Morphological Changes and Pathology of Mouse Glomeruli Infected with a Streptococcal L-Form or Exposed to Lipoteichoic Acid

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The morphology and pathology of cultured mouse glomeruli were examined at the cellular and subcellular levels after infection with a physiological isotonic L-form of Streptococcus pyogenes type 12 or exposure to streptococcal lipoteichoic acid. These changes, as viewed by light microscopy, were identical regardless of the method used to induce glomerular cytotoxicity. They were characterized by an initial reduction in the outgrowth of cells, some cellular granulation, and later, destruction of the confluent monolayer. Once initiated, cytotoxicity could not be reversed by refeddings, and complete glomerular destruction resulted after 2 weeks. Electron microscope studies revealed that the basement membrane of intact glomeruli exposed to streptococcal lipoteichoic acid had become greatly thickened (two- to fourfold) and electron dense. Our recent biochemical findings have shown that streptococcal lipoteichoic acid increases the amount of collagen formed and retained by mouse fibroblasts in tissue culture as well as causing a reduction in the hydroxylation of proline in both intracellular and secreted collagenous material (Leon and Panos, Infect. Immun. 40:785–794, 1983). These results, together with the present findings, suggest that the thickening of the glomerular basement membrane may be due to defective collagen biosynthesis as a result of streptococcal lipoteichoic acid. The use of cultured glomeruli as a model system for studying the earliest basement membrane alterations in the absence of an immune response as a result of streptococcal lipoteichoic acid is suggested.

An osmotically fragile L-form of Streptococcus pyogenes type 12, after being quickly rendered osmotically stable by reducing the sodium chloride content of the growth medium with the temporary use of oleic acid, readily attaches to cultured human kidney cells (1, 7). Therefore, a rigid cell wall is not a prerequisite for host attachment in vitro. Also, lipoteichoic acid (LTA) from S. pyogenes and its L-form, in addition to preventing attachment of S. pyogenes to human cell monolayers, is cytotoxic for a variety of human cells in vitro (1, 6, 7). Finally, the morphological changes and eventual destruction of kidney cell monolayers by this L-form are also caused by the LTA from this organism and its parental streptococcus, which are structurally different (1).

This paper is a sequel to our recent biochemical findings showing that a small amount of LTA from S. pyogenes results in the intracellular production and accumulation of defective (practically hydroxyproline-free) collagen by mouse fibroblasts (6). Likewise, collagenous protein secreted by this cell line shows a significantly reduced content of hydroxyproline as compared with control cells unexposed to LTA (6). This study utilizes cultured mouse glomeruli to detail morphological changes at the cellular and subcellular levels after infection with a physiological isotonic L-form of S. pyogenes or after exposure to LTA from this coccus. We attempt to coordinate these results with our recent biochemical findings mentioned above (6). Also, we show that the subcellular morphology and pathology of glomeruli exposed in vitro to streptococcal LTA are similar to those observed in poststreptococcal glomerulonephritis (2, 15).

MATERIALS AND METHODS

Organism and media. A physiological isotonic L-form of S. pyogenes type 12 described earlier was used.
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This organism was grown in regular brucella broth (Pfizer Diagnostics, Brooklyn, N.Y.) with bovine serum albumin, fraction V (8 g/liter; Armour Pharmaceutical Co., Chicago, Ill.), and a total sodium chloride content of 0.85% (wt/vol). Viable counts were obtained as detailed previously (7).

The tissue culture medium (TCM) used throughout was Eagle basal medium with 10% (vol/vol) inactivated fetal calf serum plus 100 U of penicillin per ml and 1 U of amphotericin B (GIBCO Laboratories, Grand Island, N.Y.) per ml.

LTA. LTA was obtained from S. pyogenes type 12 as detailed previously (14) and stored at 70°C in the dry state with a desiccant. A stock solution of LTA in TCM without serum, adjusted to pH 7.2 with 5.6% sodium bicarbonate, was used to dispense the appropriate amount(s) of this amphiphile as needed for each experiment. Large amounts of LTA (1 to 10 mg) were always necessary to obtain reproducible protein values of purified LTA preparations by the method of Lowry et al. (8). These values ranged from 2 to 5% (1). However, amino acid analyses by the procedure of Gerber and Hare (3) as modified by L. Milakofsky (personal communication), using o-phthalaldehyde and fluorescence detection, showed an amino acid content in such preparations of only 0.45%. At present there is no explanation for this difference.

Isolation, preparation, and growth of glomeruli. A modification of the procedure of Grant et al. (4) was utilized to obtain the glomeruli. Wherever possible, sterile equipment was used and all operations were performed in a sterile hood. Newborn Swiss-Webster outbred mice, less than 5 days old, were killed by decapitation, and their kidneys were removed and placed in ice-cold (4°C) TCM. Kidneys from 20 to 30 mice were minced and pressed through a 150-mesh sieve (Collector Tissue Sieve; Collector Pestle and Collector Screen 150; Bellco Glass, Inc., Vineland, N.J.), and the sieve was washed with portions of cold TCM (5% volume, 30 ml) to disengage kidney “pieces” adhering to the underside of the sieve. The minced kidney suspension was divided into two 15-ml conical centrifuge tubes and placed in crushed ice. After 20 min, the upper 3/4 layer of each tube was discarded, and the remainder was combined, resuspended in TCM (15 ml), and allowed to settle in ice for 20 min as before. The supernatant was then removed, and the pellet was resuspended in 1 ml of 12% (wt/vol) Ficoll in TCM. This suspension was layered onto a discontinuous Ficoll gradient in TCM (see below) and centrifuged at 300 x g for 2.5 min. The glomeruli were visible as a band at the 15 to 18% interface. The glomerular band was removed with a capillary pipette, suspended in TCM (5 ml at 5°C), and centrifuged at 3,000 x g for 15 min. The pellet obtained constituted the glomerular preparation for these studies.

Glomerular monolayers were obtained by resuspending the glomerular preparation in a minimal volume of TCM and seeding 107 glomeruli into plastic 2-cm² wells or 3.5-cm² petri dishes. Glomerular quantitation, viability, and subsequent death after detachment were established by the use of erythromycin B (10). Glomerular seeding was done in such a way as to permit glomeruli to cover the bottom of the well or dish in the presence of a minimal amount of TCM.

When done properly, this resulted in a liquid interface between the plastic surface and glomeruli but without sufficient fluid to allow for flotation of the glomeruli, thereby permitting maximum attachment after 24 h. To prevent drying, wells or dishes were incubated (37°C) at 90% humidity in an atmosphere of 5% CO₂-95% air. After 24 h of incubation, TCM was added to cover the now attached (cultured) glomeruli. Glomeruli were refed weekly.

Preparation of Ficoll discontinuous gradient. Various concentrations of Ficoll (Sigma Chemical Co., St. Louis, Mo.) were prepared in TCM: 12, 15, 18, and 20% (wt/vol). The gradient was prepared just before use by placing 1.5 ml of 20% Ficoll on the bottom of a 15-ml centrifuge tube, followed by the layering of 1.5 ml each of 18 and 15% Ficoll. Finally, the 12% Ficoll containing the suspended glomerular preparation was overlaid onto the gradient, and the completed gradient was centrifuged (see above).

Infecting glomeruli with the physiological isotonic L form. Glomeruli in 2-cm² wells, after being seeded for 0, 2, and 5 days, were infected with 0.1 ml (108 CFU) of an 18-h culture of the L-form and incubated. After the specified incubation period (see below), the medium was removed, and the glomerular monolayers were washed twice with phosphate-buffered saline before being fixed (95% methanol for 5 min) and stained (10 min) with Giemsa (Fisher Scientific Co., King of Prussia, Pa.). All preparations were viewed with a Leitz inverted microscope.

Glomerular exposure to lipoteichoic acid. After 24 h to allow for glomerular attachment, TCM was removed from each well and replaced with 0.9 ml of fresh medium plus an amount of LTA (0.1 ml of stock solution) to be tested. After appropriate incubation periods (see below), glomeruli were stained and viewed as described above.

Electron microscopy. Wells containing attached glomeruli (after 24 h) were exposed to 125 µg of LTA per ml as before. After 4 days of incubation, medium was decanted, and the glomeruli still attached (approximately 50% of control) were gently removed by scraping with a rubber policeman into phosphate-buffered saline (pH 7.2) and centrifuged at 2,000 x g for 5 min at 4°C. The glomerular pellet was fixed in 2% (wt/vol) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 2 h at 4°C. After a wash with sodium cacodylate buffer, a second fixation was done with 1% (wt/vol) osmium tetroxide in the same buffer for 1 h at room temperature. The pellet was washed with distilled water, dehydrated, and stained en-bloc with uranyl acetate (2% [vol/vol]) in 50% ethanol and embedded in Epon 812. Thin sections were double stained with lead citrate and uranyl acetate (7).

Collagenase treatment of glomeruli after lipoteichoic acid exposure. Glomeruli treated with LTA were fixed, dehydrated, and embedded as described above for electron microscopy, except that the en-bloc staining step was omitted. Thin sections were deposited onto uncoated copper grids and treated with 1 drop of 5% hydrogen peroxide for 10 min at 23°C. After a wash with distilled water, grids were incubated in a solution of collagenase (0.3% [wt/vol]; bacterial; no. C-0773; EC 3.4.24.3; Sigma) in 0.1 M CaCl2-2.5 mM N-ethylmaleimide-30 µM phenylmethylsulfonyl fluoride in Tris-hydrochloride buffer, 0.05 M (final pH, 7.6), for 15 min at 25°C. All grids were washed with distilled water and stained with uranyl acetate and lead citrate (7).
RESULTS

Glomeruli infected with the L-form or exposed to streptococcal LTA. These mouse glomeruli readily attached to the plastic surface, with maximum attachment and rapid cellular outgrowth being evident after 24 h. Also, they remained morphologically identifiable throughout the course of these studies when examined by light microscopy. The cellular outgrowth around each glomerulus was comprised of large irregular and spindle-shaped cells. These cells appeared to be crowded, overlapped, and several layers deep when closest to the darkened center of the organelle. Their cytoplasm was not vacuolated (Fig. 1).

Earlier studies from this laboratory had established that this physiological isotonic L-form, although remaining viable in tissue culture for days, was incapable of growth in terms of detectable cellular division (7). By comparison, the parental coccus quickly overgrew tissue culture monolayers when used as the infectious agent (7). Therefore, glomeruli were infected only with the L-form on days 0, 1, and 5 after attachment to the plastic surface. Glomeruli infected at day 0 did not attach, even after 48 h of incubation. However, when incubation was continued for 4 days after infection and the glomeruli were examined microscopically, only 2 or 3 of the $10^4$ glomeruli seeded were found attached. These attached glomeruli were characterized by minimal cellular outgrowth, with their cytoplasm being granular in appearance and containing heavily staining (dense) nuclei. Also, many of the cells closest to the center of the glomerulus appeared enlarged and dinucleated when viewed at a higher magnification (Fig. 2). These few glomeruli became detached and nonviable 7 days after L-form infection. Heavily staining nuclei in human heart cells and dinucleated human kidney cells had also been observed previously in tissue culture after infection with the L-form (1, 7).

Glomerular monolayers infected with the viable L-form 2 days after attachment and examined 24 h postinfection showed a cytotoxicity characterized by a greatly reduced outgrowth of cells which were otherwise normal except for some cytoplasmic granulation, as compared with control monolayers. In contrast to infectivity at day 0, approximately 50% of these glomeruli were still able to attach to the plastic surface. No additional morphological changes occurred after incubation for 48 h. However, these infected cultures showed the greatest change, as compared with control monolayers, when examined by light microscopy 4 days after infection. The
FIG. 2. Glomerular monolayer infected with the physiological isotonic L-form at day 0 and examined after 4 days. Arrows show dinucleated cells. Giemsa stain. Bar, 19 μm.

FIG. 3. Glomerular monolayer infected with the L-form 24 h after seeding and examined 4 days later. Monolayers exposed to 125 μg of LTA per ml for the same time period were identical. Giemsa stain. Bar, 35 μm.
cellular outgrowth surrounding the center of the glomerulus, although appearing normal, had now become minimal. Also, no semblance of a monolayer was evident, even though the glomeruli were still attached (Fig. 3). The dark-staining nuclei so apparent in cultures infected at day 0 and examined 4 days later were not observed here. Finally, there was no decrease in the number of attached glomeruli, and no further morphological changes were evident 7 days after infection. These observations were the same for glomerular monolayers infected with the L-form 5 days after attachment and examined 24 and 48 h and 7 days thereafter. Refeeding did not lead to recovery, and all glomeruli became detached after 2 weeks.

Twenty-four hours after seeding, glomeruli were exposed to various concentrations of streptococcal LTA (125, 175, 200, and 250 μg of LTA per well) for 1, 2, and 4 days, and changes in their morphology were recorded. The changes noted were identical to those following infection with the physiological isotonic L-form (Fig. 1 to 3). Also, and regardless of the time of exposure to LTA (maximum of 4 days), 50% of the glomeruli seeded continued to attach to the plastic surface. This percentage remained constant and did not change when the concentration of LTA was varied. Finally, glomerular destruction was complete when incubation was extended for 2 weeks. As above, refeeding to remove residual amounts of LTA did not reverse this cytotoxic effect. By comparison, glomerular controls were easily maintained for 1 month when reseeded at weekly intervals.

Glomeruli exposed to 125 μg of LTA per well for 4 days were also examined by electron microscopy. This concentration and time of exposure were selected because they afforded the maximal morphological changes without in-
increased glomerular detachment. Figures 4 and 5 show thin sections of a typical glomerulus before and after exposure to LTA, respectively. In addition to the usual subcellular entities visible, the basement membrane of the unexposed glomerular control appears rather uniform in width and not deeply stained (Fig. 4, arrows). However, after exposure to LTA, a two- to fourfold thickening of the basement membrane was observed, with the cells within the glomerulus remaining morphologically normal. In addition, this thickened membrane was electron dense (Fig. 5, arrows). The subsequent disappearance of this electron-dense layer after treatment with the enzyme collagenase proved this abnormally thickened structure to be basement membrane (Fig. 6).

DISCUSSION

The destruction of an organelle by a physiological isotonic L-form of S. pyogenes expands our previous results, which first established the ability of this organism to infect and destroy a variety of human cell monolayers in tissue culture (1, 6, 7). In the present study, most rapid glomerular destruction occurred when infection preceded surface attachment. This effect had been noted before with human heart cells in vitro (7). This may be due to an alteration of surface charges preventing glomerular attachment or to an increased detrimental metabolic effect by the infecting L-form as a result of the greater surface area available to it before organelle attachment. Glomerular destruction by the L-form after its attachment, although slower, was total, with half of the glomerular population being rapidly eradicated and the other half dying with time. Therefore, L-form cytotoxicity appears to be equally as effective for cultured mouse glomeruli and human cell monolayers.

Glomerular morphological changes induced by streptococcal LTA were the same as those observed after L-form infection. This confirms
an earlier study that had documented the lack of any apparent difference in the destructive ability of the LTA isolated from either the L-form or its parental S. pyogenes for human kidney monolayers in tissue culture despite differences in the length and composition of this amphiphile from these two organisms (13, 14). Finally, and as opposed to an earlier study with cultured human cell monolayers (1), glomerular destruction did not increase when the concentration of LTA was increased. Possible explanations for this include a slower penetration of the amphiphile into the glomerulus and/or the appreciably slower growth rate of this organelle when compared with cultured human cell lines. Nevertheless, the irreversible cytotoxicity of streptococcal LTA, as evidenced by treatment of glomeruli at day 0 and their failure to attach after 48 h, suggests a rapid and permanent binding of LTA with possible direct metabolic intervention, membrane perturbation(s), or both. Earlier studies with mouse fibroblasts in tissue culture had shown that streptococcal LTA is indeed capable of eliciting a subtle cytotoxic effect (without release of protein) or initiating rapid cell death, depending on the concentration used (6).

This laboratory had established that LTA from S. pyogenes caused mouse fibroblasts to increase their content of collagenous protein by an astounding 450% and that this collagenous protein was defective, being practically hydroxyproline-free. In addition, although LTA did not affect the amount of collagenous protein secreted, it did cause a reduction in the hydroxylation of its proline content (6). It has been established that hydroxyproline formation occurs almost exclusively in mammalian collagen (11, 12). It is known that collagen is a major macromolecule and component of the basement membrane of the kidney. This membrane has a profound role in glomerular filtration. Also, poststreptococcal glomerulonephritis is often a sequela of group A, type 12 infection. Therefore, the finding of an electron-dense and greatly thickened basement membrane after LTA exposure may be indicative of intracellularly defec-
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Acute glomerulonephritis in children may be accompanied by swelling of the basement membrane and an impairment of the glomerular filtration process (9). An electron microscopic study of four sequential biopsies of a young patient with acute glomerulonephritis has shown that the glomerular basement membrane markedly thickens, cells within the glomerulus remain normal, but dense deposits of electron-dense material "between epithelium and basement membrane and partially residing within the basement membrane" do occur (15). In another study of the glomerular structure in glomerulonephritis, "the original basement membrane appeared thicker and large masses of newly formed basement membrane-like material were seen" (2). These findings seemingly parallel our results with glomeruli in tissue culture after infection with the L-form or exposure to streptococcal LTA. Although the increased electron density of the basement membrane in biopsies of patients with glomerulonephritis is similar to that observed in these studies, a reason for this increase is unknown (15).

The excretion of LTA by the group A streptococci is well established (5). Also, details of the very earliest changes in acute glomerulonephritis are not well established primarily because of the lack of an accepted experimental model system. Our recent biochemical studies (6), together with the thickening of the basement membrane and the eventual destruction of the entire glomerulus in these studies, have permitted a possible correlation of morphological, biochemical, and pathological changes as a result of L-form infectivity or exposure to LTA. This was done in vitro and in the absence of complement or specific antibody and suggests that a nonimmune LTA-mediated mechanism for kidney damage may be related to disturbed collagen biosynthesis.

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LITERATURE CITED


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