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Cytotoxicity and Inhibition of Normal Collagen Synthesis in Mouse Fibroblasts by Lipoteichoic Acid from *Streptococcus pyogenes* Type 12

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The toxicity of lipoteichoic acid (LTA) from *Streptococcus pyogenes* type 12 was investigated by using mouse fibroblasts in culture in the absence of serum. Morphologically, while low concentrations of LTA elicited a subtle effect characterized by progressive cellular degeneration with practically no release of protein, larger concentrations (>50 μg/ml) of this amphiphile resulted in rapid death of cell monolayers. Metabolic studies utilized a concentration of LTA (17.5 μg/ml) which caused the smallest change in cell morphology in the least number of mouse fibroblast cells per monolayer. Under these conditions, cell monolayers showed an increase of 450% in their content of collagenous protein after exposure to LTA. However, the amount of such material secreted remained unchanged. Also, changes in the type of collagenous protein formed were observed after exposure to LTA. Collagenous protein accumulating intracellularly was found to be practically hydroxyproline-free. However, collagenous protein secreted by this cell line showed a significantly reduced content of hydroxyproline as compared with control cells unexposed to this coccal membrane component. Column chromatographic studies confirmed that the collagenous protein secreted by monolayers exposed to LTA was defective (under hydroxylated). It was concluded that LTA does not affect the amount of collagenous protein secreted. However, it does increase the amount of this protein formed and retained by this cell line as well as causing a reduction in the hydroxylation of proline in both intracellular and secreted collagenous material. A possible relationship between abnormal basement membrane morphology and disturbed collagen synthesis in post-streptococcal glomerulonephritis as related to LTA is discussed.

Much has been written about the content, composition, and characterization of lipoteichoic acid (LTA) from bacteria and of its role as the "adhesion factor" in group A streptococcal pathogenesis (10, 14, 27). Also, this laboratory has shown that major differences exist in the cellular content, structure, and composition of LTA from *Streptococcus pyogenes* type 12 and its stabilized L-form (23, 24). In addition, documented for the first time was the adherence of this coccus and its stabilized L-form to human kidney cell monolayers in tissue culture by virtue of this membrane component (4). However, very little has been published concerning the cytotoxicity of this important streptococcal membrane component. Nephropathy in rabbits has been associated with immunization to group A streptococcal LTA (26), and bone resorption has been attributed to this amphiphile (6). Also, it has been reported that LTA from *S. pyogenes* and its L-form were capable of destroying monolayers of primary and established human cell lines with a predilection for the group A streptococci (4). Our cytotoxic studies utilized media containing serum, an ingredient necessary for the continued viability of these respective cell lines but one also capable of binding this amphiphile, thereby reducing its cytotoxicity. This latter property made the use of seemingly larger amounts of LTA in these investigations necessary. More recently we have observed that mouse fibroblast monolayers can remain morphologically normal, viable, and able to produce collagenous material for an extended period of time in tissue culture without serum. This study extends our previous cytotoxic results by detailing changes induced by LTA from *S. pyogenes* type 12 on cellular morphology and on biosyn-

thesis of collagenous material by monolayers of this mammalian cell line in the absence of serum.

MATERIALS AND METHODS

Tissue culture cells and medium. Mouse fibroblast cells (L-929; American Type Culture Collection, Rockville, Md.) were grown in Eagle minimal essential medium supplemented with 10% (vol/vol) fetal bovine serum, 100 U of penicillin per ml, and 100 μg of streptomycin (GIBCO Laboratories, Grand Island, N.Y.) per ml. This medium hereafter will be referred to as MEM-10. All isotope incorporation and cytotoxicity studies utilized this medium without fetal bovine serum, hereafter designated MEM-0. All incubations were at 37°C in an atmosphere of 95% air plus 5% CO_2 at 90% humidity.

LTA. This anionic polymer was isolated from *S. pyogenes* type 12 and purified as detailed elsewhere (23, 24). Alanine was the only ninhydrin-positive material detected by paper chromatography in acid hydrolysates of this preparation. LTA was dessicated and stored in vacuo over P_2O_5-NaOH at −70°C. When needed, appropriate amounts were weighed and used immediately.

Cytotoxicity studies with LTA. A confluent layer of mouse fibroblasts grown in MEM-10 was trypsinized (0.25% trypsin [wt/vol] in MEM-0), and the viable cell count was determined by the dye exclusion method with Erythrosin B (Fisher Scientific Co., King of Prussia, Pa.) and a hemacytometer. Viable cells in 0.5 ml of MEM-10 were seeded into multiple wells (10^5 cells per well) (Linbro plate no. 76-033-05; well area, 2.0 cm^2; Flow Laboratories, Inc., Hamden, Conn.) and incubated for 24 h as above. All cell monolayers were then refed with 0.5 ml of MEM-10 containing 25 μg of ascorbic acid (necessary for collagen synthesis) (1, 15). After incubation for an additional 24 h to achieve cell confluency, medium was removed, and each monolayer was washed three times with MEM-0 before the addition of fresh MEM-0 (0.5 ml) and ascorbic acid (25 μg in 0.1 ml of MEM-0). Increasing concentrations (see Fig. 1) of LTA (in 0.05 ml of MEM-0) were then added to a series of wells in triplicate. Because of their acidic nature, solutions of ascorbic acid and LTA were adjusted to pH 7.2 (with HCO_3^-) before use. LTA-exposed and -unexposed cell monolayers were incubated as above for 24 h before being photographed with a Leitz inverted microscope at a final magnification of ×350.

A modification of a standard method for determining the toxicity of substances for target cells in tissue culture (3) was used to assess the cytotoxicity of LTA. Unlabeled cell monolayers were trypsinized and seeded into multiple wells (1.5×10^5 cells per well) containing MEM-10 (0.25 ml) as above and incubated for 3 h. L-[^{75}Se]selenomethionine (2.5 μCi per well in 0.25 ml of MEM-0; specific activity, 0.6 to 4 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) was then added, and incubation was continued overnight. All cell monolayers were then washed three times with phosphate-buffered saline, pH 7.2 and refed with fresh MEM-0 (0.5 ml), and the same concentrations of LTA (in 0.05 ml of MEM-0) described above were added. After 24 h of incubation, medium was removed, and each monolayer was washed three times with phosphate-buffered saline to remove all residual radioactivity. Each washed cell monolayer was then digested with 1 N NaOH for 1 h, and portions (0.1 ml) were counted in a gamma counter. Each experiment was done in triplicate and two portions were counted from each well. Therefore, each point in Fig. 3 represents six determinations with percent toxicity calculated as:

$$\% \text{ toxicity} = \left(1 - \frac{\text{counts per minute in wells with LA}}{\text{counts per minute in wells without LTA}}\right) \times 100$$

Since the mechanism of cell death during exposure to LTA is not known, the percent toxicity includes both cell death (detachment) and cell leakage of labeled protein when concentrations of LTA above 50 μg per well were used (Fig. 2).

Effect of LTA on the synthesis and secretion of collagenous material by mouse fibroblasts. A confluent monolayer of fibroblasts was trypsinized and counted as described above, and each of 12 75-cm^2 tissue culture flasks containing 20 ml of MEM-10 was seeded with 3×10^6 viable cells. After 24 h of incubation as above, each flask was refed with the same amount of MEM-10 medium containing 0.5 mg of ascorbic acid (pH 7.2). After an additional 24-h incubation period, the medium was decanted, and each cell monolayer was washed three times with MEM-0 (5 ml). Next, 18 ml of MEM-0 plus 0.5 ml of ascorbic acid (1.0 mg, pH 7.2) and 0.5 ml of L-[U-^{14}C]proline (5 μCi; specific activity, >250 mCi/mmol; Amersham) was added. Six flasks received 350 μg of LTA dissolved in 1 ml of MEM-0 (pH 7.2), with the remainder serving as controls. After incubation for 24 h, the cells (obtained by scraping with a rubber policeman into 5 ml of phosphate-buffered saline) and medium from each flask were collected for analyses. All medium and cell samples were heated in a boiling water bath for 30 min to inhibit possible protease activity, and each cell sample was homogenized in a ground-glass tissue homogenizer (10 ml size). Cell homogenates and medium samples were then dialyzed (12,000-molecular-weight cutoff; Fisher Scientific Co.) against running water for 72 h and distilled water for 24 h at 4°C for the removal of all residual labeled proline. Portions were then taken from all samples to determine the extent of labeled proline in the high-molecular-weight protein remaining after dialysis.

The most widely used method for assessing collagen synthesis involves measuring the formation of hydroxyproline, which is known to occur almost exclusively in mammalian collagen. The following is a modification of an established procedure (8, 17). Briefly, each sample in 6 N HCl was sealed in a glass ampoule and hydrolyzed in an autoclave (120°C) overnight. All hydrolysates were evaporated to dryness and suspended in distilled water. Appropriate samples were transferred to test tubes (20 by 1.5 cm) equipped with ground-glass stoppers; 1 ml containing L-proline (10 mg) and L-hydroxyproline (1 mg) was added, and the volume was adjusted to 9 ml with distilled water. The pH was adjusted to 8 with 0.1 N NaOH, with phenolphthalein as the indicator. After the addition of 4 ml of 0.2 M sodium pyrophosphate, pH 8, each sample was oxidized by the addition of 1 ml of freshly prepared 0.2 M Chloramine-T in water. Oxidation was

terminated after 20 min at room temperature by the addition of 2 M sodium thiosulfate (0.5 ml), and the pH was again adjusted to 8 as described above. Each sample was saturated with NaCl, and the labeled proline oxidation products were extracted by multiple toluene extractions (10 ml each, 5 min of vigorous shaking). Extractions were carried out until constant radioactivity was obtained. Each extract (10 ml) was mixed with 10 ml of Bray's solution (2) and counted. The total counts obtained represented the amount of labeled proline incorporated.

After removal of the proline oxidation products, each sample was tightly sealed and placed in a boiling water bath for 20 min. Tubes were allowed to cool to room temperature, and three extractions as described above were required to obtain all of the labeled hydroxyproline oxidation products. The combined radioactivity represented the total hydroxyproline content in each sample. The ratio of labeled hydroxyproline to proline calculated from these combined determinations (×100) is reported as percent hydroxylation (Tables 1 and 3).

Collagenase treatment of proline-labeled high-molecular-weight protein. The labeled, high-molecular-weight protein obtained after dialysis of the medium and homogenized fibroblasts from cultures with and without exposure to LTA was subjected to collagenase digestion. Each sample was lyophilized and suspended in 0.5 ml of 0.05 M Tris-hydrochloride buffer, pH 7.6. Digestion was achieved by the addition of 50 μg of bacterial collagenase (EC 3.4.24.3) (highly purified, C-3267, type VI; Sigma Chemical Co., St. Louis, Mo.) and 2.5 mM *N*-ethylmaleimide, 30 μM phenylmethylsulfonylfluoride, 0.15 M NaCl, and 0.5 mM $CaCl_2$, all in 1 ml of 0.05 M Tris-hydrochloride buffer, pH 7.6. Controls included determination of the extent of collagen (type I bovine achilles tendon, insoluble; Sigma) digestion by collagenase to ascertain enzyme potency before every experiment. Digestion was always greater than 80%. After incubation for 2 h at 37°C, the reaction was stopped by the addition of 0.15 ml of 0.2 M disodium EDTA, and a portion (0.05 ml) was taken for assessing total radioactivity. Each sample was then dialyzed twice. The first dialysis was done against 10 volumes of 0.15 M NaCl in 0.05 M Tris-hydrochloride buffer, pH 7.6 (overnight, 4°C). The labeled peptides recovered from outside the dialysis bag (12,000-molecular-weight cutoff) were hydrolyzed, and the percent hydroxylation of each sample was determined from the hydroxyproline-to-proline ratio as described above. Each sample was dialyzed further (second dialysis) against large volumes of running tap water as described above, and the final reduction in the radioactivity (after collagenase treatment and both dialyses) of the retained volume was taken as a measure of total collagenous protein in each preparation. Finally, the residual content of each dialysis bag was hydrolyzed and its hydroxyproline-to-proline ratio was determined, as before, to verify the digestion of all collagenous protein by collagenase. This final step always showed a hydroxylated content of less than 0.01% remaining.

Column chromatography of secreted collagen. Labeled collagenous protein in dialyzed medium (2 ml) was chromatographed on BioGel A-1.5, 200–400 mesh (column size, 1.5 by 90 cm). The column was equilibrated and the sample was eluted with 1 M $CaCl_2$ in 0.05 M Tris-hydrochloride buffer, pH 7.6. A flow rate of 15 ml/h was used, and 3.5-ml fractions were collected. The exclusion limit of this column was 1.5×10^6. Total radioactivity was determined by counting 0.2-ml portions from each fraction in 10 ml of Bray's solution (2).

RESULTS

Cytotoxicity of streptococcal LTA for mouse fibroblasts. Initially, these studies suffered from the compromising effect of serum on the biological activity of LTA. Serum has been shown to lessen the cytotoxicity of this amphiphile for cells in vitro (I. Ginsburg, personal communication). In confirming this observation, we found that confluent monolayers of mouse fibroblasts remained viable, intact, and without any apparent morphological change for 48 h in the complete absence of serum. Therefore, this cell line was used throughout these studies.

Changes in the microscopic morphology of fibroblast monolayers as a result of various concentrations of LTA, after 4 h in the absence of serum, were recorded (photographs not shown). At concentrations of 175 μg per well or higher, 95% of the cell population became free floating and spherical in appearance. At 150 μg per well, most cells were still spherical but remained anchored to the surface. Finally, whereas monolayers remained intact at an LTA concentration of 75 to 100 μg per well, they were no longer confluent, with about half of the cell population being pulled away from one another and spherical in appearance. LTA concentrations of 50 μg per well or less had no effect on the morphology of this cell line after 4 h of incubation.

The cytotoxic effect of LTA on these fibroblasts intensified significantly after 24 h in the absence of serum. The same morphological changes were observed over an LTA concentration of from 75 to 200 μg per well, the highest concentration tested, i.e., most cells becoming detached from the plastic surface with those still adhering appearing spherical (typical example, Fig. 1F, at 175 μg per well). But various degrees of change were also evident with the lower concentrations of LTA tested. This change was a progressively increasing number of spherical cells appearing but with a decreasing number remaining attached (Fig. 1B through E). Monolayers treated with 5 μg per well or less were normal when compared with control cell layers. Of special interest was the finding that these changes, over an LTA concentration of from 1 to 200 μg per well, did not vary when the incubation period was extended to 48 h, the maximum time for maintenance of normal cell morphology without serum. There was no apparent difference in the proportion of cells becoming spherical or those still adhering to the plastic surface

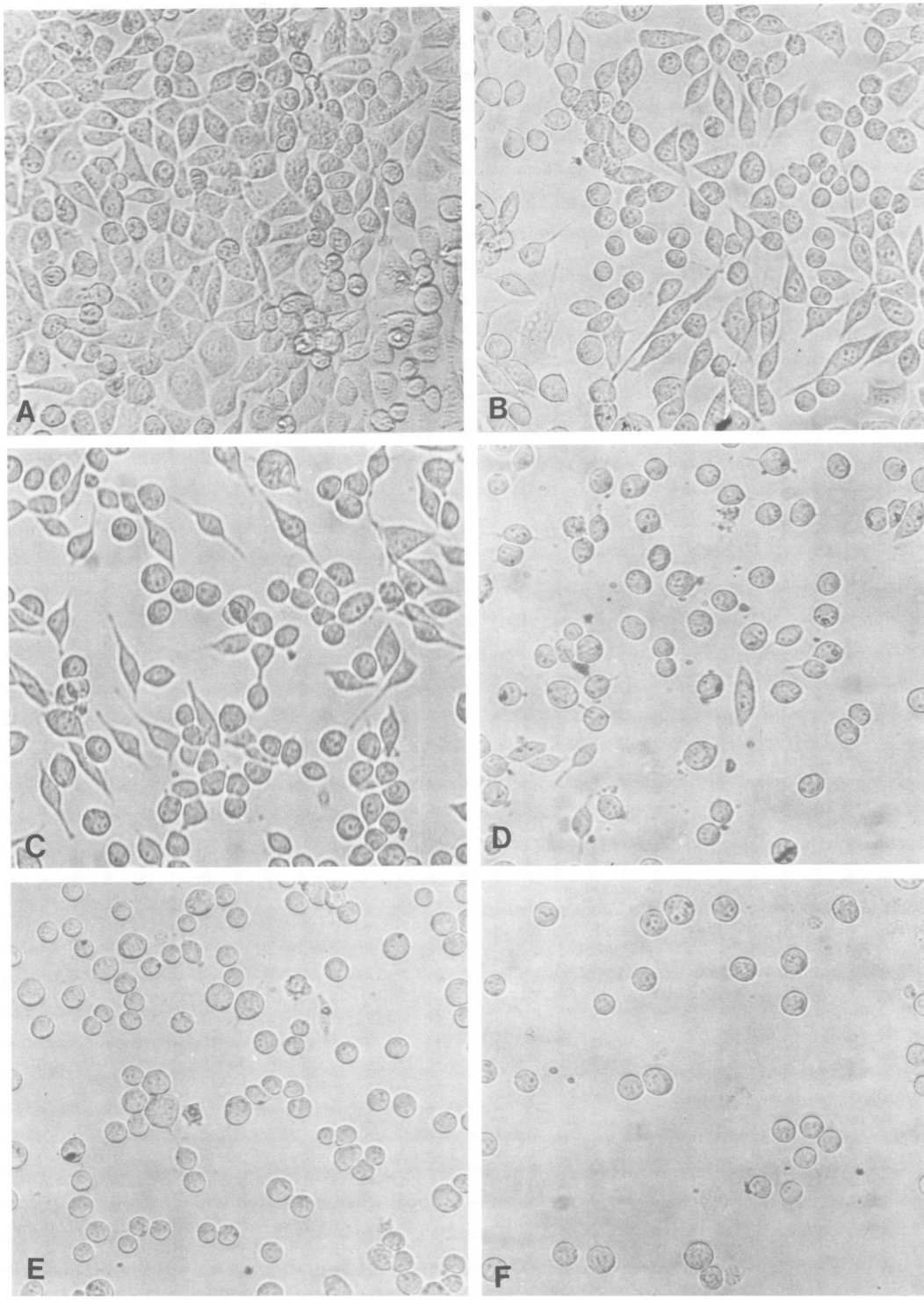

FIG. 1. Cytotoxicity of increasing concentrations of LTA for mouse fibroblast monolayers after 24 h in the absence of serum. (A) Untreated control; (B) 10 μg per well; (C) 15 μg per well; (D) 20 μg per well; (E) 50 μg per well; (F) 175 μg per well. Unstained preparations; final magnification, ×350.

(as in Fig. 1) after 24 and 48 h.

The effect of serum on LTA cytotoxicity in these studies was noticeable. For example, at the highest concentration of LTA tested (200 μg per well), greater than 50% of the cells in each monolayer became spherical after only 0.5 h of incubation in the absence of serum, suggesting a physical effect. No effect was noted after this time period with serum. Overnight incubation was required for comparable effects in the presence of serum (10% [vol/vol]). Also, the degree of destruction was mediated by this parameter, with almost complete detachment (>95%) occurring only in the absence of serum after 24 h. Finally, morphological changes (similar to Fig. 1B through E) with low concentrations of LTA (10 to 50 μg per well) were only apparent in the absence of serum after this incubation period.

Figure 2 shows the cytotoxicity of increasing concentrations of streptococcal LTA after 24 h in the absence of serum, using mouse fibroblasts preloaded with L-[^{75}Se]selenomethionine. As is apparent, significant toxicity (above 10%) for attached fibroblast cells was evident when the concentration of LTA added was 75 μg per well; a maximum toxicity of 80% was realized at a concentration of 175 μg per well. Also, a reproducible plateau in toxicity (ca. 47%) was observed when concentrations of LTA from 100 to 150 μg per well were tested. Finally, a comparison of these quantitative results with our visual observations (Fig. 1) showed that a much greater change in morphology than occurred in the release of cellular protein (ca. 6%, Fig. 2) was evident with concentrations of LTA up to and including 20 μg per well.

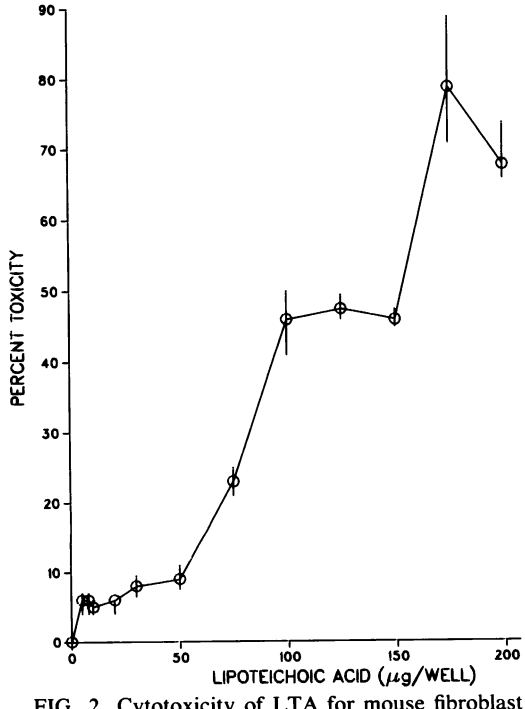

FIG. 2. Cytotoxicity of LTA for mouse fibroblast monolayers as determined by prelabeled L-[^{75}Se]selenomethionine cells after 24 h in medium without serum. Each point represents the average of six determinations ± standard deviation.

Effect of LTA on synthesis of collagenous material. These experiments were performed in bottles containing LTA (17.5 μg/ml) equivalent to that (10 μg per well) used in the morphology studies (see above). This concentration was determined from the above studies and represents the smallest amount capable of causing a minimal morphological change (spherical cell formation) in the least number of mouse fibroblast cells per monolayer (see Fig. 1B). Table 1 shows that fibroblast monolayers, after LTA treatment and dialysis, contained 28% more labeled high-molecular-weight protein than control cells. However, this increased protein content contained less hydroxyproline (by 60%) than the untreated control. An intracellular percentage of hydroxylation of approximately 1.5 for normal L-929 mouse fibroblasts has been reported by others in such experiments (15). From these data it was not possible to state unequivocally whether this decreased hydroxylation was due to the formation or presence of excess protein(s) or to the actual formation of a decreased or nonhydroxylated collagenous material being retained within these cell monolayers when exposed to LTA.

Approximately 10% of the labeled high-molecular-weight protein formed by this cell line was secreted as compared with that found intracellularly, regardless of the presence or absence of LTA. However, it had a much greater hydroxyproline content (Table 1) than intracellular collagen-containing material. Again, of particular interest was the finding that the percentage of hydroxylation of this secreted material was reduced when it was obtained from monolayers exposed to LTA, it being under hydroxylated by 30%. Our earlier results with L-[^{75}Se]selenomethionine had shown that very little, if any, significant leakage of protein occurred from cells exposed to a concentration of LTA equivalent to that utilized in this study, even though a morphological change was apparent after 24 h. This is confirmed in Table 1, which shows that the amount of high-molecular-weight material secreted by fibroblasts remained constant with and without LTA. Therefore, a low concentration of LTA seemingly does not affect the secretion of this material. But it does affect the amount of labeled collagenous material within these cells as well as decreasing the percent hydroxylation of this intracellular and secreted protein.

TABLE 1. Effect of LTA on high-molecular-weight protein synthesis by mouse fibroblast monolayers[a]

Culture	Cells		Medium	
	High mol wt protein (cpm × 10^5)	% Hydroxylation[b]	High mol wt protein (cpm × 10^4)	% Hydroxylation[b]
Fibroblast culture (control)	6.18 ± 0.45	1.96 ± 0.46[c]	6.14 ± 0.97	20.01 ± 1.98[c]
Fibroblast culture exposed to LTA (17.5 μg/ml)	7.90 ± 0.34	0.79 ± 0.49[c]	6.58 ± 0.72	13.99 ± 2.06[c]

[a] Determined after 24 h in the absence of serum. Each value is the average of six experiments ± standard deviation; six bottles per experiment. Each bottle contained 5 μCi of [*U*-^{14}C]proline and 6.8 × 10^6 ± 0.4 × 10^6 viable cells (equivalent to 1.8 ± 0.2 mg of protein).

[b] Hydroxyproline/proline × 100.

[c] Statistically significant differences between values for the two cultures (Student *t* test; $P < 0.05$).

Collagenase treatment of proline-labeled high-molecular-weight protein from cells and medium. Enzymatic proof was sought to verify that labeled high-molecular-weight protein produced by mouse fibroblasts contained collagenous material. Therefore, the radioactivity of this labeled protein in cells and medium from cultures with and without LTA was determined before and after collagenase digestion and dialysis. As is known, the extent of proline hydroxylation does not affect the extent of collagen digestion by collagenase (16). Table 2 shows that control cells retained most of their radioactivity (94%) as compared with cells exposed to LTA (73%) after collagenase digestion, indicating only a 6% collagen content in normal cell monolayers as compared with a 27% content in cells exposed to LTA, an increase of 450%. It is known that under-hydroxylated collagen is secreted at a markedly reduced rate (18). Conversely, the amount of collagen secreted into the medium by fibroblast monolayers before and after exposure to LTA was equal, approximately 60% of the labeled protein synthesized and secreted being collagenous material.

Studies were undertaken to also confirm that the reduced hydroxylation of labeled high-molecular-weight protein from cells and medium of fibroblast cultures exposed to LTA was due to altered collagenous protein. This was achieved by comparing the hydroxyproline-to-proline ratio of pooled preparations before and after collagenase treatment plus dialysis (Table 3). Surprisingly, we found that the high-molecular-weight protein accumulating within the fibroblast cells after collagenase treatment and dialysis was practically free of hydroxyproline (2.8% hydroxylation) as compared with the small but poorly hydroxylated collagenous content (7.7% hydroxylation) in control cells. Also unexpected was the finding that the secreted collagenous material from cells exposed to LTA was significantly less hydroxylated (by 24%) than that released by control cells. A percent hydroxylation of 40.6 for collagenous material from the medium of control cultures (Table 3) is in agreement with that reported by others for this particular cell line (40 to 50% hydroxylation) (15). The increase in the individual percent hydroxylation after collagenase digestion of cells and medium reflects the removal of extraneous labeled protein by the collagenase-dialysis treatment. Finally, the ratio of the percent hydroxylation of secretable collagenous material from LTA-exposed cells to that of control cells (0.70) (Table 1) was not significantly altered when these

TABLE 2. Collagenous protein synthesis and secretion by mouse fibroblast cells before and after exposure to streptococcal LTA[a]

Fraction	Radioactivity		% Nondialyzable protein
	Before collagenase digestion (cpm)	After collagenase digestion and dialysis (cpm retained)	
Fibroblasts (control)	3.1 × 10^5 ± 0.1 × 10^5	2.9 × 10^5 ± 0.2 × 10^5	93.5 ± 2.0
Fibroblasts exposed to LTA (17.5 μg/ml)	4.1 × 10^5 ± 0.2 × 10^5	3.0 × 10^5 ± 0.0	73.2 ± 2.5
Spent medium (control)	4.3 × 10^4 ± 0.2 × 10^4	1.6 × 10^4 ± 0.2 × 10^4	37.2 ± 2.5
Spent medium from cells exposed to LTA	4.7 × 10^4 ± 0.3 × 10^4	1.9 × 10^4 ± 0.0	40.4 ± 2.7

[a] All values are the average of two determinations in duplicate ± standard deviation. Growth and labeling conditions are described in Table 1, footnote *a*.

TABLE 3. Radioactivity and percent hydroxylation of dialyzable, collagenous protein (pooled samples)[a]

Culture	Radioactivity (cpm) in:		% Hydroxylation in:	
	Fibroblasts	Spent medium	Fibroblasts	Spent medium
Control	5,204 ± 425	13,432 ± 842	7.7 ± 0.5	40.6 ± 3.0
Exposed to LTA	10,380 ± 620	12,876 ± 750	2.8 ± 0.2	31.2 ± 2.0

[a] All values are the average of two determinations in duplicate ± standard deviation. Growth and labeling conditions are described in Table 1, footnote *a*.

sources were subjected to collagenase digestion (0.76) (Table 3). Likewise, this same ratio for intracellular collagenous material after and before exposure to LTA (0.40) (Table 1) remained similar to that determined after collagenase digestion (0.36) (Table 3). These data confirm that the results in Table 1 are due to changes in collagenous material.

Molecular weight estimation. Attempts were made to estimate the molecular weight of secreted but labeled collagenous material from this cell line, treated with and without LTA, by column chromatography. As is apparent from Fig. 3, spent medium from untreated cells (control) contained most of its labeled proline in material eluted as a single peak corresponding to a molecular weight somewhat larger than 200,000. However, similarly spent medium from cells treated with LTA showed a drastic decrease in radioactivity in this high-molecular-weight region, with labeled material instead being distributed over the entire chromatographic profile. Therfore, the molecular weight of collagenous material after exposure to LTA could not be estimated by this method, even though a percent hydroxylation of approximately 14 had been established by chemical analyses (Table 1) and the presence of collagen confirmed by collagenase digestion. A similarly altered chromatographic profile was obtained by others for secreted collagen from

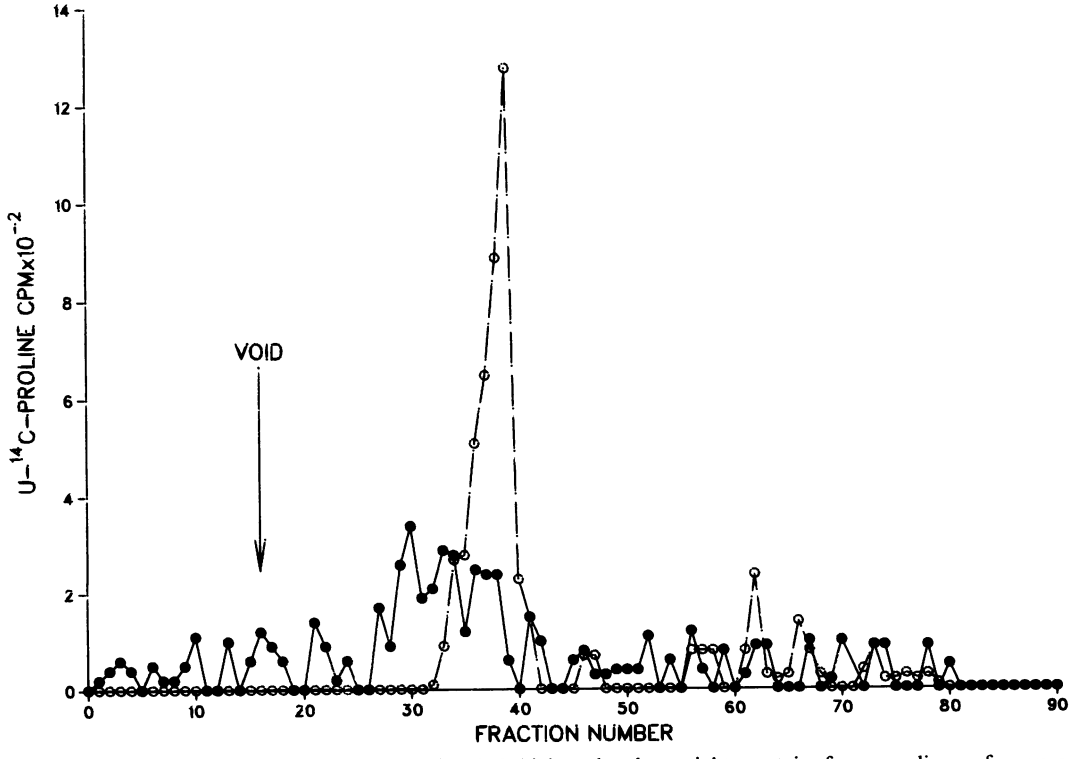

FIG. 3. Gel chromatography of proline-labeled high-molecular-weight protein from medium of mouse fibroblast monolayers with (17.5 μg/ml) and without (control) LTA for 24 h in the absence of serum. BioGel A-1.5 column, 200–400 mesh. Sample size, 2.0 ml, eluted with 1 M $CaCl_2$–0.05 M Tris-hydrochloride buffer, pH 7.6; 3.5-ml fractions collected. Symbols: ○, from untreated culture (control); ●, from culture exposed to LTA.

3T6 mouse fibroblasts whose hydroxylation had been reduced by exposure to α,α′-dipyridyl for 8 h (20).

DISCUSSION

These studies have emphasized the destructive capabilities of LTA from *S. pyogenes* type 12 for mouse fibroblast monolayers in tissue culture in the absence of serum. While low concentrations of LTA elicited a subtle effect characterized by progressive cellular degeneration with practically no release of protein, larger concentrations (>50 μg/ml) resulted in cell death (marked morphological changes and cell detachment after 24 h). Under appropriate conditions, this cell line is also capable of synthesizing collagenous material (15), indicating that growth and collagen formation are not inseparable in mouse fibroblast cells. This normal formation of collagenous protein was also inhibited by LTA (see below). Thus, these collective results illustrate a biochemical and morphological destructive capability unreported thus far for any amphiphile. Earlier, it was also shown that LTA was responsible for the attachment of this coccus and its L-form to various human cell lines in tissue culture and for their rapid destruction (4). Therefore, these combined activities suggest a role for this microbial membrane component which is unique in the pathogenesis of infectious diseases caused by *S. pyogenes* in humans.

The group A streptococcus used in this study also secreted LTA (R. Marshall and C. Panos, unpublished data), indicating that coccal cell lysis is not obligatory for the initiation of tissue damage by this amphiphile. Also, serum interacts with free LTA to reduce its cytotoxic properties (I. Ginsberg, personal communication) (see above). However, this interaction becomes minimized when the streptococcus is in direct contact with the plasma membrane of the host. Under these conditions, secreted LTA may pass from donor to recipient without exposure to serum, thereby permitting relatively small amounts of LTA to remain localized to express a maximal cytotoxic effect during pathogenesis.

Differences exist between the LTA from *S. pyogenes* and its L-form, the latter having a markedly shorter polyglycerol-phosphate chain length and lacking D-alanine (23, 24). Both of these LTAs contained glucose and both lost their cytotoxic activity for human cell monolayers in tissue culture and their ability to prevent binding of this coccus to human kidney cells when chemically deacylated (4). Group A coccal LTA is known to induce nephrocalcinosis in rabbit kidneys, whereas stripped LTA (free fatty acids and backbone polymer only) does not (26). Therefore, although major structural changes and omissions in the hydrophilic portion of LTA from this streptococcus seemingly do not alter its cytotoxic or binding capabilities, removal or detachment of its lipid moiety does. At variance with these data are the findings of Simpson et al., who reported that deacylated LTA killed heart cells (21).

The production and intracellular accumulation of collagenous protein which is practically hydroxyproline-free as well as the secretion of under-hydroxylated collagen by monolayers of this cell line when exposed to a low concentration of LTA were unexpected. The structure and function of collagen is dependent, in large part, upon its hydroxyproline content (7, 11, 18, 19). For example, it is known that the hydroxyl group of hydroxyproline has an essential role in the stabilization of the triple helix conformation of collagen and that inhibition of proline hydroxylation leads to the synthesis of biologically nonfunctional protein. α,α′-Dipyridyl inhibits proline hydroxylation, resulting in the formation of defective collagen (20). The column chromatographic profile of collagenous material obtained from mouse fibroblast monolayers after exposure to LTA was indistinguishable from that shown by others for collagen after exposure to α,α-dipyridyl (20). Therefore, our chromatographic and chemical results leave little doubt as to the damaging effect (reduced hydroxylation) of LTA on collagenous material synthesized by this cell line. In this regard, ascorbic acid is necessary for normal collagenous protein synthesis by mouse fibroblasts (1, 15). Also a deficiency in ascorbic acid prevents the synthesis of hydroxyproline, resulting in the production of collagen with an unstable triple helix conformation at body temperature. This in turn leads to improperly formed collagenous fibers, which are the cause of the skin lesions and blood vessel fragility so prominent in scurvy (19). Under these conditions, defective collagenous protein is accumulated intracellularly and is released or secreted slowly as a nonfunctional entity unsuited for the assembly of collagen fibrils (7, 18). Therefore, in the presence of appropriate ions, LTA may cause the removal, by chelation, of ascorbic acid or metal ions so necessary for the synthesis of normal collagenous material by mouse fibroblasts in tissue culture.

As indicated, the concentration of LTA used here (10 μg per well) caused very little morphological change and insignificant protein leakage after 48 h. But the results were clearly indicative of a metabolic disturbance, as evidenced by the greatly increased production of intracellular collagenous material which was practically free of hydroxyproline, a finding closely mimicking the result of ascorbic acid deficiency in mammals.

Therefore, group A coccal LTA apparently increases the synthesis but not the hydroxylation of this protein by mouse fibroblast monolayers in tissue culture. This latter aspect probably accounts for the greatly elevated intracellular accumulation of this defective protein. LTA has also been shown to stimulate cell division (21). It has been stated that inhibition of hydroxylation prevents the normal secretion of collagenous material from the cell and that portions of this material are subsequently degraded and then excreted (20). By comparison, the total amount of collagenous material secreted by this cell monolayer with and without exposure to LTA remained similar, suggesting that a 9% decrease in hydroxyproline content is not enough to significantly alter the total amount released. A possible difference in the rate of secretion after exposure to LTA, however, must await definitive kinetic studies.

Collagen is a major macromolecule of most connective tissue and a component of the basement membrane of the kidney, a membrane with a profound role in glomerular filtration. Poststreptococcal glomerulonephritis is often a sequelae of group A, type 12 infections, a malady thought to be a form of immune complex nephritis developed during the course of the immune response to a streptococcal infection anywhere in the body. To our knowledge, no information is available on possible biochemical defects in the collagenous component of the glomerular basement membrane in this streptococcal disease. Mainly found in children, acute glomerulonephritis may be accompanied by swelling of the glomerular basement membrane and an impairment of the glomerular filtration process, possibly as a result of deposition of immune complexes (12). In these current studies we have shown the synthesis and accumulation of large amounts of abnormal intracellular collagenous material in the presence of small amounts of coccal LTA by mouse fibroblast monolayers in tissue culture. In a concurrent study to be detailed elsewhere, we have also shown the destruction of mouse glomeruli growing in tissue culture by LTA from this *S. pyogenes* and its L-form. However, preceding this destruction was the finding, by electron microscopic and enzymological techniques, of a greatly increased thickening or swelling of the glomerular basement membrane. No region of the membrane remained normal, with increases in thickness being irregular and ranging from two- to fourfold. Therefore, this morphological change plus the eventual destruction of the intact glomerulus in the absence of complement or specific antibody suggests a nonimmune LTA-mediated mechanism for kidney damage, which is probably related to disturbed collagen biosynthesis.

Alluded to earlier was the reducing effect of serum on LTA cytotoxicity. The failure of streptolysin S to affect cells has also been ascribed to the delaying effects of mammalian sera used to maintain various human cell lines in tissue culture (5). It is known that cell-bound streptolysin S may become complexed to carrier molecules such as serum, albumin, RNA, etc., and be transferred from one carrier to another. The complex formed is composed of a nonspecific carrier moiety and a specific moiety synthesized by the streptococcus (5). More recently, it has been shown that LTA may also function as a carrier of streptolysin S (25). In similar fashion, secreted LTA in vivo may be bound to and transferred from various nonspecific carriers until finally accepted by the kidney, with the intermittent complexed form of LTA being less toxic. The binding of streptococcal LTA to serum albumin has been documented (22). Upon sufficient accumulation of this amphiphile, the initiation of glomerular damage and retarding of the healing process then occurs. This situation may be accelerated by the use of penicillin, which is known to exacerbate the release of LTA by *S. pyogenes* (9). In addition, repeated infection could result in the accumulation of "particulate" LTA released during phagocytosis to initiate and hasten the formation of detrimental immune complexes to continue or intensify the initial lesion. It is known that streptococcal fragments pose problems for lysosomal enzymes and are not readily digested during phagocytosis (13). Should this possibility prove tenable then the secretion of LTA, especially during the latent period of the disease, may be as important as the immune response in helping to elucidate the evasive mechanism(s) evoking streptococcal glomerulonephritis.

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