Effect of Streptococcal Lipoteichoic Acid on Prolyl Hydroxylase Activity as Related to Collagen Formation in Mouse Fibroblast Monolayers

OFRA LEON and CHARLES PANOS*

Department of Microbiology, Jefferson Medical College, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Received 3 June 1985/Accepted 4 September 1985

Dried and wet mouse fibroblast monolayers with labeled collagenous substrate were used to study the effects of lipoteichoic acid (LTA) on cellular prolyl hydroxylase activity. LTA is a scavenger of cations, and Fe^{2+} is essential for prolyl hydroxylase activity. Surprisingly, addition of LTA to dried monolayers resulted in increased prolyl hydroxylase activity, whereas preincubation of Fe^{2+} with LTA only negated this increase. However, significant inhibition of enzyme activity by wet monolayers occurred whether LTA was added directly to the test system or whether it was used after preincubation with Fe^{2+}. These data suggest that LTA causes membrane perturbations. Also, that the binding of LTA to the membrane of dried and wet monolayers appears to be decidedly different when based on the subsequent availability of Fe^{2+} for cellular prolyl hydroxylase activity. The ability of LTA to act as a cationic exchanger and the presence of intracellular Fe^{2+} inaccessible to LTA probably accounted for the lack of complete inhibition of prolyl hydroxylase activity by this amphiphile in the wet cell system. Considerably less iron was needed to negate the partial inhibition of prolyl hydroxylase activity by LTA in viable cells than was needed to restore the increased enzyme activity by this amphiphile in equivalent dried preparations. These and other results showed that, although LTA does not affect collagen polypeptide chain formation in wet monolayers, its involvement at the molecular level does result in a marked decrease in the hydroxylation of collagenous peptidyl prolines residues through LTA interaction with Fe^{2+}. This reduction in prolyl hydroxylase activity equaled the reduction in hydroxylation of collagenous protein in fibroblast monolayers caused by LTA reported earlier (O. Leon and C. Panos, Infect. Immun. 40:785–794, 1983). Therefore, these data suggest that partial inhibition of prolyl hydroxylase activity is directly related to the synthesis of defective collagen by wet fibroblast monolayers exposed to minute amounts of group A, type 12 streptococcal LTA. Use of LTA also showed that complete inhibition of hydroxyproline formation is not required for the continued formation and accumulation of defective collagenous protein by these monolayers.

Lipoteichoic acid (LTA) is responsible for group A streptococcal adherence (3, 10, 17, 28). Also, in addition to being a membrane component, LTA is secreted during growth of Streptococcus pyogenes type 12 (8, 25). Earlier work from this laboratory had dealt with various chemical, biochemical, and enzymological aspects of LTA from S. pyogenes type 12 and its derived L-form (2, 22, 23). Later, LTA from these organisms was shown to be cytotoxic for a variety of human and animal cell lines in tissue culture (3, 11, 12). In more recent studies, mouse fibroblasts in culture in the absence of serum were shown to produce and retain a significantly increased amount of underhydroxylated (decreased hydroxyproline content) collagenous material when treated with low concentrations of LTA from this streptococcus (12). Likewise, subsequent electron microscope studies revealed that the basement membrane of intact, cultured mouse glomeruli exposed to streptococcal LTA was greatly thickened and electron dense (26). These morphological changes seemingly paralleled those observed in biopsies of glomerular material from young patients with acute glomerulonephritis (4, 16, 24). Since under- or nonhydroxylated collagen is known to remain within the cell and to be excreted very slowly, a possible relationship between these basement membrane changes and defective collagen biosynthesis as a result of streptococcal LTA was proposed. These studies were done in vitro and in the absence of complement or specific antibody, indicating that a nonimmune LTA-mediated mechanism for kidney damage by a streptococcal product was also possible (12).

Prolyl hydroxylase is responsible for the hydroxylation of specific peptidyl prolines in polypeptide precursors of collagen (19). This enzyme has a requirement for ferrous ion (19). LTA is known to function in cation control and to have relative affinities for cations for its anionic sites (6, 15). This paper describes the different effects of LTA on dried and wet fibroblast monolayers. Also, it emphasizes that the reduction of prolyl hydroxylase activity in wet fibroblasts by LTA leading to abnormal collagen formation is due to interaction between required Fe^{2+} and this amphiphile.

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 per ml (GIBCO Laboratories, Grand Island, N.Y.) per ml (MEM-10). All incubations were at 37°C in an atmosphere of 95% air plus 5% CO_{2} at 90% humidity.

LTA. This polymer was isolated from S. pyogenes type 12 and purified as described before (23, 26). This preparation
had an amino acid content of 0.43%, which included alanine, when examined by amino acid analyses with ophthalaldehyde and fluorescence detection. The protein content of this preparation was 0.35%. A stock solution of LTA (500 µg/ml) in 0.05 M Tris hydrochloride (pH 7.2) was kept frozen until needed.

Preparation of unhydroxylated collagenous substrate. A modification of the procedure of Peterkofsky and Diblasio was used (18). Fibroblast cells were grown in plastic tissue culture flasks (surface area, 75 cm²; 250 ml; Corning Glass Works, Corning, N.Y.), each seeded with 1.5 × 10⁶ cells and containing 20 ml of MEM-10. At confluency (approximately 4 to 5 days), medium was decanted, and cells were refed with 20 ml of MEM-10–200 µCi of [3,4-3H]-proline (specific activity, 55 Ci/mmol; ICN Pharmaceuticals, Inc., Irvine, Calif.) and 0.5 mM a,a'-dipyridyl (Sigma Chemical Co., St. Louis, Mo.) added to each flask. After 24 h of incubation as described above, cells were detached with a rubber policeman, and cells plus medium were homogenized with a 746 LEON cutoff, 1-in. diameter; A. H. Thomas Co., Philadelphia, Pa.) twice (for 24 h each time) against 3.5 liters of 0.4 M NaCl in 0.1 M Tris hydrochloride (pH 7.6) at 4°C. After dialysis, the homogenate was incubated for 1.5 h at 37°C to precipitate fibrils of unlabeled collagen, and all fibrils and cell debris were removed by centrifugation (20,000 × g, 10 min) at 4°C. The clear supernatant contained the unhydroxylated collagen substrate and exhibited a radioactivity of 1.2 × 10⁵ cpm/ml. This substrate was 90% collagenase digestible and completely unhydroxylated as determined by the method of Juva and Prockop (7). This radioactive collagen substrate in the above buffer was stored at 0 to 5°C, since one cycle of freezing and thawing totally destroyed its ability to be hydroxylated in the prolyl hydroxylase assay system (see below). A commercially available substrate (L-prolyl-1-glycyl-2-proline)₃ labeled at L-prolyl-1-4-H(N) with a specific activity of 2.8 µCi/mg (NET-729; molecular weight range, 720 to 2,528; New England Nuclear Corp., Boston, Mass.) was also used for comparative purposes. It arrived dissolved in 2% ethanol and packed in dry ice with instructions to store at 4°C.

Prolyl hydroxylase assay. Enzyme activity in mouse fibroblast monolayers was assessed in two ways: (i) by adding unhydroxylated collagen substrate to dried monolayers and (ii) by using wet monolayers containing unhydroxylated collagen substrate in situ.

Use of the dried monolayer method required a modification of the procedure of Trupin et al. (27). Mouse fibroblasts (10⁵ cells) were seeded into 30-ml culture flasks (surface area, 25 cm²; Falcon; A. H. Thomas) containing 5 ml of MEM-10 and incubated for 6 days as described above, with refeeding being done on the third day. On the sixth day, medium was removed, and fresh medium (5 ml) containing ascorbic acid (50 µg/ml) was added. Incubation was continued for an additional 24 h. After the seventh day, medium was removed, and the monolayers were washed three times with phosphate-buffered saline. After the last phosphate-buffered saline washing was removed by aspiration, monolayers were dried for 0.5 h in a stream of nitrogen, and each flask was stored in an evacuated dessicator in the freezer (–20°C). We have found that such preparations retain full prolyl hydroxylase activity for 3 months. Others have shown enzyme stability for many weeks when dried monolayers were stored in a dessicator below 0°C (27).

The reaction mixture for assaying prolyl hydroxylase activity contained 1 mM ascorbic acid, 1 mM alphaketoglutarate, 0.1 mM ferrous ammonium sulfate, 0.5 mM dithiothreitol, and (depending on the monolayer used [see below]) 5 × 10⁴ cpm of prepared, unhydroxylated collagen substrate, all in a final volume of 1 ml of 0.05 M Tris hydrochloride (pH 7.6) buffer. One milliliter of this mixture was added to each 7-day-old dried monolayer, and the flasks were placed in a shallow water bath (30°C) for 30 min. Controls included flasks with dried monolayers with and without 1 ml of buffer, immersed in boiling water (0.5 h) before use, and reaction mixtures without monolayers. After the addition of 0.05 ml of 8% (wt/vol) albumin (fraction V; Armour Pharmaceuticals, Kankakee, Ill.), the reaction was stopped by adding 0.2 ml of 25% (wt/vol) trichloroacetic acid and keeping the flask at 5°C for 10 min. The protein precipitate was removed by centrifugation in a Microfuge B (Beckman Instruments, Inc., Fullerton, Calif.) for 1.5 min and then washed twice with 0.1-ml portions of trichloroacetic acid (5% [wt/vol]). All washes were combined and added to the original supernatant. Tritiated water released by the prolyl hydroxylase reaction was separated by column chromatography (AG 50W-X8; column size, 3.0 by 0.5 cm; Bio-Rad Laboratories, Richmond, Calif.) as described by Peterkofsky and Diblasio (18). The effluent and two additional water washes (0.5 ml each; total volume, approximately 2.2 ml) were placed in a scintillation vial, 10 ml of Bray fluid was added, and the mixture was counted. The total radioactivity obtained represented the amount of collagenous proline hydroxylated per monolayer.

For the preparation of wet monolayers with labeled, unhydroxylated collagen in situ, cells were grown to confluency in 75-cm² bottles, trypsinized, and seeded into 30-ml culture flasks as described above. After 3 days of incubation, cells were refed with 5 ml of medium and, after an additional 3 days, the medium was removed and the cells were refed with 2 ml of MEM-10 containing 1 µCi of [3,4-3H]-proline and 0.5 mM a,a'-dipyridyl. The medium was again removed after an additional incubation of 24 h, and all confluent monolayers now containing labeled, unhydroxylated collagen were washed four times, three times with 2-ml portions of 0.05 M Tris hydrochloride buffer, (pH 7.6) and once with 1.5 ml of buffer which overlaid each monolayer for 0.5 h at 4°C before being aspirated. This washing procedure ensured the removal of all medium components, including residual labeled proline, accumulated tritiated water, and a,a'-dipyridyl from wet monolayers to be used in the assay procedure. Total radioactivity per monolayer was 42,696 ± 983 cpm (n = 5). For assessing proline hydroxylase activity, buffer was removed, ascorbic acid, alphaketoglutarate, dithiothreitol, and ferrous ammonium sulfate in 1 ml of Tris hydrochloride buffer, as described above, were added to each monolayer, and all flasks were incubated for 30 min at 30°C. The reaction was stopped and processed, and radioactivity in each supernatant was determined as described before. Protein was determined by the method of Lowry et al. (14) with bovine serum albumin as standard.

LTA addition studies. The effect of LTA on prolyl hydroxylase activity in dried and wet monolayers was assessed by (i) adding LTA directly to the reaction mixture and (ii) preincubating each component of the reaction, except Fe²⁺, with LTA (room temperature, 0.5 h) in buffer before adding
that particular combination to the reaction mixture. Likewise, various concentrations of Fe" were preincubated with LTA in 0.05 M Tris hydrochloride buffer (pH 7.2) containing alpha-ketoglutarate and dithiothreitol (see above) to prevent oxidation before addition to the assay system. The concentrations of LTA tested were 50, 100, and 250 μg per final volume (1 ml) of reaction mixture.

Monolayer disruption. Washed, confluent fibroblast monolayers (30 mg of protein) were suspended in 10 ml of 0.05 M Tris hydrochloride buffer (pH 7.6) at 0°C and sonicated in a Branson Sonic Power Co. (Danbury, Conn.) model S75 Sonifier equipped with a 3-mm needle probe. Sonication was done four times for 10 s each at a setting of 2 A, followed by a repeat of this sequence at a 4 A. After each sonic exposure, samples (approximately 1 mg of protein) were removed for determination of prolyl hydroxylase activity as described above and assessment of cell breakage by light microscopy. Maximum breakage (>95%) was observed after a total exposure of 30 s regardless of the setting used. Disruption by alumina grinding was done precisely as detailed elsewhere for our preparation of bacterial membrane fragments (20).

RESULTS

Hydroxylase assay system. Use of a,α'-dipyridyl did not result in obvious morphological changes to cell confluency as determined by examination of unstained monolayers by light microscopy. Also, cell yields (i.e., protein) were comparable to cells not exposed to this chelator. All dried, washed confluent monolayers had a protein content of 1.02 ± 0.15 mg (n = 4) per 25-cm² flask. Finally, increasing the incubation period of the assay from 30 min to 3 h did not result in increased release of tritiated water.

Both a commercially available nonhydroxylated collagen substrate and one synthesized by us (see Materials and Methods) were used concurrently, with identical results being obtained. However, the commercial preparation (received frozen; molecular weight range, 720 to 2,528) steadily lost its ability to be hydroxylated when kept frozen, being only 60 and 30% as active in the assay when frozen for 1 and 3 months, respectively. Since our preparation was not dialyzable, retained its maximum ability to be hydroxylated for one month at 4°C, and lost only 20% of this ability after storage for 3 months, it was the substrate of choice for most of these studies.

Cell age and enzymatic activity. Figure 1 illustrates the increase in enzyme activity with cell age, with the greatest activity occurring at day 7. However, changes in the viable count did not parallel enzyme activity, with a greater increase in viable count than in enzyme activity occurring from day 4 through day 7. Also, cell numbers increased as prolyl hydroxylase activity decreased after day 7. Under these growth conditions, monolayer confluency was achieved by day 4, with cells layering, as a result of overgrowth, thereafter. Although a precipitous drop in enzyme activity resulted by day 8, monolayers appeared normal when unstained preparations were examined by inverted light microscopy. Others have noted similar results.

FIG. 1. Prolyl hydroxylase activity and viable count as related to mouse fibroblast cell age. Each point is an average of four determinations. Standard deviations for viable counts were minimal and, therefore, are not all apparent.
Therefore, only 6- to 7-day-old monolayers were used in these studies.

Increased prolyl hydroxylase activity in mouse fibroblast cell monolayers after cell disruption has been reported (27). However, no increased activity was observed in our preparations after sonic disruption. Also, all preparations obtained by alumina grinding were devoid of prolyl hydroxylase activity.

**Ferrous ion and enzymatic activity.** Because of the ability of LTA to bind divalent cations and the requirement of Fe$^{2+}$ for prolyl hydroxylase activity, the concentration of Fe$^{2+}$ needed for maximal enzymatic activity by dried mouse fibroblast monolayers was determined. Figure 2 confirms the dependency of prolyl hydroxylase on Fe$^{2+}$ and shows that maximum enzymatic activity was achieved at an Fe$^{2+}$ concentration of 0.1 mM. The fact that some activity (approximately 33%) continued in the absence of added Fe$^{2+}$ suggests residual ion being present within the fibroblast monolayer. A similar observation was noted previously (13). Identical results were obtained whether 6- or 7-day-old cells were used or whether labeled collagen substrate was added exogenously to a dried monolayer or was present within a wet monolayer. Finally, omission of ascorbic acid, alphaketoglutarate, or dithiothreitol resulted in loss of approximately 60 to 70% of prolyl hydroxylase activity.

**Effect of LTA on prolyl hydroxylase activity in dried cell monolayers.** Earlier, a concentration of 10 μg of LTA per 2-cm$^2$ well had been used to study the deleterious effect of LTA on collagen biosynthesis in mouse fibroblasts. Each well contained a confluent monolayer equal to $4.2 \times 10^5 \pm 0.2 \times 10^5$ viable cells (12). In the present study, confluent monolayers equal to $5.3 \times 10^6 \pm 0.1 \times 10^6$ viable cells in 25-cm$^2$ flasks were used. Because of a nearly equivalent increase in volume and viable cell count between these studies (approximately 12 times), a concentration of 100 μg of LTA per flask was used routinely for these new findings to be comparable with previous results. Studies with more (250 μg) or less (50 μg) LTA per flask did not alter the results obtained.

Table 1 shows the results observed with dried monolayers when LTA was added directly to the complete assay system and also when it was preincubated with individual components of the assay system before use. As is apparent, hydroxylation of nonhydroxylated collagen substrate increased by 323% when reaction mixture served in lieu of

![Graph showing relationship between prolyl hydroxylase activity and Fe$^{2+}$ concentration in 7-day-old dried fibroblast monolayers.](http://iai.asm.org)

**FIG. 2.** Relationship between prolyl hydroxylase activity and Fe$^{2+}$ concentration in 7-day-old dried fibroblast monolayers. Average of four determinations.

### TABLE 1. Dual effect of streptococcal LTA on prolyl hydroxylase activity in dried fibroblast monolayers with exogenous, nonhydroxylated collagen as substrate

<table>
<thead>
<tr>
<th>Hydroxylase assay system*</th>
<th>Enzyme activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer (no reaction mixture)</td>
<td>442 ± 36</td>
</tr>
<tr>
<td>Complete (control)</td>
<td>1,428 ± 153</td>
</tr>
<tr>
<td>Complete + LTA</td>
<td>1,977 ± 84*</td>
</tr>
<tr>
<td>Complete, but Fe$^{2+}$ preincubated with LTA</td>
<td>1,537 ± 65</td>
</tr>
<tr>
<td>Complete, but ascorbic acid preincubated with LTA</td>
<td>1,995 ± 76*</td>
</tr>
<tr>
<td>Complete, but alphaketoglutarate preincubated with LTA</td>
<td>2,288 ± 125*</td>
</tr>
<tr>
<td>Complete, but dithiothreitol preincubated with LTA</td>
<td>1,957 ± 80*</td>
</tr>
</tbody>
</table>

* Concentration of LTA used per experiment; 100 μg/ml; collagen substrate added, 5 × 10$^6$ cpm per monolayer.

* Tritiated water released, cpm (± the standard deviation) per mg of dry weight of protein. Each value is the average of four determinations. For controls, assays without cell monolayers resulted in a nonspecific background of $99 ± 17$ cpm ($n = 5$). Also, assays with dried (or wet) monolayers previously immersed in boiling water (0.5 h) gave a specific activity of only $150 ± 25$ ($n = 3$).

* Statistically significant differences when compared with control (unpaired t test; $P < 0.05$), but not significantly different when compared among themselves ($P = 0.05$).
buffer. Surprisingly, prolyl hydroxylase activity increased by 38% when LTA was added directly to the complete system. Conversely, preincubation of Fe\(^{3+}\) with LTA in buffer or reaction mixture prevented this increased activity.

**TABLE 2.** Prolyl hydroxylase activity by wet fibroblast monolayers with normal or nonhydroxylated collagen in situ

<table>
<thead>
<tr>
<th>Hydroxylase assay system(^a)</th>
<th>Enzyme activity(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Monolayers containing normal collagen</strong></td>
<td></td>
</tr>
<tr>
<td>Buffer (no reaction mixture)</td>
<td>1.074 ± 199(^c)</td>
</tr>
<tr>
<td>Complete (control)</td>
<td>1.230 ± 129(^c)</td>
</tr>
<tr>
<td>Complete + LTA</td>
<td>1.282(^c)</td>
</tr>
</tbody>
</table>

| **Monolayers containing nonhydroxylated collagen** |                         |
| Buffer (no reaction mixture) | 967 ± 123\(^d\) |
| Complete (control)            | 2,439 ± 274 \(^d\) |
| Complete + LTA                | 1,503 ± 155\(^d\) |
| Complete, but reaction mixture preincubated with LTA | 1,607 ± 155\(^d\) |
| Complete, but Fe\(^{3+}\) preincubated with LTA | 1,610 ± 155\(^d\) |
| Complete, but ascorbic acid preincubated with LTA | 2,420 ± 260\(^d\) |
| Complete, but alphaketoglutarate preincubated with LTA | 2,250 ± 274\(^d\) |
| Complete, but dithiothreitol preincubated with LTA | 2,600 ± 260\(^d\) |

\(^a\) Concentration of LTA per experiment. 100 \(\mu\)g/ml of total volume.

\(^b\) Tritiated water released, cpm (± the standard deviation) per mg of dry weight of protein. Total radioactivity per monolayer, 42,696 ± 983 cpm. With one exception, each experiment is the average of from 4 to 8 determinations.

\(^c\) Controls, same as in Table 1.

\(^d\) Statistically significant difference from control monolayers containing nonhydroxylated collagen (unpaired \(t\) test; \(P < 0.05\)).

\(^e\) Not statistically different from control monolayers containing nonhydroxylated collagen (unpaired \(t\) test; \(P > 0.05\)).

**FIG. 3.** Return of increased prolyl hydroxylase activity in dried monolayers previously inhibited by preincubation of Fe\(^{3+}\) with LTA, with increasing concentrations of Fe\(^{2+}\). One hundred percent activity equals dried monolayers without LTA. Each point is an average of four determinations.

enzyme activity remaining comparable to that of the complete system without LTA. However, this lack of increased activity could be negated by the addition of excess Fe\(^{2+}\). Figure 3 shows that when increasing concentrations of Fe\(^{2+}\) were preincubated with a constant amount of LTA, enzyme activity increased to that observed after addition of LTA directly to the complete assay system; i.e., an increase in prolyl hydroxylase activity of nearly 40% (complete system plus LTA; Table 1). Finally, preincubation of ascorbic acid, alphaketoglutarate, and dithiothreitol with LTA in buffer before addition to the reaction mixture also resulted in significantly increased enzymatic activity (average of 46%) compared with the control.

**Effect of LTA on prolyl hydroxylase activity in wet monolayers.** Wet monolayers were used because of the increase (instead of the expected decrease) in prolyl hydroxylase activity observed with dried monolayers after addition of LTA. Impetus for this approach came from the following. (i) Dried monolayer activity depended on the penetration of exogenous substrate into the cell, whereas wet monolayers synthesized and contained this nonhydroxylated substrate in situ, and (ii) these wet monolayers had been used earlier to detail the formation of defective collagen by LTA (12). Also, they had a collagenous content of 33% when grown with a, a'-dipyridyl. This compared favorably with a collagen content of 27% (determined earlier; reference 12) after exposure to LTA. Mouse fibroblast monolayers normally contain approximately 6.5% collagenous protein (12).

Table 2 shows that the amount of hydroxyproline formed by wet fibroblast monolayers containing normal (control) or nonhydroxylated collagen in buffer is identical. This continued activity is probably due to necessary residual cofactors remaining within, or attached to, the cells after the wash procedure. These monolayers with nonhydroxylated collagen substrate in situ exhibited considerably more prolyl
TABLE 3. Restoration of prolyl hydroxylase activity with excess Fe$^{2+}$ in wet fibroblast monolayers after inhibition by LTA

<table>
<thead>
<tr>
<th>Complete hydroxylation assay system with</th>
<th>Enzyme activity $\pm$ cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 mM Fe$^{2+}$ (control)</td>
<td>2.731 ± 274</td>
</tr>
<tr>
<td>0.1 mM Fe$^{2+}$ + LTA</td>
<td>1.524 ± 155</td>
</tr>
<tr>
<td>0.2 mM Fe$^{2+}$ + LTA</td>
<td>2.936 ± 274$^c$</td>
</tr>
<tr>
<td>0.3 mM Fe$^{2+}$ + LTA</td>
<td>2.681 ± 274$^c$</td>
</tr>
</tbody>
</table>

$^a$ Same as in Table 2.
$^b$ Same as in Table 2. Each value is the average of two determinations.
$^c$ Statistically, no difference from control value (unpaired t test; $P > 0.05$).

hydroxylase activity (by 70%) than did dried monolayers with exogenous substrate (Table 1). Also, they contained a total of 14,090 cpm attributed to nonhydroxylated collagen substrate (42,696 cpm x 33%), of which approximately 17% was eventually hydroxylated (Table 2; control monolayers containing nonhydroxylated collagen). The addition of control reaction mixture or reaction mixture plus LTA in lieu of buffer to monolayers containing normal collagen (i.e., grown without a,a’-dipyridyl) failed to change the extent of hydroxyproline formation. This indicates that the amount of hydroxylation already completed is not altered by addition of LTA. Monolayers containing nonhydroxylated collagen in complete reaction mixture instead of buffer displayed a marked increase in hydroxylation activity (by 252%), emphasizing the need of necessary cofactors for synthesis. Also, hydroxyproline formation increased by almost 100% when nonhydroxylated collagen replaced normal collagen in situ (Table 2; controls in both assay systems). Unlike the results with dried monolayers, addition of LTA directly to, or when preincubated with, reaction mixture did not increase enzyme activity. Instead, hydroxylation remained inhibited by 38%, the resulting activity being equal to that only observed with normal collagen (Table 2; control monolayers containing normal collagen). Only preincubation of Fe$^{2+}$ with LTA in buffer or reaction mixture resulted in decreased prolyl hydroxylase activity (by 34%). Likewise, neither inhibition nor increased enzyme activity was observed (Table 2) after preincubation of the remaining components of the reaction mixture with LTA in buffer. No inhibition of enzyme activity was ever noted when LTA was added to dried monolayers (Table 1).

As indicated, increased prolyl hydroxylase activity by LTA in dried monolayers could be prevented by preincubating Fe$^{2+}$ with LTA. Full activity was restored in the presence of excess iron (0.5 mM; Fig. 3). By comparison, considerably less Fe$^{2+}$ was needed to override the inhibitory effect on prolyl hydroxylase activity by LTA with the wet monolayer system (0.2 mM Fe$^{2+}$; Table 3).

**DISCUSSION**

The use of monolayers in this study permitted better simulation of an in vivo environment for pursuing the mechanism of LTA involvement in abnormal collagen biosynthesis. Cofactors remaining within each monolayer did permit some marginal prolyl hydroxylase activity to continue in the absence of exogenously added requirements. However, this did not affect the results obtained. Likewise, serum albumin was omitted in these studies. Albumin has a high affinity for LTA, and its presence would have necessitated a greater amount of this amphiphile for comparable cytotoxic and biochemical results than its absence would have (12, 21). Also, this protein had been added to reaction mixtures by others to increase prolyl hydroxylase activity (1, 18). However, we found that the amount of tritiated water released in our assay system without albumin was no different than that with this protein. Finally, differences in hydroxylation activity after storage between the two substrates tested is probably related to a difference in molecular weight, with greater stability of activity with time being observed for the larger molecule. Others had come to a similar conclusion (9).

These studies utilized dried and wet fibroblast monolayers. The former differed from the latter in that they were nonviable and incapable of cellular metabolic regulation and exhibited considerably less prolyl hydroxylase activity than did equivalent wet fibroblast monolayers. However, they still retained an ability to utilize exogenous substrate, confirming the earlier work of others (27). Air drying of these intact fibroblast monolayers had been shown to destroy permeability barriers between enzyme and substrate (27), undoubtedly leading to irreversible membrane damage. LTA also seems to cause membrane alterations as well as being responsible for abnormal collagen biosynthesis. For example, low concentrations of LTA caused morphological changes and some leakage of labeled cell or protein from cells in tissue culture (12). In addition, higher concentrations (as in this study) resulted in changes in monolayer morphology (equivalent to those appearing in Fig. 1B; reference 12) within 0.5 h, the incubation period for these enzymatic studies. Finally, the cytotoxicity of LTA as a result of possible membrane alterations had been demonstrated with HeLa cell monolayers pre- and postlabeled with selenomethionine (J. Sredy, Ph.D. thesis, Thomas Jefferson University, Philadelphia, Pa., 1981).

The effects of LTA on prolyl hydroxylase activities in dried and wet monolayers were decidedly different. LTA is a scavenger of cations (6, 15), and Fe$^{2+}$ is required for prolyl hydroxylase activity (1, 9, 18). Therefore, the increased prolyl hydroxylase activity exhibited by dried monolayers on addition of LTA was unexpected. This may be a reflection of even greater membrane perturbation by this amphiphile than that resulting from the drying of these monolayers. This probably permits more rapid influx of exogenous high-molecular-weight substrate (and cofactor penetration) directly into the cisternae, the site of prolyl hydroxylation (19), resulting in increased enzymatic activity. This increase implies that very little, if any, Fe$^{2+}$ is removed by LTA under these conditions. Substantiating this was the finding that, after LTA addition, prolyl hydroxylase activity in dried monolayers was 95% of that exhibited by wet monolayers with substrate in situ in the absence of LTA. This further proves the membrane perturbation capabilities of this amphiphile. LTA also binds more Fe$^{2+}$ before than after its addition to dried monolayers. The inhibition of increased enzyme activity by preincubation with Fe$^{2+}$ and the results observed from the prior combination of LTA with each of the other ingredients of the assay system (Table 1) tend to confirm this. This implies that, after preincubation of LTA with iron, enough Fe$^{2+}$ is still available for continued normal prolyl hydroxylase activity. LTA is believed to act as a cation exchanger and has been shown to exhibit relative affinities for various cations for its anionic sites (6, 15). Therefore, our findings suggest that LTA may be acting as a competitive inhibitor of Fe$^{2+}$ for prolyl hydroxylase activity with the other available intracellular cations. This seems plausible, since (i) the amount of LTA utilized in these experiments contained 0.39 μmol of phosphorus (23), which was more than sufficient to bind all of the Fe$^{2+}$ (0.1 μmol) added to the reaction mixture (a ratio of 4:1) and (ii) that prolyl hydrox-
lase activity was decreased by 70% in the absence of added Fe²⁺ (Fig. 2). Finally, the restoration of increased prolyl hydroxylase activity in dried monolayers by preincubating LTA with Fe²⁺ occurred only when successively higher concentrations of Fe²⁺ were used (Fig. 3). This probably results from the increasing frequency with which Fe²⁺ becomes available from this cationic exchanger, reaching maximal activity when sufficient Fe²⁺ is added to overcome the effect of LTA itself.

By comparison, addition of LTA to resting wet monolayers with substrate already present in situ resulted in the expected reduction of prolyl hydroxylase activity. In this case, which is more closely analogous to the in vivo pathogenic process, LTA apparently is able to bind more Fe²⁺ than when added directly to dried monolayers. The identical decrease in prolyl hydroxylase activity when LTA was added to the reaction mixture as when preincubated with Fe²⁺ tends to substantiate this belief. Therefore, it would appear that binding of LTA to membranes of dried and wet monolayers is different when based on the subsequent availability of Fe²⁺ for cellular prolyl hydroxylase activity. Although wet monolayers are metabolically more active and more versatile, a reason for this difference now would be only speculative.

The ability of LTA to act as a cationic exchanger with Fe²⁺ and the other cellular ions, as well as the presence of intracellular Fe²⁺ inaccessible to this amphiphile, probably accounts for the lack of complete inhibition of prolyl hydroxylase activity by LTA in the wet-cell system. Likewise, the complete lack of enhanced enzyme activity by these monolayers (as opposed to dried cell preparations) is undoubtedly due to abundant substrate already present in situ. This would circumvent a need for the cellular penetration of exogenous substrate required with dried monolayers.

Considerably less iron (two and one-half times less) was needed to reverse the partial inhibition of prolyl hydroxylase activity in viable cells than to restore increased enzyme activity in equivalent dried cell preparations with the use of LTA. This was so even though the extent of change in each direction was equal (38%). Although the two systems are not directly comparable (because only one required preincubation of Fe²⁺ with LTA for decreased activity), these results suggest that viable cells do cause the release of some Fe²⁺ from LTA, whereas dried preparations do not. This would explain the need for a lesser amount of exogenous Fe²⁺ to overcome the inhibition of prolyl hydroxylase activity in the wet system. Without this ability, only after exceeding the relative exchange affinities of LTA for cations with excessive concentrations of Fe²⁺ would restoration of initial activity occur, i.e., as seen with dried fibroblast monolayers.

These studies, together with our earlier findings (12), confirm that LTA does not affect collagen polypeptide chain formation. However, it does decrease markedly the extent of hydroxylation of collagenous peptides through its interaction with Fe²⁺. This would prevent formation of a stable triple helix and result in the accumulation and slow secretion of nonfunctional collagenous protein (19). Earlier, we had shown marked accumulation of abnormal collagen (by 450%) in growing mouse fibroblast monolayers after exposure to LTA (12). Also, we had speculated that increased thickening of the basement membrane of mouse glomeruli in tissue culture after exposure to this amphiphile would result from the accumulation (26). Expressing some of our earlier data (Table 3; reference 12) in a different manner has now shown that the percent hydroxylation of all (i.e., retained and secreted) collagenous protein synthesized by these same mouse fibroblast monolayers before and after treatment with LTA was 31.4 and 18.5%, respectively, a reduction in hydroxylation ability by LTA of 41%. In agreement with this is the almost identical reduction in prolyl hydroxylase activity (by 38%) observed after addition of LTA to wet fibroblast monolayers in this study.

Our findings indicate that complete inhibition of prolyl-proline formation is not required for continued formation and accumulation of defective collagenous protein by mouse fibroblast monolayers in tissue culture. Also, that while LTA reduces proline hydroxylation, a,a'-dipryridyl results in the formation of completely unhydroxylated collagen.

Finally, these previous studies (12) have resulted from the exposure of cell monolayers to as little as from 2 \times 10^{-3} to 5 \times 10^{-3} µg of LTA per cell. Thus, partial inhibition of prolyl hydroxylase activity, defective collagenous protein synthesis, and increased thickness of mouse glomerular basement membrane may, indeed, be manifestations of minute amounts of group A, type 12 streptococcal LTA.

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LITERATURE CITED


