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Persistence, Pathogenesis, and Morphology of an L-Form of *Streptococcus pyogenes* Adapted to Physiological Isotonic Conditions When in Immunosuppressed Mice

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Evidence obtained using nonimmunosuppressed and newborn mice suggests that the immune response of the host plays a role in the rapid removal of a physiologically isotonic L-form of *Streptococcus pyogenes*, since its inability to persist in vivo was not due to osmotic lysis. With mice immunosuppressed with methylprednisolone sodium succinate, viability and detection of this L-form by fluorescent antibody was prolonged for at least 2 weeks, the approximate duration of immunosuppression in these mice. However, heat-killed L-form cells only persisted for 3 days in such mice. Therefore, persistence of a viable L-form in these treated mice was not simply due to the lack of removal of L-forms by a compromised host. At no time was there any indication of illness in nonimmunosuppressed or immunosuppressed mice after L-form injection, and all internal organs, when examined macroscopically, remained normal. Thus, overt pathogenesis was not a characteristic of this L-form in a suitable host even when its immune response had been compromised. The microscopic morphology of the L-form after isolation from immunosuppressed mice changed drastically. It was typically micrococcal in appearance and exemplified the cellular variability achievable by this organism in vivo. Also, streptolysin S production was increased markedly by passage of the L-form in immunosuppressed mice. However, M protein, as a cellular component, was not detected serologically, nor was any reformation of a rigid cell wall apparent by electron microscopy after isolation of this streptococcal L-form from such mice.

An L-form of *Streptococcus pyogenes* has been shown capable of being rapidly adapted to grow in physiological isotonic medium and to destroy human heart cells in tissue culture (15). Subsequently, electron spin resonance spectroscopy revealed that this rapid adaptation was due to changes in the lipid chain rigidity of the L-form membrane (1). However, the fact that osmotically fragile L-forms can be adapted to grow in physiological conditions does not necessarily imply that their capability for survival in vivo will be comparable to that of the parental bacterium. Conflicting reports have appeared. For example, Clasener et al. (2), employing L-forms of the group A streptococci seemingly adapted to physiological conditions, found that these L-forms were unable to persist in mice beyond 40 and 60 min after intraperitoneal or intravenous injection, respectively, suggesting a clearing by the immune defenses of the animals. However, in an earlier study Schmitt-Slomska et al. (25), using similar but osmotically fragile streptococcal L-forms injected intraperitoneally into mice, were able to isolate these organisms up to 25 days after inoculation, the maximum time tested. It is known that the group A streptococci can persist in the tissues of rabbits in the absence of bacteremias, for up to 104 days after intravenous injection (6). By comparison, both animal and human sera have been found to be lethal for L-forms, protoplasts, and spheroplasts of certain gram-positive and gram-negative pathogenic bacteria but to be without effect upon the parental form (18, 20). This probably accounts, in part at least, for the inability of past investigators to recover viable L-forms with regularity as early as a few hours after animal inoculation (2, 17, 25). Therefore, there is no doubt that the defense mechanisms of a host are instrumental in helping to rapidly eliminate bacterial L-forms in vivo.

Suppression of a host's immune response before infection would aid in establishing any overt pathogenic or persistent capability by an L-form. However, at present no such detailed information is available. Therefore, these stud-

ies were undertaken to determine the pathogenicity, persistence, and change in morphology of an L-form of *S. pyogenes* capable of growing under physiological conditions when injected into immunosuppressed mice.

(This investigation was presented, in part, in preliminary form at the Annual Meeting of the American Society for Microbiology, Atlantic City, May 1976.)

MATERIALS AND METHODS

Organism and media. The L-form of *S. pyogenes* (type 12) recently adapted to grow in physiologically isotonic media was again used (15). This organism was grown in brucella broth (Pfizer Diagnostics, Brooklyn, N.Y.) supplemented with 0.5% (wt/vol) NaCl (total NaCl content, 0.85%) and 0.8% (wt/vol) bovine serum albumin (fraction V, Armour Pharmaceutical Co., Chicago, Ill.). When a culture reached its mid-logarithmic phase of growth as determined by turbidity (optical density at 660 nm, approximately 0.2) and viable count (colony-forming units [CFU]), cells were spun down and washed (4°C) once each with saline and distilled water before determination of cellular protein by the method of Lowry et al. (16), with bovine serum albumin as standard. Viable counts were performed on freshly prepared Trypticase soy agar plates supplemented with 10% (vol/vol) horse serum and containing 0.85% (wt/vol) NaCl. Blood agar plates were made by adding 5% (vol/vol) defibrinated sheep blood to supplemented Trypticase soy agar medium.

Immunosuppression procedure. Initially, prednisolone (Upjohn Co., Kalamazoo, Mich.) was dissolved in saline and injected intraperitoneally into mice (0.5 mg/mouse) for 5 days. However, due to toxic side effects such as weight loss, loss of hair, lethargy, etc., and a 5% mortality rate between 3 to 5 days after initial injection, use of this drug was discontinued in favor of methylprednisolone sodium succinate (Solu-Medrol, Upjohn Co., Kalamazoo, Mich., lot no. 160DF), which is less toxic. The dosage schedule used was based upon earlier studies by others (10, 12) in which cortisone was used to augment bacterial infection; i.e., cortisone acetate (2.5 mg/mouse per 5 days) was injected intramuscularly before infection with *S. pyogenes*. Since Solu-Medrol is four times as effective as cortisone, on a weight basis, in decreasing the inflammatory response and in causing leukopenia in animals and man (8), the dose of Solu-Medrol given was reduced proportionately. Solu-Medrol in Mix-O-Vials was dissolved according to the manufacturer in the diluent supplied, each milliliter, when mixed, containing 40 mg of the drug plus additives (lactose, alcohol, phosphate). Three-week-old Swiss Webster male mice were immunosuppressed by intraperitoneal injection as follows: days 1 to 5, 0.63 mg; days 6 and 7, 0.31 mg; days 8 and 9, 0.16 mg; and day 10, 0.0 mg per 10-g mouse. All animals survived the immunosuppression schedule and exhibited no ill effects.

Mice were assayed for immunosuppression on days 9 and 16 after initial injection of Solu-Medrol. Sheep erythrocytes (2×10^8/mouse in 0.5 ml of saline) were injected intraperitoneally into mice 3 days before assaying, animals were sacrificed on the two days indicated, and their spleens were removed and weighed. Each spleen was assayed for the number of immunoglobulin M (IgM) antibody-producing cells per 10^6 cells by the Jerne plaque technique as modified by Cunningham and Szenberg (5).

Injection of the physiologically isotonic L-form. In all of these experiments, mice, after day 5 of immunosuppression, were injected intraperitoneally with 0.5 ml of mid-logarithmically grown cultures (typically, 1.5×10^6 CFU; 16 μg of L-form protein) of the L-form, while drug administration was slowly being decreased to prevent shock due to sudden withdrawal. Controls included the injection of sterile medium (0.5 ml) and portions of heat-killed (70°C, 30 min) L-form cultures (15 μg of L-form protein/0.5 ml) into immunosuppressed mice for assessing toxic effects and the persistence of antigen. Nonimmunosuppressed and newborn mice were injected intraperitoneally but only with viable L-form cultures (0.5 and 0.1 ml, respectively). In a separate study, the former were also injected intravenously (tail inoculation, 0.25 ml of culture).

Detection of the L-form. Nine- and twenty-eight-day experiments were conducted. Typically, the former utilized 50 mice, 40 immunosuppressed as described above and 10 nonimmunosuppressed. Of these, 20 immunosuppressed and all of the nonimmunosuppressed mice were inoculated with the L-form. The remaining immunosuppressed mice served as controls: they were injected with either heat-killed L-form cultures (15 mice) or with uninoculated medium (5 mice). For twenty-eight-day experiments, 75 mice were utilized in similar fashion. For the shorter-term experiments, mice were sacrificed daily by cervical dislocation, whereas for the long-term studies they were sacrificed on alternate days. From each animal (i) peritoneal fluid, both undiluted (0.1 ml) and after a 10-fold dilution in supplemented brucella broth, (ii) pieces (average weight, approximately 40 mg) of left and right kidney, spleen and liver, after being macerated with knife and forceps, and (iii) heart blood (0.1 ml) were each plated in duplicate for detection of viable L-form cells. All plates were incubated aerobically and examined after 3 and 7 days for L-form growth before being discarded. Impression smears of the various organs mentioned and slides of the peritoneal exudate from these 3-week-old mice were fixed in ice-cold methanol and stored at −40°C until needed. All such preparations were examined microscopically by the direct and indirect fluorescent-antibody technique for the presence of the L-form. All experiments were repeated in toto three times, and each value given is an average of at least six different determinations or examinations.

Finally, experiments with newborn mice were repeated five times, with at least 12 mice used in each study. The same organs and the peritoneal exudate of these mice were examined for viable L-forms as above.

Preparation of antisera and fluorescent-antibody techniques. Mid-logarithmic cultures of the L-form were centrifuged and washed with saline (two

times, 4°C), mixed with Freund complete adjuvant, and injected subcutaneously into eight New Zealand white rabbits (6 to 8 lb [ca. 3 to 4 kg] each). Each animal received 0.2 ml of L-form suspension (2.4×10^{11} CFU/ml) plus 0.2 ml of the adjuvant (total L-form protein injected, 80 μg). Injections were repeated twice, 7 days apart, and all animals were sacrificed by cardiac puncture after 21 days. Sera were tested for antibody by the agglutinin reaction using intact L-form cells suspended in 0.85% saline and titers of from 1:64 to 1:512 were obtained. Sera were pooled and absorbed with fluorescent-antibody mouse powder (Difco Co., Detroit, Mich.) for removal of nonspecific staining in the direct and indirect fluorescent-antibody techniques. For the direct fluorescent-antibody technique (21), the absorbed rabbit antiserum was conjugated with fluorescein isothiocyanate on Celite as described by Rinderknecht (24) and diluted 1:10 with buffer (0.145 M NaCl, 0.01 M phosphate, pH 7.1). Control slides of the L-form gave optimal fluorescence when treated with sera at this dilution and viewed with an American Optical Corp. Fluorostar microscope (with vertical illuminator). Likewise, for indirect fluorescent-antibody tests (21), unlabeled but absorbed rabbit sera were similarly diluted 1:10 and employed together with fluorescein-conjugated IgG fraction of goat anti-rabbit gamma globulin (lot no. 39071, Cappel Laboratories, Inc., Dowington, Pa.) at a dilution of 1:32, which was also absorbed with mouse powder and eluted with the above buffer from a Sephadex G-25 column to remove free fluorescein. Tissue cells fluoresced nonspecifically with the indirect fluorescent-antibody technique; therefore, to mask nonspecific fluorescence slides were stained with Evan blue (22). When stained by this procedure, the L-form appeared yellow-green against the orange-red color of the tissue cells. Although both the direct and indirect fluorescent-antibody techniques were equally as sensitive in our hands, the former was preferred for black and white photography. Finally, use of labeled normal rabbit serum in the direct fluorescent-antibody and of unlabeled serum in the indirect fluorescent-antibody techniques (as controls) was negative.

Morphological studies. L-form colonies were examined with a Leitz inverted microscope. For phase-contrast microscopy, the L-form was isolated from immunosuppressed mice after 6 days by plating the peritoneal exudate onto agar, incubating for 4 to 6 days, and subtransferring, by the Dienes agar block technique (7), to broth for from 2 to 3 days. Such broth cultures were transferred once again (for only 8 h) to obtain photomicrographs of cellular morphology at the early stationary phase of growth using a Zeiss Universal microscope. For electron microscopy, broth cultures of the L-form before and after passage in immunosuppressed mice were grown as above and fixed in situ using the 5% glutaraldehyde-0.2 M cacodylate buffer (pH 7.2 to 7.3) technique described by Cole (4) before being embedded in Epon. Thin sections were cut, stained with uranyl acetate and lead citrate and viewed with a Hitachi HS-8 electron microscope.

Streptolysin S. Hemolytic units were determined by the method of Younathan and Barkulis (30) utilizing 1 ml of 0.7% (vol/vol) washed sheep erythrocytes added to increasing dilutions of culture supernatant (1 ml). The amount of hemoglobin released was measured at a wavelength of 550 nm. Streptolysin S titers and cellular protein content were determined over the entire growth phase of the L-form before and 6 days after passage in immunosuppressed mice.

M protein. Samples of the L-form, before and after passage in immunosuppressed mice for 6 days, were grown in 1 liter of the supplemented brucella broth for 18 h, and the M protein extraction procedure of Lancefield (13) was performed on sedimented, washed cells. The extracts obtained were concentrated to one-tenth their volume in vacuo and examined for M protein by the capillary precipitin technique (3) with M protein (type 12) antiserum supplied by E. Fox (LaRabida, Chicago). These results were also confirmed by the slide double-diffusion test. The protein content of these concentrated extracts was 1.9 to 2.0 mg/ml.

All morphological and physiological studies were repeated at three different times, in duplicate, with fresh isolates.

RESULTS

Recovery of the L-form from nonimmunosuppressed mice. With regard to 3-week-old mice, viable L-forms were only occasionally recovered from 2 to 24 h, but were easily demonstrable by the indirect fluorescent-antibody technique in the peritoneal cavity up to 72 h after intraperitoneal injection. For the most part, however, viability in the peritoneal cavity was greatest up to but dropped appreciably 2 h after injection (from 1.2×10^6 to 1×10^2 CFU/ml). Likewise, no viable organisms were recovered from the liver, spleen, or kidneys, and each of these was devoid of antigen by indirect fluorescent antibody when examined from 2 h to 1 month after intraperitoneal injection. With mice injected intravenously, these same organs were positive by indirect fluorescent antibody up to 72 h, but some viable L-forms could be obtained, on occasion, from these and blood at 24 h but not thereafter. During these experiments, all mice remained active and well in appearance and their organs were normal when examined visually at autopsy.

Newborn mice, after intraperitoneal injection of the L-form (10^4 to 10^5 CFU), also remained normal and displayed no obvious symptoms indicative of illness up to 1 month, at which time these experiments were terminated. Again, all internal organs were normal by visual inspection. Only a few viable L-form cells were sporadically recovered from the peritoneal cavity after 8 h, and none was recovered from any of the internal organs 2 h after L-form injection. Fluorescent-antibody studies were not performed with these animals.

Recovery of the L-form from immunosuppressed mice. In preliminary experiments, L-forms were injected intravenously or intraperitoneally into 3-week-old mice. In all studies, the L-form was detected for a longer period of time and isolated in far greater numbers and with relative ease from liver, spleen, and kidneys after intraperitoneal injection. Therefore, this route of injection was preferred in all subsequent studies. Table 1 tabulates the results from immunosuppressed mice over a 9-day period. It is apparent that the L-form was recoverable in large numbers from the peritoneal cavity up to 9 days after injection. They were also isolated consistently from the various organs indicated from days 2 through 8, with these numbers decreasing after day 8. Also, this organism was present in blood from the heart up to 3 days after injection. Finally, although large numbers of the L-form persisted in such mice for the duration of these studies, all animals appeared healthy and active and their internal organs, macroscopically, were normal at autopsy.

To determine to what extent viability could be maintained in immunosuppressed mice, longer-term experiments were conducted. Table 2 tabulates the results obtained over a period of 28 days. Viable L-forms continued to persist in the peritoneal cavity up to day 10, at

TABLE 1. *Viable count and fluorescent-antibody results of tissues from immune-suppressed mice over a 9-day period*[a]

Days after L-form injection	L-form CFU					
	Peritoneal cavity (per ml of fluid)	Heart blood[b]	Organs[c]			
			Liver	Spleen	Left kidney	Right kidney
1	2×10^5	2+	(−)	(−)	(−)	(−)
2	3×10^5	1+	1+	2+	1+	1+
3	3×10^6	1+	2+	2+	2+	1+
4	4×10^5	−	2+	2+	1+	2+
5	1×10^7	1+	2+	2+	1+	2+
6	2×10^6	−	2+	3+	1+	1+
7	3×10^4	−	1+	3+	1+	1+
8	5×10^6	−	1+	2+	1+	1+
9	4×10^4	−	1+	2+	−	−

[a] Inoculum was 1.5×10^6 L-form CFU per mouse intraperitoneally (16 μg of protein). Same section of segmented organ was examined by impression onto glass slides for fluorescent antibody and on agar for CFU.

[b] −, No colonies; 1+, 1 to 2 colonies per plate; 2+, 3 to 4 colonies per plate; 3+, 5 to 6 colonies per plate.

[c] For organs only: (−), no CFU and fluorescent antibody negative; −, no CFU but fluorescent antibody positive.

TABLE 2. *Viable count and fluorescent-antibody results of tissues from immune-suppressed mice over a 28-day period*[a]

Days after L-form injection	L-form CFU					
	Peritoneal cavity (per ml of fluid)	Heart blood[b]	Organs[c]			
			Liver	Spleen	Left kidney	Right kidney
2	1×10^4	2+	2+	2+	1+	1+
4	1×10^5	1+	2+	2+	2+	1+
6	1×10^5	−	2+	2+	2+	2+
8	2×10^6	−	2+	2+	2+	2+
10	5×10^4	−	1+	2+	−	1+
12	1×10^2	−	1+	1+	−	1+
14	1×10^3	−	1+	1+	−	−
17	−[c]	−	−	−	−	−
19	(−)	−	(−)	(−)	(−)	(−)
21	(−)	−	(−)	(−)	(−)	(−)
28	(−)	−	(−)	(−)	(−)	(−)

[a] Inoculum was 8.0×10^6 L-form CFU per mouse intraperitoneally (17 μg of protein). Same section of segmented organ examined by impression onto glass slides for fluorescent antibody and on agar for CFU.

[b] −, No colonies; 1+, 1 to 2 colonies per plate; 2+, 3 to 4 colonies per plate.

[c] For organs only: (−), no CFU and fluorescent antibody negative; −, no CFU but fluorescent antibody positive.

which time their numbers decreased more rapidly. On day 17 viable L-forms were no longer recoverable from the peritoneal cavity. Similarly, no viable L-forms were detectable in the internal organs examined after 14 days, but antigen was detected by indirect fluorescent antibody up to 17 days after injection. Figure 1 illustrates a typical result of the L-form plus macrophages from the peritoneal exudate and indicates the concentration of organisms present with time as visualized by the direct fluorescent-antibody technique. As is apparent, the L-form was still easily demonstrable 14 days after injection.

Results of the Jerne plaque assay for monitoring the humoral response to sheep erythrocytes in mice 6 days after initiation of immunosuppression clearly showed that treated mice contained approximately 10 times less IgM antibody-producing cells than nonsuppressed mice and that their spleens were considerably reduced in weight (Table 3). However, similar studies with mice 13 days after L-form injection and 9 days after cessation of Solu-Medrol administration revealed that their spleen weights and numbers of antibody-producing cells had returned to normal.

Heat-killed L-forms injected into immunosuppressed mice were detectable by indirect fluorescent antibody up to 3 days after injection in the peritoneal exudate, liver, and spleen and only up to day 2 in the kidney.

Morphology. Mid-logarithmically growing L-form cells, 7 to 9 days after isolation from immunosuppressed mice, remained predominately and rather uniformly coccal in shape

TABLE 3. *Humoral immune responses to sheep erythrocytes in normal and methylprednisolone-treated mice*[a]

Determination	Normal mice	Methylprednisolone-treated mice
Plaque-forming units[b]	117 ± 10	12 ± 15
Avg spleen weight (mg)	137	89

[a] On day 9 of immunosuppression. Results were from 24 mice; each value was an average of 48 determinations.

[b] Per 10^6 spleen cells, Jerne plaque technique.

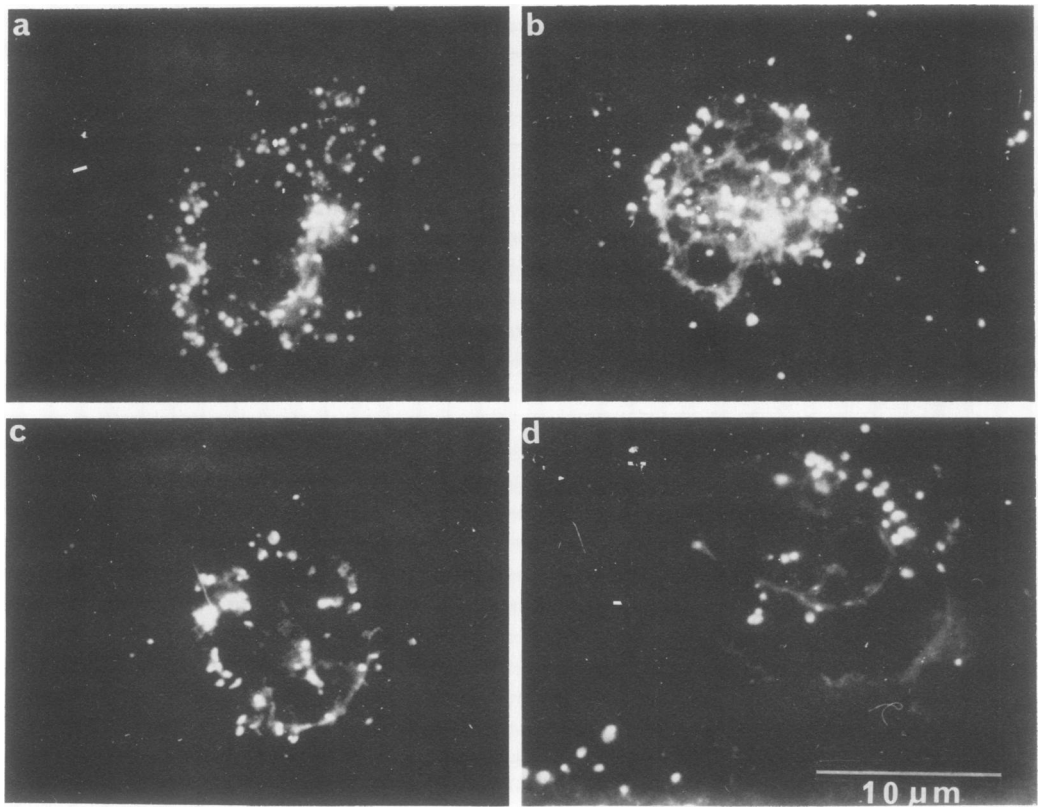

FIG. 1. *Macrophage-containing peritoneal exudate from immunosuppressed mice as visualized by the direct fluorescent-antibody technique. (a) Day 2, (b) day 8, (c) day 12, and (d) day 14 after L-form infection.*

and contained many filaments between the granular elements (Fig. 2 and 4) (7). This was especially so when maintained, for the most part, on agar. No major change in this morphology was apparent over the entire growth cycle of such fresh isolates when in broth for 3 days. However, after this time the typical microscopic L-form morphology returned (Fig. 3a to f). Also, typical and striking in growth after mouse passage (Fig. 2) was the lack of significant numbers of vacuolated and/or bizarre-shaped large bodies so characteristic of this L-form during its growth cycle before mouse passage, as is typified in Fig. 3b to f, and for which a corresponding growth curve appears in Fig. 7. In these cultures of L-forms not passaged in mice, this predominant and seemingly uniform coccal morphology is only present for 1 to 2 h

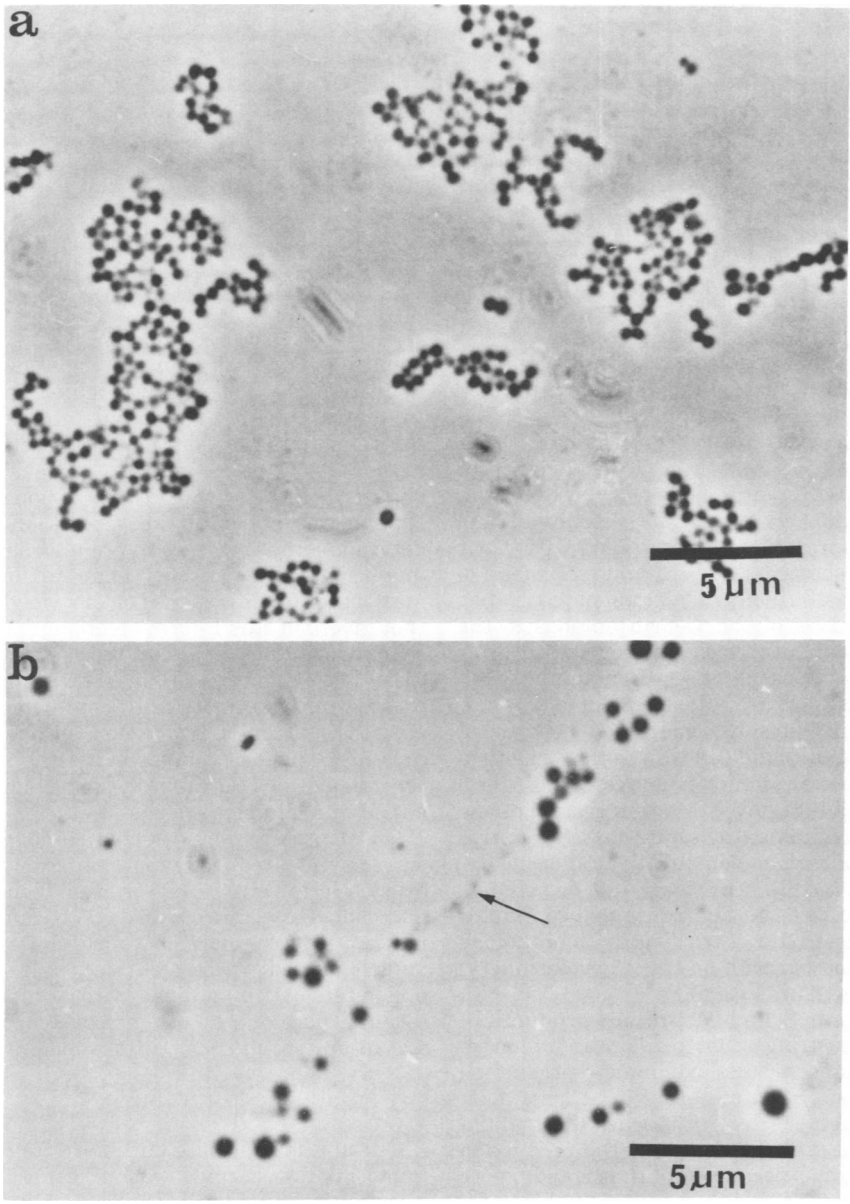

FIG. 2. *Phase-contrast microscopy of broth cultures of the L-form from late logarithmic to the early stationary phases of growth after passage in immunosuppressed mice. (a) Typical uniform coccal morphology and (b) filaments (arrow) between the L-form granular elements (7).*

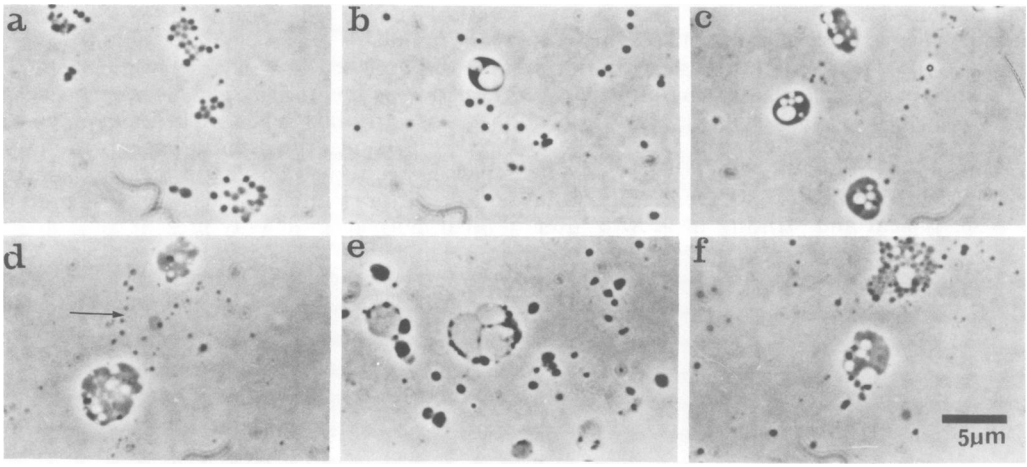

FIG. 3. *Cellular morphology of the L-form in liquid medium before passage in immunosuppressed mice after (a) 2 h, early logarithmic phase; (b) 4 h, approximately mid-logarithmic phase; (c) 5 h, late logarithmic phase; (d) 6.5 h, early stationary phase (arrow indicates filament); (e) 8 h, early stationary phase; and (f) 16 h, late stationary phase. Corresponding growth curve in Fig. 7.*

during the early logarithmic phase of growth (Fig. 3a). Also typical of laboratory-maintained L-form cultures is the presence of some filaments, more often toward the mid-logarithmic to the end of the growth cycle (easily seen in Fig. 3d). Finally, large bodies of the L-form in liquid medium before and after isolation from immunosuppressed mice decreased in opacity at their respective late stationary phases of growth (Fig. 3f). In Fig. 4, electron micrographs of thin sections of the L-form emphasize the morphological entities comprising the cultures after mouse passage, i.e., filaments, coccal-shaped cells, and granular elements (Fig. 4a). Also, it illustrates the continued absence of formation of the rigid cell wall in this organism after being in an immunosuppressed host for 6 days (Fig. 4b). Figure 5 is a thin section of one such typical filament, illustrating its relative size and ribosomal content. Macroscopically, the size of the L-form colony did not change after mouse passage and continued to exhibit the characteristic "fried-egg" morphology which is due to growth on (i.e., lacy periphery) and into the agar (Fig. 6).

Streptolysin S and M protein production. Figure 7 illustrates that passage in immunosuppressed mice results in enhanced production of streptolysin S, culminating in at least a 16-fold rise when the L-form reaches the stationary phase of growth. However, the production of streptolysin S decreased to that noted before mouse passage after being cultured in broth for 3 days. Also, based on cellular protein, the rise in hemolytic units per mg of L-form protein is equally as impressive, increasing from 59 to 160 after from 2 to 10 h of growth in liquid medium. In contrast, hemolytic activity in the L-form before mouse passage was only detected at the stationary phase of growth, increasing only slightly thereafter (Fig. 7). As is apparent, no change was noted in the generation time as determined by increases in protein (not shown) and from optical density changes, or in the maximal growth response or dry weight yields (not given) of this L-form before and after passage in immunosuppressed mice. Finally, colonies of the L-form isolated from immunosuppressed mice were strongly beta-hemolytic on agar. The hemolytic zone had an average radius of 25 mm as compared with 0.7 mm for controls not passed in mice, as measured from enlarged photomicrographs.

No M protein was detected serologically in extracts prepared from approximately 80 mg of dried L-form cells before or after being in immunosuppressed mice for 6 days. Because of the nature of the undefined medium employed (supplemented with albumin), false-positive precipitates were obtained with normal rabbit serum. Therefore, no attempt was made to detect M protein in spent culture medium.

DISCUSSION

Our ability to isolate an L-form from nonimmunosuppressed mice was in general agreement with that of earlier investigators: consistent recoveries up to 2 h, with an occasional isolate up to 24 h, but none thereafter (2, 17,

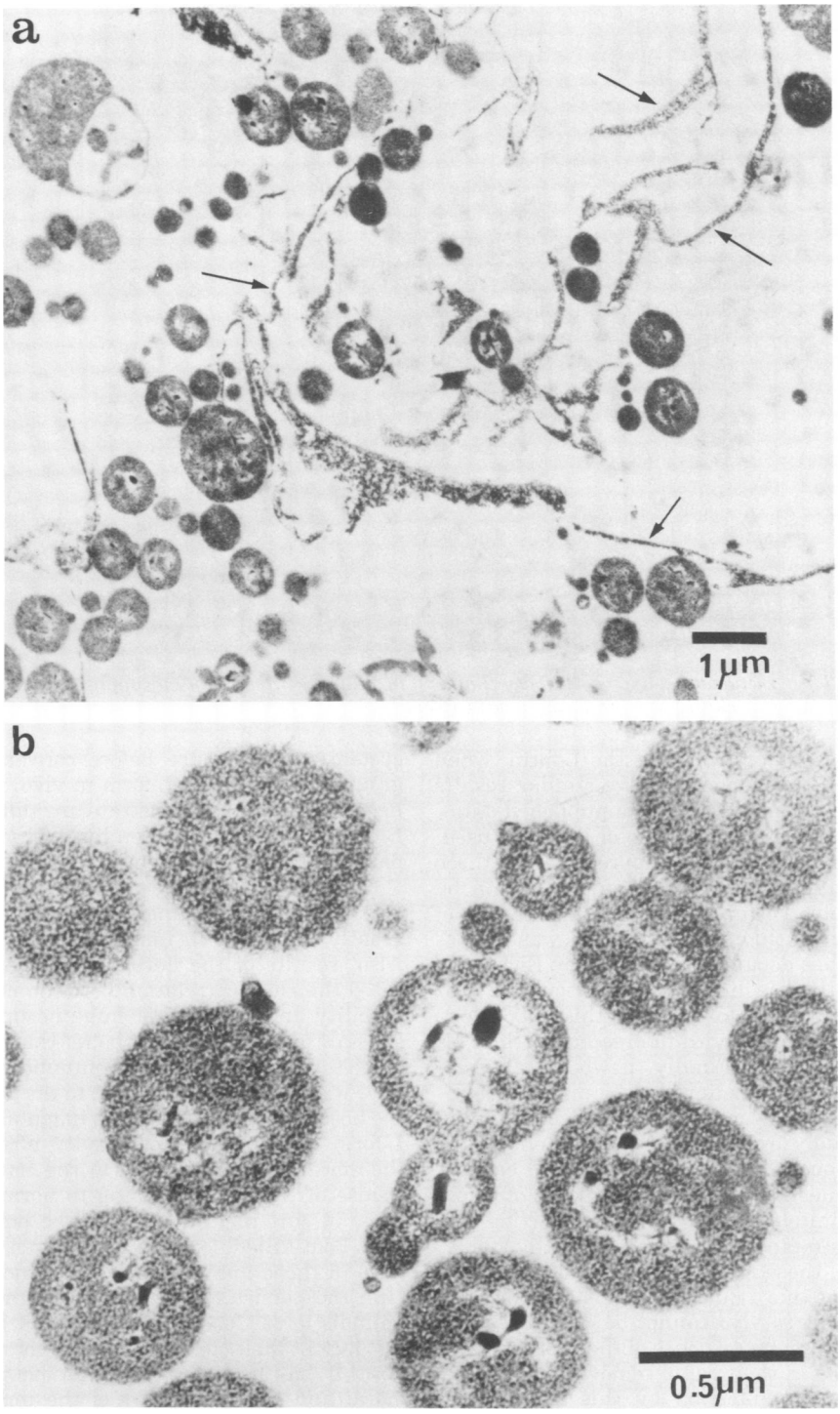

FIG. 4. *Thin section of the L-form at its mid-logarithmic phase of growth after isolation from an immunosuppressed mouse. (a) Predominating filament (arrows), coccal, and granular element components of the culture. (b) Higher magnification showing lack of any visible reformation of the rigid cell wall.*

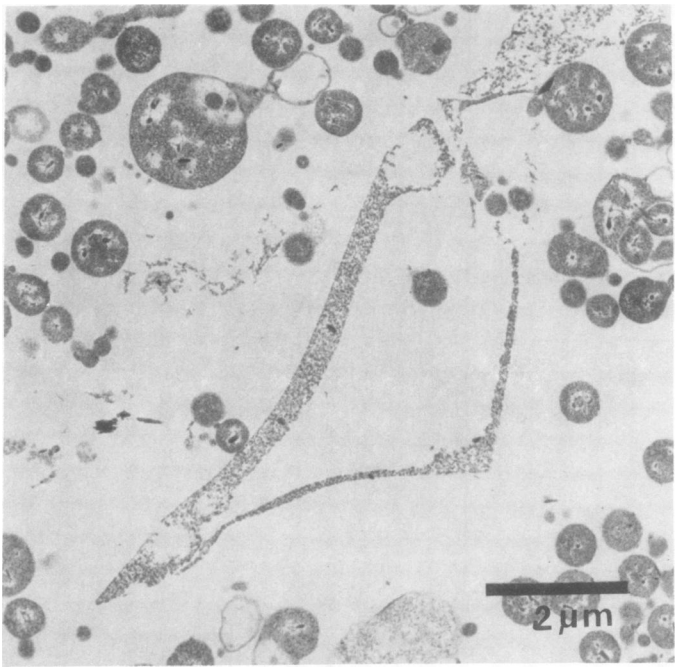

FIG. 5. *Thin section of a typical L-form filament in cultures from immunosuppressed mice showing filament thickness, relative size, and ribosomal content.*

25). Mixtures of mucin plus the L-form, when injected intraperitonally, gave similar results (M. Chevion and C. Panos, unpublished data). Also, regardless of the route of injection used, L-forms were detectable approximately three times longer by fluorescent antibody than by viable count. The maximum time of detection of the L-form by fluorescent antibody was comparable to that documented earlier by Rickles et al. (23) with mice injected intramuscularly with a streptococcal L-form from this laboratory after being labeled with fluorescein isothiocyanate, i.e., 3 days. Finally, newborn mice, although generally believed to be more susceptible to infection, were still able to clear viable L-forms rapidly from the peritoneal cavity after intraperitoneal injection. As indicated earlier, these studies used an L-form capable of growing indefinitely in all media supporting the parental streptococcus. It is osmotically stable. Therefore, these results suggest that, since the inability of this L-form to persist for long periods of time in vivo cannot be due to osmotic lysis, the immune response of the host probably plays a role in their rapid removal.

To augment infection by this L-form, the mouse's immune response was compromised by the use of the methylprednisolone sodium succinate, a corticosteroid. Earlier, Mortimer (19) had also used an immunosuppression drug (hydrocortisone) to affect the reticuloendothelial system of the mouse before conversion of a group A coccus to its L-form in vivo. However, he was unable to demonstrate any difference in L-form production between hydrocortisone-prepared and nonprepared mice.

In the present study, viability and detection of the L-form by fluorescent antibody was greatly prolonged in immunosuppressed mice. Also, results of the Jerne plaque assay indicated the close correlation between decreasing viability and loss of host immunosuppression, substantiating our initial belief that the rapid decrease in viability in nonimmunosuppressed mice was due, at least in part, to the defenses of the host. The precipitous drop in viability of the L-form in the peritoneal cavity after day 8, therefore, is probably due to the immune response of the host returning to normal. However, in this regard, it should be pointed out that heat-killed L-forms only persisted for 3 days in these immunosuppressed mice. Therefore, the persistence of viable L-forms in such animals for at least 2 weeks cannot be due to just lack of their clearance by this compromised host. It has been reported that both the humoral and cellular factors of the immune response are depressed by corticosteroids (8). The ability of immunosuppressed mice whose antibody-forming capacity has been decreased to clear heat-killed L-forms indicates that cellular factors are not significantly depressed. Thus,

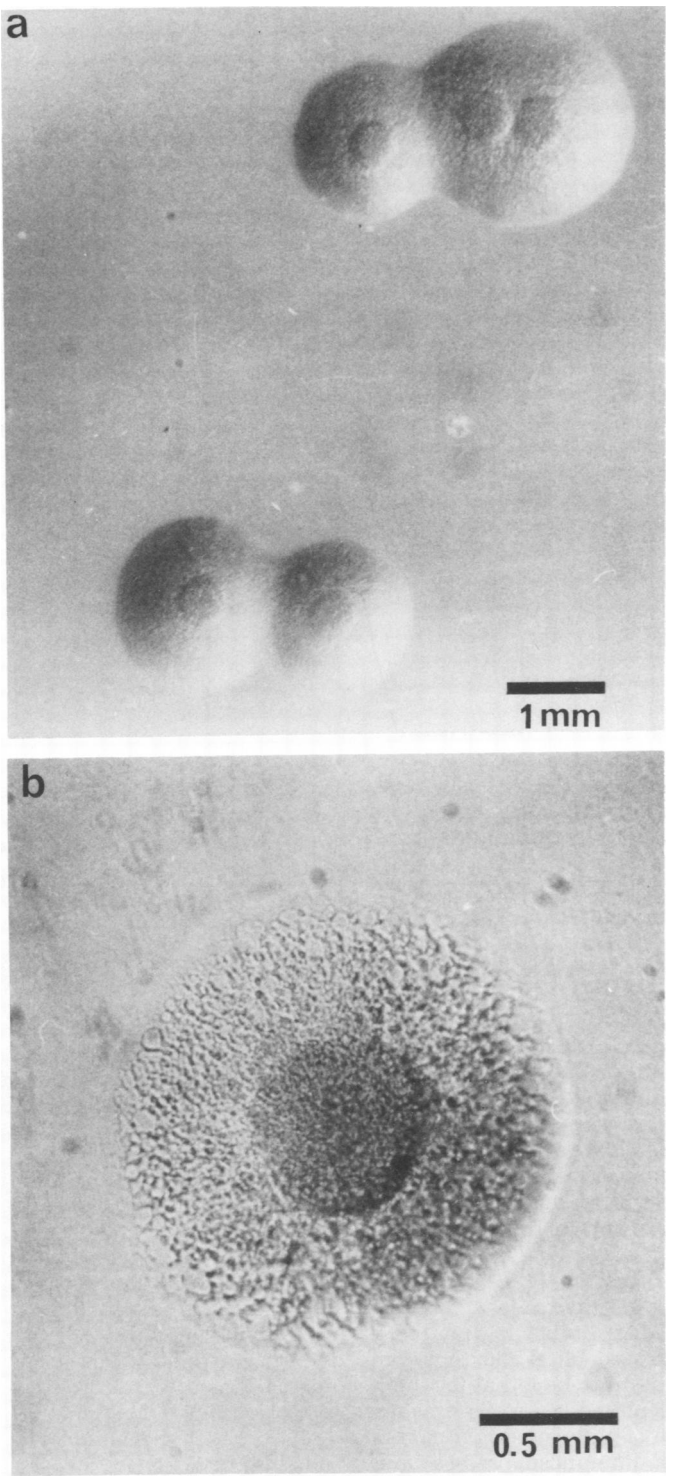

FIG. 6. *Typical colonial morphology of the stabilized, physiologically isotonic L-form after passage in immunosuppressed mice for 6 days. Note retention of typical "fried-egg" morphology with its characteristic lacy periphery.*

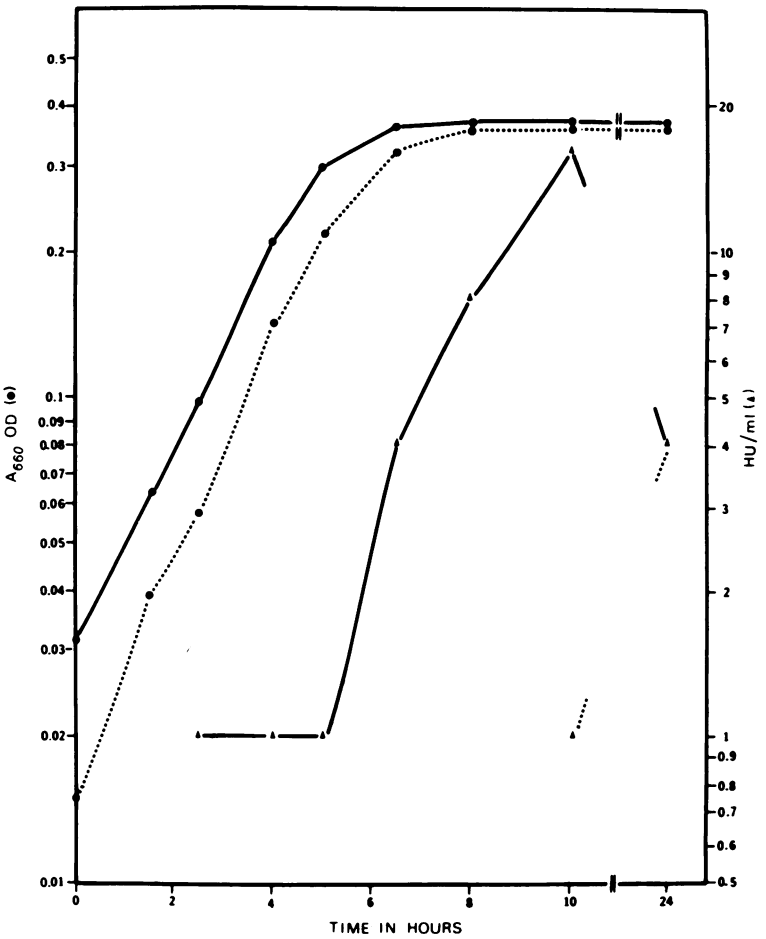

FIG. 7. *Growth curves of the physiologically isotonic L-form before and after isolation from immunosuppressed mice and the comparative increase in hemolytic units (HU) with time between such cultures; (-----) before and (——) after passage in mice.*

humoral factors are necessary for elimination of viable L-forms. Other investigators have also shown that humoral factors such as antibody and complement are cidal to L-forms (18, 20). These cidal factors are probably not necessary for elimination of killed L-form cells, since they were rapidly cleared even in the immunosuppressed host.

Of interest was isolation of L-forms from heart blood after intraperitoneal injection. This indicates the wide distribution of L-forms in these immunosuppressed mice and that complement activity, known to be lethal for the L-form, is probably decreased (8). At no time was there any indication of pathogenesis in nonimmunosuppressed or immunosuppressed mice after L-form injection, and all internal organs examined remained normal. Therefore, overt pathogenesis is not a characteristic of this L-form in a suitable host even when its immune response has been compromised. However, the pathogenicity of *S. pyogenes* in mice is reviewed elsewhere (9).

Of a striking nature was the change in the microscopic morphology of the L-form from immunosuppressed mice; i.e., L-forms were predominately and typically micrococcal in appearance. This change persisted for several days after initial isolation from these treated mice and exemplifies the morphological variability that this L-form is capable of. Although most L-forms of gram-positive bacteria need an osmotic support for growth, the support necessary for their survival in vivo could come from mucine (in 17) or from spermine (11), which is in many tissues and purported to have a stabilizing effect on protoplasts. Therefore, this requirement for osmotic stabilization may be less

important in vivo than in vitro since at many sites the osmotic pressure is higher than that of serum. Considering a need for an osmotic stabilizer and these morphological results, one can only wonder in what form or state the L-form is capable of existing in the body. In this respect it is of interest to point out that stabilized L-forms of certain gram-negative bacteria reverted to the bacterial form only after injection into rat kidney (29). At present, no information is available to indicate whether prolonged or continued passage of this particularly adapted, stable coccal L-form in immunosuppressed mice might result in its reversion to the parental *S. pyogenes*.

The L-form obtained after mouse passage reacted to the same extent with antisera prepared against it before injection. Also, the numbers of CFU obtained before and after mouse passage were similar. No L-forms were found in mice before inoculation. Therefore, these results, together with comparative growth characteristics and nutritional requirements and microscopic and macroscopic examinations of this L-form before and after passage, leave little doubt as to their common lineage.

Immunosuppressive drugs are in wide use in medicine, and these results suggest that bacterial L-forms may survive in immunologically suppressed individuals. However, whether an L-form may become pathogenic because of a reversion to the bacterial form, by reformation of a particular pathogenic component (i.e., M protein) or by sensitizing appropriate tissues to future streptococcal infections, remains to be studied.

It is known that the tissue-damaging properties of streptolysin S may be attributed to degranulation of lysosomes (27, 28). Also, treatment of animals with cortisone decreases the susceptibility of their isolated lysosomes to streptolysin S (9). Finally, some evidence is at hand of the possible participation of streptolysin S in the induction of renal damage in humans (26). Therefore, the dramatic increase in the production of this hemolysin after mouse passage, if occurring in vivo and for a long period of time, might prove to be of great consequence to a host, even though, because of its affinity for cell membranes (9), streptolysin S is readily inactivated. This increased production may also be a reason for the rapid destruction of heart cells in tissue culture by this L-form (15). It has been reported that streptolysin S production by the group A streptococci is either not affected by mouse passage or may even be decreased (9, 14). Thus, this increased production of streptolysin S by the L-form after passage in immunosuppressed mice may be indicative of yet another abnormality after permanent inhibition of cell wall formation.

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