml) saline. Isotonicity was restored after 45 s with 0.88 ml of 3% saline followed by 5 ml of Hanks balanced salt solution (HBSS; Microbiological Associates, Bethesda, Md.). Centrifugation at 200 × g was repeated, and the pellet was resuspended in HBSS or Ca^{2+}-free Krebs-Ringer phosphate buffer. The resulting leukocyte suspensions contained 75 to 85% PMN and 15 to 25% mononuclear leukocytes. For certain experiments, 95% pure PMN were obtained from normal human venous blood by Ficoll (Sigma Chemical Co., St. Louis, Mo.)-Hypaque (Winthrop Laboratories, New York, N.Y.) separation (2) followed by dextran sedimentation and hypotonic lysis of the remaining erythrocytes.

Assays of leukotoxicity. (i) Phase-contrast microscopy. PMN monolayers were prepared by allowing 0.2 ml of human venous blood to clot on a cover slip for 30 min at 37°C. The clot was washed off with warm HBSS, and the cover slip was inverted onto 0.1 ml of a suspension of 5 × 10^8 CFU of leukotoxic or nonleukotoxic streptococci per ml of HBSS with 10% fresh autologous serum. The preparation was then observed and photographed on a temperature-controlled stage of a Zeiss Photomicroscope with phase optics fitted with a 16-mm movie camera and stroboscopic flash.

(ii) Trypan blue uptake by PMN as an indication of PMN death. PMN (2.5 × 10^6) obtained by Ficoll-Hypaque sedimentation and streptococci (5 × 10^8 CFU) were suspended in HBSS and centrifuged at 200 × g for 15 min at room temperature to prepare a button. After incubation of the button at 37°C for 30 min, the supernatant fluid was decanted and frozen for later granule enzyme analysis (see below). Trypan blue (0.5% in 0.9% saline) was added to the button, and viable PMN were identified by trypan blue exclusion.

To test leukotoxicity in suspensions, 0.4 ml of Ca^{2+}-free Krebs-Ringer phosphate buffer with 1,000 nmol of glucose containing 4 × 10^7 PMN/ml were placed in 20-ml glass vials, and 0.1 ml of opsonized leukotoxic or nonleukotoxic streptococci in phosphate-buffered saline was added to the vials. The vials were then placed in a 37°C shaker bath at 20 rpm for 30 min; 0.5 ml of 1% trypan blue in 0.9% saline was added, and the percentage of PMN excluding the stain from the nucleus was enumerated.

Samples were also taken for measurements of trypan blue exclusion from O_2 consumption and bactericidal experiments (see below).

Virulence in mice. White male mice (strain DUB-ICR; Flow Laboratories, Inc., Rockville, Md.) were injected either intraperitoneally (0.3 ml) or intravenously (0.1 to 0.15 ml) with leukotoxic or nonleukotoxic streptococci. The 48-h 50% lethal dose and confidence limits were calculated by the method of Litchfield and Wilcoxon (12).

Phagocytosis of streptococci. Ingestion of streptococci was evaluated by three techniques. (i) Preparations were examined with phase-contrast microscopy to determine whether cell-associated organisms were ingested. (ii) Removal of viable organisms from the supernatant of a bacteria-PMN suspension was determined by using differential centrifugation and serial dilution methods described below. (iii) Neutrophils (2.4 × 10^6/ml) in HBSS were mixed with ^{14}C-labeled leukotoxic streptococci, heated leukotoxic streptococci, or nonleukotoxic streptococci at 1 to 200 CFU/PMN in polypropylene tubes (12 by 75 mm; Falcon Plastics, Oxnard, Calif.) at a final volume of 3 ml with or without 10% fresh autologous serum. The tubes were tumbled at 12 rpm at 37°C, and at 0, 5, 10, and 20 min, 0.2-ml samples were placed in 4 ml of HBSS (with 10 U of heparin/ml) and centrifuged at 200 × g for 5 min. The supernatant was discarded, and the pellet was washed again as above. The final pellet was suspended in 0.1 ml of protosol (New England Nuclear Corp.). Brays scintillation fluid (2.5 ml) was added, and the mixture was poured into a glass scintillation vial and counted in a liquid scintillation counter (model LS250, Beckman Instrument Co., Inc., Fullerton, Calif.). The results are expressed as the percentage of bacteria that were cell associated.

PMN oxidative metabolism. (i) Leukocyte oxygen consumption. Neutrophils (6 × 10^6) and 9.6 × 10^7 streptococcal CFU were incubated at 37°C in 3 ml of HBSS with 10% serum or no serum for 8 to 10 min in the chambers of a polarographic oxygen monitor (model 53; Yellow Springs Instrument Co., Yellow Springs, Ohio). The oxygen consumption of PMN incubated alone and streptococci incubated alone was measured under each set of experimental conditions. The results were reported as the maximal rate of O_2 consumed per hour after the consumption by the bacteria alone was subtracted.

(ii) HMP. Reactions were carried out in 20-ml glass vials containing 5 × 10^6 PMN, 5 × 10^8 opsonized streptococcal CFU, 0.05 ml of [1-^{14}C]glucose (2 μCi/ml; New England Nuclear Corp.), and 1,000 nmol of nonradioactive glucose in a total volume of 0.5 ml of Ca^{2+}-free Krebs-Ringer phosphate buffer. The vials were stoppered, and the stoppers were pierced by a center well (Kontes Glass Co., Vineland, N.J.) containing a strip (2 by 0.5 cm) of filter paper previously saturated with fresh 10% KOH.

The reaction was initiated by adding 0.1 ml of the streptococcal suspension to the vial with a syringe fitted with a 20-gauge needle. The vials were incubated in a shaking 37°C water bath for 30 min. The reaction

was stopped, $^{14}CO_2$ was released by addition of 0.2 ml of 2 N H_2SO_4 with a needle and syringe to each vial, and the solution was incubated for an additional 45 min at 37°C. The strips of filter paper and the center well were then placed in 10 ml of scintillation fluid (4% Liquiflor [New England Nuclear Corp.], 66% toluene, 30% absolute methanol) and counted in a scintillation counter.

(iii) Iodination of protein. A modification of the method of Klebanoff was employed (9) for iodination of bacteria. PMN (5×10^6) and 5×10^8 streptococcal CFU were incubated in 0.5 ml of Ca^{2+}-free Krebs-Ringer phosphate buffer plus 0.10 μCi of $Na^{125}I$ (New England Nuclear Corp.) and nonradioactive NaI at a final total concentration of 0.08 mM. The samples were tumbled at 12 rpm for 30 min at 37°C, and the reaction was stopped by the addition of 0.1 ml of 0.01 M sodium thiosulfate. Iodinated protein was precipitated and washed 3 times with 1 ml of 10% trichloroacetic acid, with centrifugation of the protein suspension at 2,000 × *g* for 5 min after each trichloroacetic acid addition. The final button was taken up in 1 ml of 10% trichloroacetic acid and counted in a gamma counter (Beckman Biogamma II; Beckman Instruments, Inc., Fullerton, Calif.).

Degranulation. Preparations were prepared as described below.

Beta-glucuronidase activity was assayed by determination of the generation of phenolphthalein from phenolphthalein-glucuronic acid (5).

Lysozyme was quantitated by measurement of changes in the optical density of a suspension of *Micrococcus lysodeikticus* (16) after addition of the granule preparation.

Enzyme activity released into the surrounding medium was compared to enzyme released from PMN after disruption with 0.1% Triton X-100.

PMN bactericidal activity. Streptococci at ratios of 1 to 100 CFU/PMN were added to 2×10^7 PMN in 4 ml of HBSS with 10% normal autologous serum or without serum. The mixtures were tumbled at 12 rpm at 37°C in polypropylene tubes (12 by 75 mm) for 120 min. Samples were removed after 0, 30, 60, and 120 min. Total counts of viable bacteria were performed after hypotonic lysis of the PMN and serial dilution and pour plate counts. Samples (0.5 ml) were taken from mixtures of the 1-CFU/PMN samples, placed in 4.5 ml of normal saline, and centrifuged at 200 × *g* for 5 min. Serial dilutions and pour plate counts were done on the supernatant and on the pellet after hypotonic lysis to determine numbers of viable cell-free and cell-associated bacteria.

Statistical analyses. Data were analyzed with Student's *t* test (unpaired data). Results are expressed as mean ± standard error of the mean.

RESULTS

Assays of leukotoxicity. (i) Phase-contrast microscopy. About 1 PMN out of 10 that ingested a single chain of leukotoxic streptococci (5 to 7 bacteria) showed obvious morphological signs of damage. The first manifestation of leukotoxicity was loss of normal motility. The PMN then became round, cytoplasmic blebs formed, and granules disappeared. Finally, the cell, including the nucleus, swelled, and all other internal structures except for the ingested organisms disappeared. If the PMN survived ingestion of the first chain of streptococci, ingestion of more bacteria frequently resulted in death (Fig. 1). When trypan blue was added to preparations after interaction with leukotoxic streptococci, all PMN that were morphologically damaged took up trypan blue. In addition, occasional normal-looking PMN that had ingested streptococci took up trypan blue.

(ii) Trypan blue uptake by PMN as an indication of PMN death. The trypan blue uptake experiments quantitated leukotoxicity when the bacteria and PMN were in direct contact in a pellet as in the degranulation experiments. Leukotoxic streptococci spun into a pellet with PMN in the absence of serum at a ratio of 200 CFU/PMN killed 92.8 ± 2.0% (mean ± standard error of the mean) of the PMN in 30 min. Nonleukotoxic streptococci killed only 9.0 ± 1.2% of the PMN, and heated leukotoxic bacteria killed only 8.0 ± 0.9% of the PMN. There were 3.4 ± 0.9% dead PMN in preparations that had no streptococci.

Other experiments quantitated leukotoxicity in suspensions of opsonized bacteria. Figure 2 shows the relationship of leukotoxicity to the streptococcal CFU/PMN ratio. These were the same conditions used for assays of HMP shunt and protein iodination (see below).

Virulence in mice. Virulence in mice of the leukotoxic and nonleukotoxic strains was equivalent when the bacteria were injected intraperitoneally. After intravenous injection both strains were significantly less virulent, but the toxic strain was more virulent than the nontoxic strain (Table 1).

Phagocytosis of streptococci. The phagocytosis experiments examined the ability of PMN to phagocytize leukotoxic and nonleukotoxic streptococci. Phase-contrast microscopy confirmed the intracellular location of nearly all cell-associated organisms (Fig. 1). PMN that were morphologically damaged did not continue to ingest bacteria.

After 120 min of incubation at 1 CFU/PMN equal numbers of leukotoxic (99.8 ± 0.1%) and nonleukotoxic streptococci (99.8 ± 0.1%) were removed from the supernatant ($P = 0.389$).

Experiments done with ^{14}C-labeled bacteria showed that at 1 CFU/PMN the leukotoxic and nonleukotoxic strains became equally cell associated in the presence of serum (Fig. 3). At high CFU/PMN ratios (70 to 200) equal numbers of leukotoxic (4.0 ± 1.0%) and nonleukotoxic (3.5 ± 0.7%) bacteria became cell associated by 20 min ($P = 0.366$). Neither the leukotoxic nor the

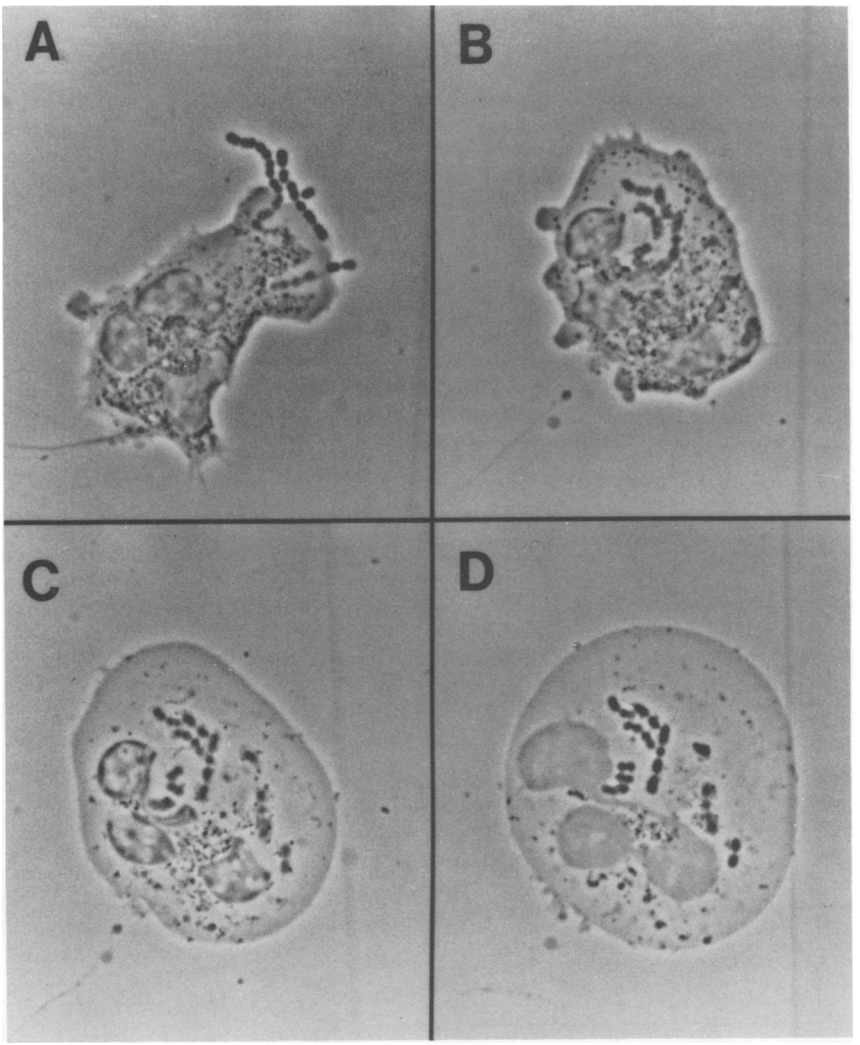

FIG. 1. *Phase-contrast micrographs of leukotoxic action of streptococci on human PMN. Elapsed time, 2 min 30 s. (A) PMN engulfing several chains of leukotoxic streptococci. (B) PMN has ceased moving. It has assumed a rounded shape and cytoplasmic blebs are being extended. (C) PMN is now swollen and immobile. (D) Most internal structures have disappeared, and nucleus is swollen.*

nonleukotoxic strain was cell-associated in the absence of serum. However, heated leukotoxic streptococci became cell associated readily without serum (Fig. 4). Heated nonleukotoxic streptococci were also cell associated in the absence of serum.

PMN oxidative metabolism. The oxidative metabolism experiments examined the effect of interactions of leukotoxic and nonleukotoxic streptococci with PMN on aspects of oxidative metabolism related to bactericidal activity.

(i) Oxygen consumption. PMN ingestion of unheated and heated leukotoxic streptococci stimulated equivalent bursts of oxygen consumption ($P = 0.150$). In addition, ingestion of either leukotoxic or nonleukotoxic streptococci stimulated equivalent bursts of oxygen consumption ($P = 0.058$) (Table 2).

(ii) HMP shunt. PMN HMP shunt activity was equivalently stimulated by the leukotoxic and nonleukotoxic strains at ratios of up to 50 CFU/PMN. However, as the numbers of leukotoxic streptococci were increased to a level that caused marked PMN damage, the shunt activity of the PMN decreased. No such decrease was noted with the nontoxic streptococci (Fig. 5). At 100 CFU/PMN the amount of HMP shunt activity was significantly lower for those PMN incubated with leukotoxic streptococci compared with PMN incubated with nonleukotoxic

streptococci, heated toxic streptococci, or toxic streptococci with no SLS activation mixture ($P < 0.001$ for all cases) (Fig. 6).

(iii) Iodination of protein. PMN iodinate protein by interactions of H_2O_2, myeloperoxidase, and iodide. This reaction is thought to kill bacteria by iodinating vital surface proteins. There was good correlation between PMN bactericidal capability and ability to iodinate protein in vitro. In the in vitro test all available appropriate proteins were iodinated, including PMN, serum, and bacterial proteins (9).

Protein iodination by PMN was equivalent after interaction with the leukotoxic and nonleukotoxic strains at ratios of up to 50 CFU/PMN. However, as the numbers of leukotoxic streptococci were increased to a level that caused marked PMN damage, protein iodination decreased. No such decrease was noted with the nontoxic streptococci (Fig. 7). At a ratio of 100 CFU/PMN the amount of protein iodination by PMN incubated with leukotoxic streptococci was significantly lower than that for PMN incubated with nontoxic streptococci, heated toxic streptococci, or toxic streptococci with no SLS activation mixture ($P = 0.007$, 0.002, and 0.003, respectively) (Fig. 8).

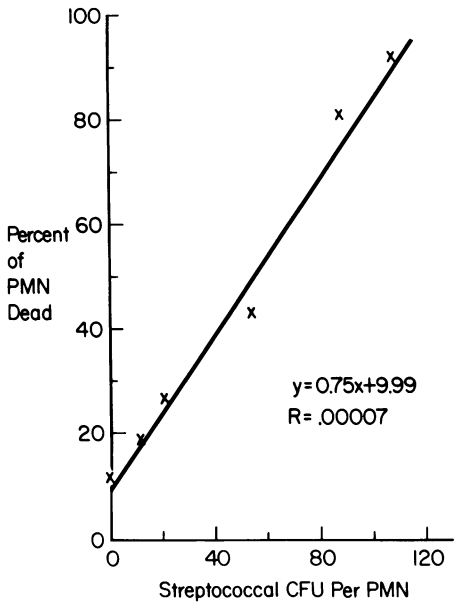

FIG. 2. *Relationship of neutrophil death to the numbers of leukotoxic streptococci per PMN. PMN were incubated with leukotoxic streptococci at varying CFU/PMN ratios. PMN death was determined by trypan blue exclusion after 30 min of incubation; non-phagocytizing PMN viability was 89.8 ± 3.5%.*

Degranulation. The phenomenon of degranulation is also related to PMN bactericidal activity. Since the streptococcal toxin causes granules to rupture, this is also an indication of damage to PMN. The leukotoxic streptococci stimulated significantly more release of beta-glucuronidase from PMN, compared with both the nontoxic and heated toxic strains ($P = 0.008$ and 0.036, respectively). There was also a significantly greater release of lysozyme by leukotoxic streptococci when compared with nonleukotoxic streptococci ($P = 0.019$) (Table 3).

PMN bactericidal activity. At ratios of 1 and 10 CFU/PMN both the leukotoxic and nonleukotoxic strains were killed equally efficiently by 120 min ($P = 0.42$ and 0.41, respectively). At a ratio of 100 CFU/PMN bactericidal activity was poor but equivalent for leukotoxic and nonleukotoxic strains ($P = 0.33$) (Table 4 and Fig. 9).

Incubation of the bacteria with 10% fresh normal serum in the absence of PMN confirmed that neither strain was serum sensitive. Streptococci did not grow in the test medium. Colony counts over a 2-h period in experiments without PMN showed no significant change.

DISCUSSION

The dramatic nature of the leukotoxicity of streptococci has attracted the attention of investigators over the years (11, 14, 23). A single phagocytized chain of *S. pyogenes* containing the leukotoxic factor can kill a neutrophil within 3 min of ingestion (23). The leukotoxic factor is cell-bound SLS (14).

Animal studies suggest that the presence of

TABLE 1. *Virulence in mice*[a]

Injection route	Virulence at 48 h (CFU)[b]					
	Leukotoxic streptococci			Nonleukotoxic streptococci		
	LD_{50}	Upper limit	Lower limit	LD_{50}	Upper limit	Lower limit
Intraperitoneal	4.3×10^7	11.3×10^7	1.6×10^7	4.3×10^7	11.3×10^7	1.6×10^7
Intravenous	2.3×10^8	3.2×10^8	1.6×10^8	3.9×10^8	4.8×10^8	3.1×10^8

[a] Mice were given intraperitoneal or intravenous injections of either leukotoxic or nonleukotoxic *S. pyogenes*.
[b] Fifty percent lethal doses (LD_{50}) and 95% upper and lower confidence limits were calculated by the method of Litchfield and Wilcoxon (12).

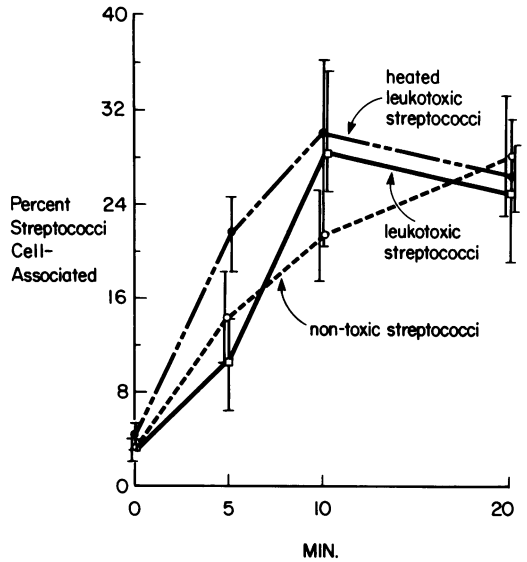

FIG. 3. *UPtake of streptococci by PMN in 10% serum. PMN were incubated with ^{14}C-labeled leukotoxic streptococci, nontoxic streptococci, or heated leukotoxic streptococci. The percentage of streptococci associated with cells was determined by performing ^{14}C counts on the washed PMN pellet.*

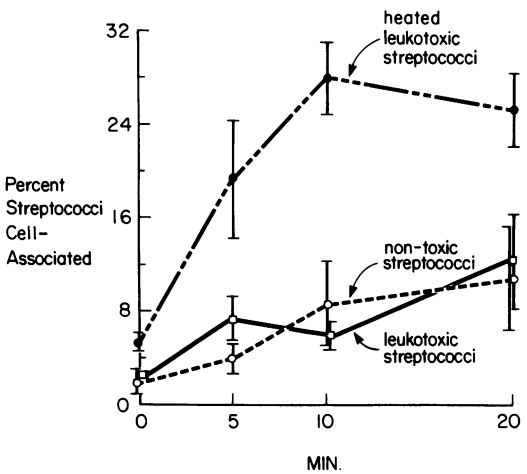

FIG. 4. *Uptake of streptococci by PMN in the absence of serum. PMN were incubated with ^{14}C-labeled leukotoxic streptococci, nontoxic streptococci, or heated streptococci.*

the leukotoxic factor does not directly correlate with the virulence of the organism. In 1921, Stevens et al. (19) observed that streptococci in which virulence had been increased by mouse passage did not produce more "hemolysins." Snyder and Hamilton (17) were able to increase SLS content in three of four group A streptococcal strains by 8 to 10 mouse passages, but these passages did not affect virulence. Leedom and Barkulis (10) were successful in increasing the virulence of a strain of group A streptococcus after 22 and 43 mouse passages. The two derived strains, although more virulent, had less SLS than did the parent strain. Stollerman and Bernheimer (20) reported that the SLS production in strains of *S. pyogenes* that caused rheumatic fever and those that caused only pharyngitis were comparable. Similarly, Potter and Moran (15) found no significant difference in SLS content of nephritic and nonnephritic M-type 12 streptococci.

M protein, which functions as an antiphagocytic substance, is a potent virulence factor for *S. pyogenes* (10, 21). M protein attached to bacteria is not leukotoxic (1). Although it would be interesting to find out if there is any synergistic activity between SLS and M protein, such

TABLE 2. *Oxygen consumption*[a]

Strain	O_2 consumed (μl/h/10^6 PMN)
Leukotoxic streptococci	32.9 ± 3.7 (13)
Nonleukotoxic streptococci	22.3 ± 5.3 (14)
Heated leukotoxic streptococci	43.9 ± 14.3 (5)
No bacteria	7.9 ± 0.9 (38)

[a] PMN (6×10^6) were incubated with 9.9×10^7 streptococcal CFU for 8 to 10 min, and the maximal rate of oxygen consumption was measured in a polarographic oxygen monitor. At this CFU/PMN ratio, 90% of the PMN remained viable. The O_2 consumption by bacteria alone was subtracted. Data are expressed as mean ± standard error of the mean (*n*). There was no significant difference between the rate of O_2 consumption by PMN with unheated and heated leukotoxic streptococci ($P = 0.150$). There was no significant difference between the rate of O_2 consumption by PMN with leukotoxic and nonleukotoxic streptococci ($P = 0.058$).

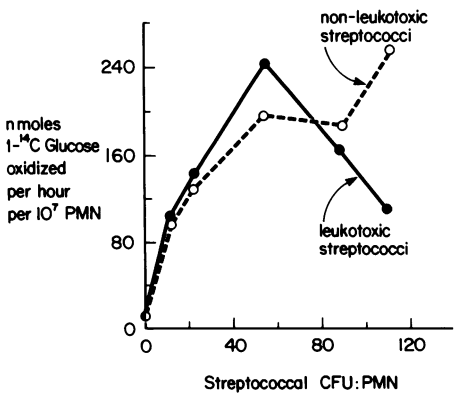

FIG. 5. *PMN HMP shunt activity. Oxidation of [1-^{14}C]glucose by PMN was determined at various ratios of CFU/PMN.*

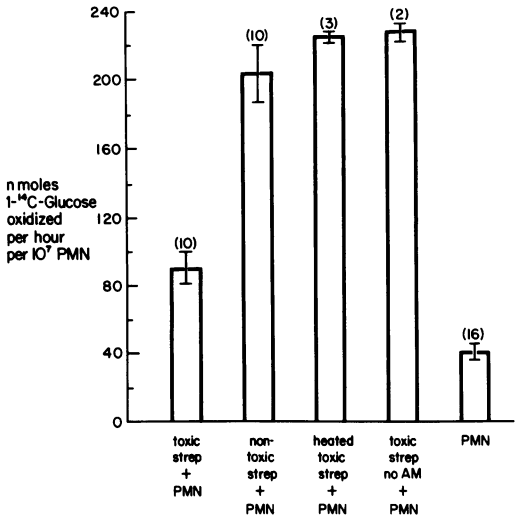

FIG. 6. *PMN HMP shunt activity after incubation with 100 CFU/PMN. Oxidation of [1-^{14}C]glucose by PMN was determined after incubation of neutrophils with streptococci (strep) as indicated on the graph. AM is activation mixture for SLS. Toxic, nontoxic, and heated toxic streptococci alone oxidized less than 12 nmol of [1-^{14}C]glucose per h. Numbers of experiments are shown over bars.*

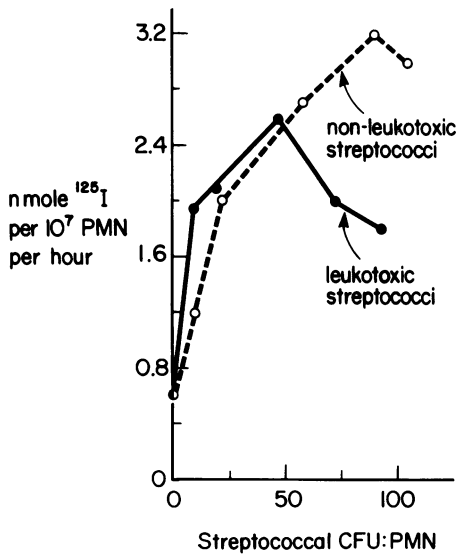

FIG. 7. *Iodination of protein by PMN incubated with streptococci. ^{125}I associated with trichloroacetic acid-precipitable protein was quantitated after incubating PMN with various numbers of either leukotoxic or nonleukotoxic streptococci.*

studies would be complicated by the antiphagocytic nature of M protein. For M protein-positive strains to be ingested so that oxidative activity, degranulation, or bactericidal capacity can be examined, the M protein-positive strains have to be first opsonized with specific antibody or the M protein has to be destroyed by other means.

The M protein-negative strains C203U and C203S were relatively avirulent for mice after either intravenous or intraperitoneal injection. Other investigators found that M protein-positive strains were lethal for mice after repeated passage in mice in numbers as low as 10^1 or 10^2 organisms (4). The slightly greater virulence of the leukotoxic strain compared with the nonleukotoxic strain when given intravenously was possibly related to hemolysis.

Careful examination of the interactions of the streptococcal strains with human neutrophils showed that leukotoxicity did not influence the ability of neutrophils to ingest the organisms. This is compatible with the data of Snyder and Hamilton (18) who found that RNA-bound SLS did not affect ingestion of heat-killed streptococci.

Both leukotoxic and nonleukotoxic streptococci equally stimulated the PMN postphagocytic metabolic burst. We noted divergent activity only when high ratios of bacteria to neutrophils were utilized. The effects of leukotoxicity could be noticed as a diminished neutrophil metabolic response due to the death of large

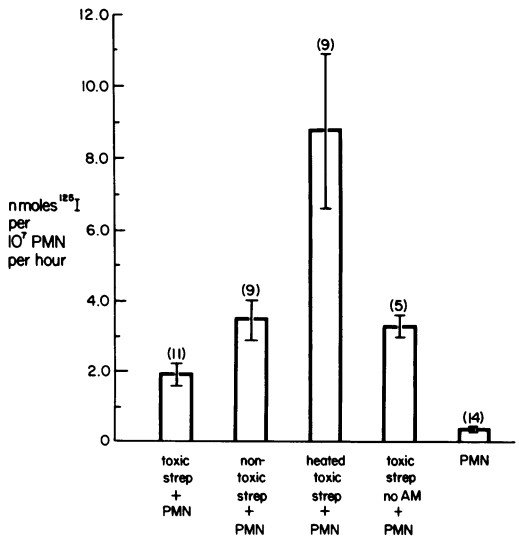

FIG. 8. *Iodination of protein by PMN after incubation with 100 CFU/PMN, which was lethal for more than 80% of PMN. ^{125}I associated with trichloroacetic acid-precipitable protein was quantitated after incubating PMN with streptococci. AM is activation mixture for SLS. Without PMN, leukotoxic streptococci, nontoxic streptococci, and heated toxic streptococci resulted in less than 0.2 nmol of ^{125}I associated with protein. Numbers of experiments are shown over bars.*

TABLE 3. *Percentage of granule enzymes released from PMN*[a]

Culture	% of enzyme released	
	Lysozyme	Beta-glucuronidase
Leukotoxic streptococci plus PMN	46.1 ± 5.5 (18)	24.7 ± 3.1 (18)
Nonleukotoxic streptococci plus PMN	15.0 ± 0.8 (3)	3.8 ± 0.6 (3)
Heated leukotoxic streptococci plus PMN	24.8 ± 0.6 (2)	6.1 ± 0.5 (2)
PMN alone	12.1 ± 2.1 (8)	6.1 ± 2.8 (8)

[a] Streptococcal CFU (5×10^8) were incubated with 2.5×10^6 PMN, and release of granule enzyme into media (as percentage of that released by 0.1% Triton X-100) was determined. Data are expressed as mean ± standard error of the mean (n). Leukotoxic streptococci induced a significantly greater release of lysozyme ($P = 0.019$) than did nonleukotoxic streptococci and a significantly greater release of beta-glucuronidase than both nonleukotoxic and heated toxic streptococci ($P = 0.008$ and 0.036, respectively). Neither strain of streptococcus had measurable endogenous lysozyme or beta-glucuronidase activity.

TABLE 4. *Percentage of streptococci killed by PMN*[a]

Strain	% of streptococci killed at CFU/PMN ratio:		
	1:1	10:1	100:1
Leukotoxic streptococci	99.5 ± 0.2 (5)[b]	63.6 ± 23.0 (5)	30.7 ± 9.8 (3)
Nonleukotoxic streptococci	99.6 ± 0.3 (3)	69.8 ± 16.8 (8)	20.5 ± 16.0 (5)

[a] Human PMN and leukotoxic or nonleukotoxic streptococci were incubated together at various ratios. Numbers of streptococcal CFU were determined by serial dilution and pour plate colony counts. Percent killing was determined for each experiment and then mean ± standatd error of the mean was calculated.

[b] Percentage of bacteria killed in 2 h, mean ± standard error of the mean (n).

numbers of the phagocytic cells. However, both the HMP shunt and iodination experiments indicate less PMN dysfunction than was predicted by the trypan blue estimates of PMN death. For example, at a ratio of 50 CFU/PMN after 30 min of incubation, 50% of the PMN were dead by trypan blue exclusion, but HMP shunt and protein iodination were still equivalent for the toxic and nontoxic streptococci. This could be explained by assuming that the metabolic burst occurred before death of the PMN. Large numbers of leukotoxic bacteria caused greater release of granular enzymes, thus reflecting the effects of the leukotoxin on the granules of the phagocytic cell (8, 22, 23).

There is evidence that heating destroyed more than SLS. Heated bacteria were cell associated even in the absence of serum, whereas neither living strain became cell associated without serum. Also, the heated strain was iodinated more avidly than both the living strains.

Knowledge of the ability or inability of PMN to kill leukotoxic streptococci is crucial to our understanding of neutrophil-streptococcus interactions. At low ratios of bacteria to neutrophils, both leukotoxic and nonleukotoxic organisms were killed efficiently. Wilson (23) observed that leukotoxic streptococci sometimes proliferated within the destroyed neutrophils, but sometimes they did not. He assumed that the latter instance represented killing of streptococci by PMN. When numbers of bacteria per neutrophil were increased so that a high percentage of neutrophils were destroyed by the leukotoxic factor, bacteria were not killed efficiently. However,

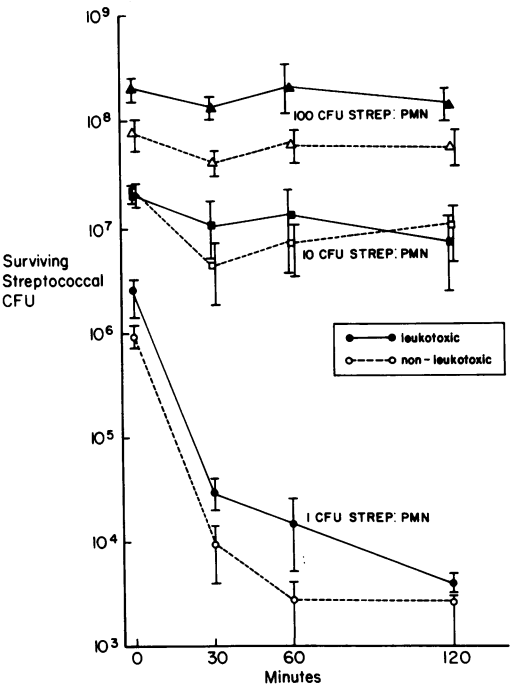

FIG. 9. *PMN bactericidal activity for streptococci. Human PMN and leukotoxic or nonleukotoxic streptococci were incubated together at varying ratios. Numbers of CFU were determined by serial dilution and pour plate colony counts. The results are reported as the percent survival of initial streptococcal inoculum. Mean ± standard error of the mean for at least three determinations for each point are shown. There was no significant difference between the bactericidal capacity of PMN for the two strains.*

even in the absence of the leukotoxic factor, those large numbers of bacteria could not be efficiently killed by normal neutrophils. Thus, we could not demonstrate a diminution in vitro of neutrophil bactericidal activity caused by or associated with streptococcal leukotoxin.

In vitro studies of leukotoxicity of streptococci suffer from several potential problems. As Wilson observed, the leukotoxicity of streptococcal chains in a culture does vary from chain to chain (23), and this variation may be different from that encountered in vivo. In addition, leukotoxicity does vary in vitro (14) and probably also in vivo according to the age of the microbe. Leukotoxicity could also be effected in vivo by PMN heterogeneity. Observation of pharyngeal smears from patients with pharyngitis demonstrated disrupted neutrophils in patients with streptococcal pharyngitis much more frequently than in patients with pharyngitis associated with other etiologies (3). This suggests that leukotoxicity does occur in the pharyngeal mucosa.

Does leukotoxicity confer any selective advantage for the organism? Theoretically, one could envision a situation in which large numbers of streptococci destroy phagocytic cells before those cells are able to destroy the bacteria. However, our in vitro data did not support the concept of leukotoxicity being an important factor in preventing bacterial destruction by neutrophils.

It appears that leukotoxicity is not an important virulence factor. Although PMN may be injured or killed by the leukotoxic streptococci, the streptococci are also killed. This is probably best explained by the ability of PMN to rapidly kill the ingested bacteria before the phagocytes succumb to the leukotoxic bacteria.

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