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Induction of L-Variants in Human Diploid Cells Infected by Group A Streptococci

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Human diploid cells in culture, infected with a balanced amount of living group A streptococci, were able to survive the infection and could be divided and propagated normally thereafter. The streptococci were rapidly phagocytized by the tissue culture cells. At the beginning, they kept their typical appearance, as well as their ability to fix dyes and group-specific immunoglobulins. After 1 to 2 days, the number of detectable streptococci decreased and they underwent important morphological changes. After some subsequent divisions of the cell line, streptococci persisted in cells only as large, isolated, swollen cocci, and no longer grew on suitable media. After six to eight divisions, a noticeable percentage of the tissue culture cells were very similar in appearance to the same cell line experimentally infected with "stable" L-variants. Cultures on L-phase media of supernatant fraction and cells, made 24 to 48 hr after inoculation, showed typical L-colonies. These grew well on media without antibiotics, as well as on media containing penicillin or vancomycin. They could be propagated on media with penicillin for months and were able to revert to group A streptococci after several subcultures on antibiotic-free media. Controls of uninoculated tissue culture cells never showed the presence of any microorganism. Group A streptococci inoculated into Eagle's basal medium, which was used for the tissue cultures, did not grow and never gave rise to L-colonies, even though the medium contained penicillin. Previous data suggest a biochemical explanation for this conversion, which otherwise is an occasional phenomenon.

The ability of tissue culture cells to withstand a bacterial contact and to control it to the extent of a true survival is indeed not a new observation. As early as 1923, Lewis demonstrated that chick histiocytes in culture were able to phagocytize and to destroy a large inoculum of *Bacillus radicicola*, a bacterium normally associated with plants (20).

Since then, many workers have investigated the behavior of various tissue cultures in the presence of various species of bacteria or related organisms (17). These attempts generally led to the destruction of the tissue culture cells, but, under certain conditions, the bacteria were able to live in the cells for a more or less prolonged period of time. This has been shown, for instance, for *Mycobacterium tuberculosis* (13, 27), *Brucella abortus* (14, 32, 36), *Haemophilus pertussis* (6, 7), and *Salmonella typhi* (37). From these experiments, however, it would seem that unmodified bacteria cannot survive within the cytoplasm of the cell unless their activity is reduced; as a matter of fact, bacteria able to multiply actively in tissue cultures quickly overwhelm the cells and destroy the culture, even though they have a very limited (if any) cytopathogenic effect by themselves. Thus, with fast-growing bacteria, fast-growing cell lines are required (26).

On the other hand, a very prolonged coexistence of the cell line and an intracellular parasite is possible when the latter belongs to the group of bacterial forms lacking a cell wall, such as *Mycoplasma*. This phenomenon has been observed also with the L-phase of certain bacteria (15, 23), and we have previously demonstrated it for the L-variant of group A streptococci (33).

Furthermore, Hatten and Sulkin demonstrated that it is possible to isolate typical L-colonies from hamster kidney cell cultures from 2 to 12 days after their infection by brucellae (8, 9). Similar observations have been reported with *S. typhi* (18).

We thought it of interest to observe the behavior, in quantitatively defined conditions, of organisms normally considered extracellular, such as group A streptococci.

MATERIALS AND METHODS

Tissue culture cells. The cell line used throughout the experiments was the human diploid line L 809 previously described (33). This line was derived from the skin and muscle of a female embryo of 12 mm. It showed, and retained, all of the characteristics of human diploid cell lines described by Hayflick and Moorhead (12). It was cultivated in Eagle's basal medium (BME) supplemented with 10% calf serum. No antibiotic was used, either during the propagation of the cell line or during the experiments. The cell line was frequently checked for possible contamination by *Mycoplasma*, in accordance with Hayflick's technique (10). The absence of such contaminants was also checked by electron microscopy several times during the propagation of this strain. The experiments were performed on subcultures of various ages between the 20th and the 40th division. The average life of this cell line was between 50 and 60 divisions.

Inoculation of group A streptococcus. The group A streptococcus strain Gl-8 (A 4514 of our collection) was used. This strain was cultivated for 6 hr in a water bath at 37 C in Trypticase Soy Broth (TSB; BBL); the culture was divided into 2-ml vials and immediately frozen at −70 C. This preparation served as stock culture.

For the inoculation of the tissue culture cells, a vial of the stock culture was thawed; 1 ml was inoculated into 10 ml of TSB and incubated at 37 C for 18 hr. A 4-ml amount of this overnight culture was then inoculated into 45 ml of TSB and incubated for 5 hr at 37 C with constant moderate agitation. The resulting culture was centrifuged at 3,000 × *g* for 10 min; the supernatant fluid was then discarded and the streptococci were suspended in BME without serum. This suspension was adjusted to an optical density of 0.5 at 500 nm in a Beckman H 14 photometer. Dilutions of 10^{-1} to 10^{-9} in BME were prepared from this suspension. The total number of streptococci was estimated for each dilution in a Helber cell, and the viable count was determined by pouring 0.1 ml of each suspension into a petri dish containing 12 ml of 5% blood-Todd-Hewitt agar (Difco).

To check the possible existence of spontaneous L-variants in the bacterial inoculum, 10 to 15 ml of the same undiluted bacterial suspension was filtered on an acetate membrane filter (pore size, 0.45 μm; Millipore Corp.), and the filtrate was inoculated on media suitable for the L-phase growth (*see below*). Unfiltered inocula were also plated on penicillin agar (TSA-P, *see below*).

Inoculum for other groups. Preliminary studies have been attempted with cultures belonging to other groups of streptococci. Strains B_1, C_{22}, D_2, F_4, G_{15}, H_3, and K_2 of our collection were grown and inoculated in the same manner described above.

Inoculation of tissue cultures. Before inoculation of the cells, all inocula were again moderately shaken for 15 min at room temperature.

The tissue cultures were inoculated, after the normal medium had been discarded, in confluent monolayers in several containers: petri dishes (diameter of 60 mm), containing approximately 4×10^5 cells; Leighton tubes, containing approximately 2×10^5 cells; and 60-ml bottles, containing approximately 2×10^6 cells. The volume of inoculum (of various streptococcal concentrations) was 0.5 ml for the petri dishes, 2.5 ml for the bottles, and 0.25 ml for the tubes.

During each experiment, the following controls were performed: (i) uninoculated cell culture, (ii) BME minus cells but inoculated with the same doses of streptococci, and (iii) diploid cells of the same cell line inoculated with group A streptococcus L-forms, obtained in vitro by penicillin action several months before the experiments and unable to revert to bacterial form even after several subcultures on media without penicillin.

Cultivation of infected cells. Incubation was performed at 37 C in stoppered bottles or tubes; for the cultures in petri dishes, an atmosphere of 5% CO_2 plus air was used. After 5 hr of incubation, the liquid was removed and the cell monolayers were thoroughly washed two to three times (approximately 10 min each time) with BME containing 2% calf serum; they were then kept in BME-10% calf serum at 37 C, under the same conditions as before.

The cells were observed twice a day, and, when the medium was cloudy or showed a change in *p*H, it was immediately removed and replaced with fresh medium.

Depending on the state of the tissue culture, the first trypsinization of cultures in bottles was performed after 24 or 48 hr in a ratio of 1:1 or 1:2. Cells cultivated in Leighton tubes or petri dishes were not divided. Thereafter, the surviving cultures in bottles were regularly divided: first, twice a week in a ratio of 1:2; then, after four or five divisions, in a ratio of 1:3 once a week. The rhythm of division for uninoculated bottles was kept parallel to that of infected ones. When performing these divisions, at least two Leighton tubes and two petri dishes were also inoculated with cells to provide material for the subsequent examinations (*see below*).

Examination of infected tissue culture cells. Examination was performed immediately at the end of the 5-hr infection period, 1, 2, and 4 days after infection, and then on new confluent cell monolayers resulting from each division.

Microscopic examination. Three techniques were used for examining the cover slides of the Leighton tubes: (i) phase-contrast microscopy, (ii) microscopy of Giemsa-stained slides, and (iii) direct immunofluorescence microscopy.

Hot Giemsa staining was performed according to the technique recommended by Giroud for the staining of *Rickettsia*, which is as follows. The cover slides are washed twice for 10 min with Dulbecco's phosphate-buffered saline and fixed for 15 min in acetone. Twenty-five drops of Giemsa stain are added to 5 ml of neutralized distilled water brought to ebullition just prior to the addition of the stain (it is important not to boil the mixture after this addition). The slides are immediately flooded with the hot dilution for 5 min; they are then washed with neutralized distilled water, differentiated for 2 to 3 sec in a mixture of absolute ethanol plus xylene (1:1, v/v), profusely washed again with neutral distilled water, and finally dried and mounted for microscopy.

Direct immunofluorescence microscopy was performed with anti-group A streptococcus immunoglobulins prepared against the bacterial phase (killed whole-cell vaccines) and L-phase variants of strain Gl-8. The immunoglobulin fraction was separated before conjugation with fluorescein isothiocyanate (FITC; 33).

Cultivation. The supernatant fractions of all cultures (bottles, tubes, and dishes) were inoculated into media for cultivation of streptococci and of L-variants. Monolayers grown in petri dishes were also cultivated, after removal of the supernatant fraction, by pouring molten agar medium over the cells.

Cultivation of streptococci was performed on Todd-Hewitt broth and on Todd-Hewitt broth plus 1.2% agar and 5% defibrinated horse blood.

Cultivation of L-variants was attempted on TSB supplemented with 1.5% NaCl, 1.5% yeast extract (Difco), 10% egg yolk extract [prepared by heating at 90 C for 30 min a suspension of 200 g of egg yolk, coagulated (Difco), and then filtering the suspension on gauze and paper until the preparation was clear], and 10% inactivated calf serum. L-variants were also cultivated on TSB enriched as above but without egg yolk extract and solidified with 0.8% agar (final concentration), referred hereafter as TSA; this medium was eventually supplemented with 1,000 units of penicillin (TSA-P) or 1,000 μg of vancomycin (TSA-V) per ml. TSB cultures were incubated at 37 C for 5 to 7 days and then were plated on TSA, TSA-P, and TSA-V. Cell monolayers in petri dishes were poured with TSA and TSA-P. Transfers from agar cultures were performed by the "sandwich" technique, taking care to wet the surface of the fresh agar with a few drops of inactivated calf serum before spreading the sandwiches on it.

All petri dishes were sealed with adhesive tape, incubated, and examined daily with a microscope ($\times$ 160 to $\times$ 400); apparently negative plates were not discarded before the 12th or 14th day. L-like colonies grown on agar media were always transplanted, for the first subculture, both on TSA and on TSA-P. Thereafter, they were checked by cross-transfer from TSA to TSA-P and vice versa.

The techniques used for identification of the isolated bacteria and L-variants were described in previous papers (33, 35).

Number of cultures. In total, 257 cultures of the L 809 cell line were used for inoculation. For cultures infected with streptococci, 15 bottles (subsequently divided as described), 15 petri dishes, and 118 tubes were used. The controls infected with "stable" L-variants were in 2 bottles (subsequently divided), 2 petri dishes, and 6 tubes; for uninfected controls, 6 bottles (subsequently divided), 7 petri dishes, and 86 tubes were used.

RESULTS

Survival of group A streptococci in BME minus cells. Table 1 shows the numbers of viable streptococci in BME plus 2% serum inoculated with a massive dose of bacteria, kept at 37 C for several days, and examined at various intervals. These organisms survived in this medium for several hours without dividing; then they decreased in number during the next few days. However, it will be seen from the Table 3 (*see below*) that a definite multiplication of the bacteria took place during the 5-hr inoculation period when they were in the presence of tissue culture cells. These results are consistent with those of Quinn and Lowry (30).

TABLE 1. *Survival of streptococci in BME plus 2% calf serum*

Time after inoculation	Streptococcal colony-forming units/ml
0 Hr	3.2×10^7
2 Hr	2.7×10^7
4 Hr	2.2×10^7
2 Days	10^3
5 Days	10^2

TABLE 2. *Relationship between the size of the bacterial inoculum and the subsequent behavior of tissue culture cells*

Inoculum[a]	Appearance of cells after		Division after 24 hr
	24 Hr	48 Hr	
$\leq 10^2$	Normal	Normal	Easy
10^3 to 10^5	Altered	Partially destroyed	Possible
10^6 to 10^7	Partially destroyed	Destroyed	Difficult
$\geq 10^8$	Destroyed		Impossible

[a] Number of colony-forming units per milliliter inoculated into 2×10^6 tissue culture cells.

Absence of spontaneous L-variants in streptococcal inoculum. All of the membrane filtrates of bacterial inocula failed to give rise to L-colonies when plated on suitable media. This conforms with the results of Mortimer (25), Rotta et al. (31), and Maxted (24), as well as with our own previous observations (35). All unfiltered inocula cultivated on TSA-P gave rise to no L-colonies, not even atypical ones.

Importance of the density of inoculum. There is an optimal relationship between the number of tissue culture cells inoculated and that of bacteria (Table 2). Taking the 60-ml bottles containing 2×10^6 cells as a reference unit, we observed that an inoculum of $\geq 10^6$ streptococci led to a rapid and irreversible destruction of the tissue culture. When the inoculum was between 10^3 and 10^5 bacteria, the cell cultures showed signs of suffering after 24 hr; however, it was frequently possible to maintain their survival and multiplication. After two or three subsequent divisions, these

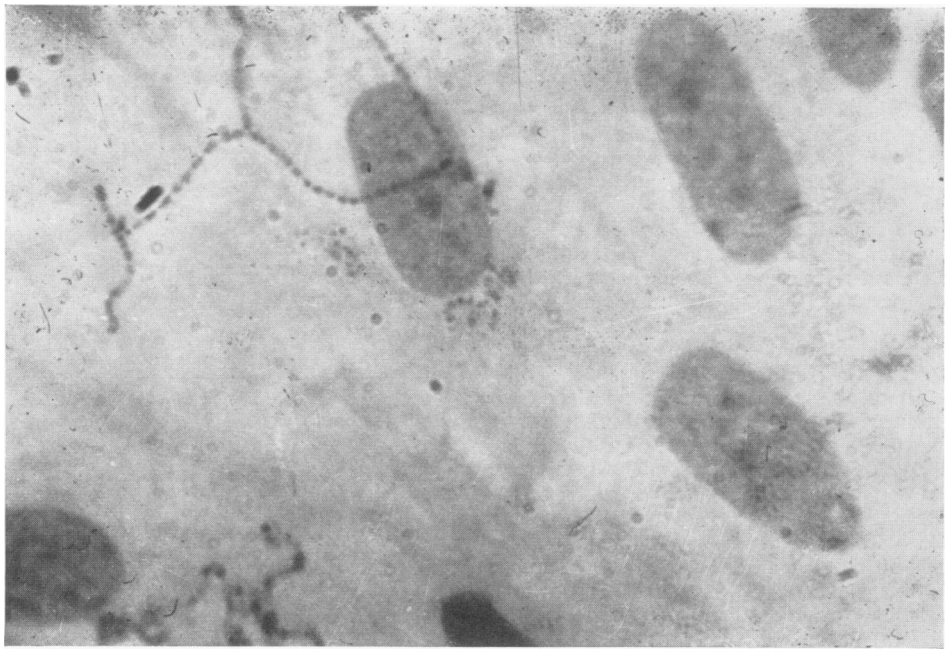

FIG. 1. *Human diploid cell line, 24 hr after contamination by group A streptococci. Numerous streptococca chains, associated with cells. Giemsa stain.* × *1,000.*

cells recovered a typical morphological aspect and a regular rhythm of growth. With a smaller bacterial inoculum, $\leq 10^2$, the streptococci disappeared rapidly and the tissue culture cells did not show any signs of damage.

Morphological changes of inoculated streptococci. Changes were easier to observe when the relative proportion of bacteria to tissue culture cells was optimal. After incubation, the streptococci were rapidly phagocytized by the cells, since a rather high percentage of these may be associated with cells at the end of the 5-hr incubation period. In the beginning, they kept their typical short-chain aspect, well stained by the hot Giemsa method (Fig. 1), and gave a strong reaction (+++) with the FITC-labeled anti-group A streptococcus immunoglobulins. We point out that, at this stage, these streptococci gave only a very faint (±) fluorescence with the FITC-labeled immunoglobulins prepared against homologous L-phase organisms. Later, the number of streptococci detectable in tissue culture cells decreased, and they underwent very important morphological changes (Fig. 2). The cocci swelled considerably, and they were single or in short but very thick chains (Fig. 3). Moreover, some of them began to give strong (+++) fluorescence with labeled anti-L-phase immunoglobulins (Fig. 4).

The conversion of streptococci to atypical intracellular organisms was progressive, and the percentage of infected cells depended on the relative number of bacteria per cell (Table 3). As we pointed out above, one may notice from the examination of Table 3 that a certain division of the streptococci could have taken place in the inoculum during the incubation period, since there were more infected tissue culture cells than there were viable bacteria (at least estimated as colony-forming units) in the original inoculum.

Isolation of streptococci from the cell cultures. Table 4 shows that streptococci were isolated 1 or 2 days after inoculation in each instance when the number of bacteria in inoculum was high: $\geq 10^6$ streptococcal colony-forming units for 2×10^6 cells. On the other hand, tissue cultures inoculated with few bacteria did not give rise to streptococcal cultures in a definite percentage of cases. Thus, one-fifth of the cell cultures infected with 10^2 streptococci were "sterile" 48 hr after inoculation. With medium inocula (10^3 to 10^6 bacteria), when the division of the cells was possible, the isolation of streptococci became less frequent with the consecutive splits. After two or three divisions, it was impossible to grow streptococci from these cultures, even though microscopic examination still revealed the presence of cocci within the cells at this stage.

Isolation of L-phase organisms from the cell cultures. At the end of the 5-hr incubation period,

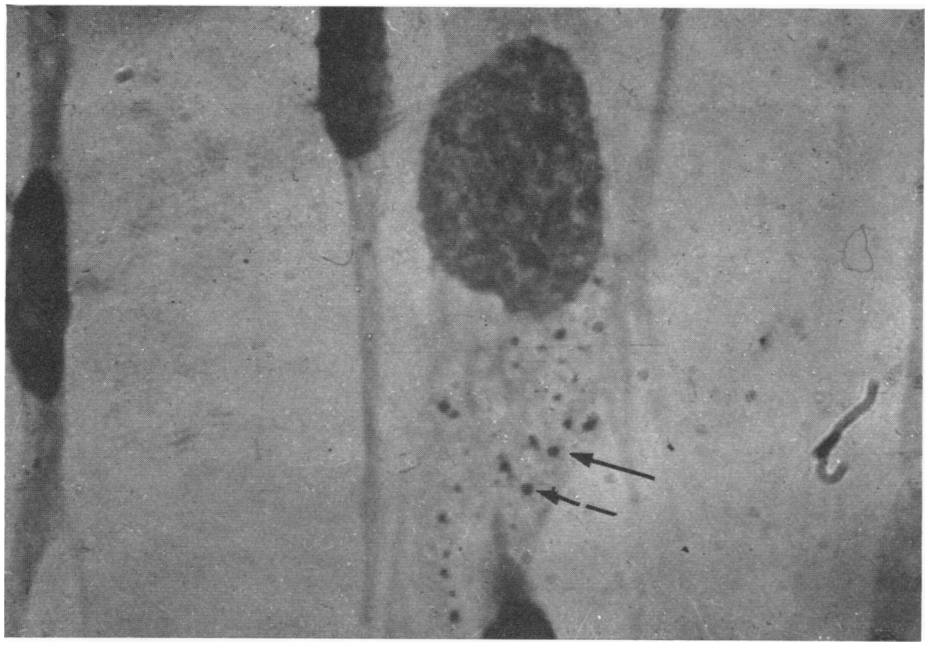

FIG. 2. *Same cell line shown in Fig. 1 after four divisions (11 days after infection). Presence within the cell of several isolated, swollen cocci (arrows). Stain, hot Giemsa; differentiation, ethanol-xylene.* × *1,000.*

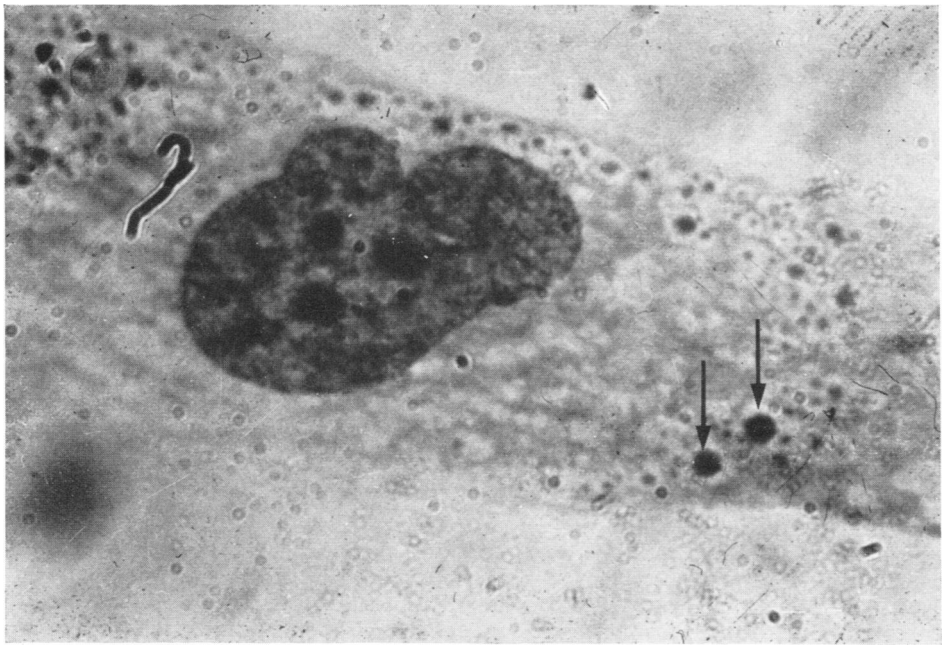

FIG. 3. *Appearance of the cell line after seven divisions (30 days after infection). Large globular elements (arrows), colored purple-red by hot Giemsa after differentiation.* × *1,000.*

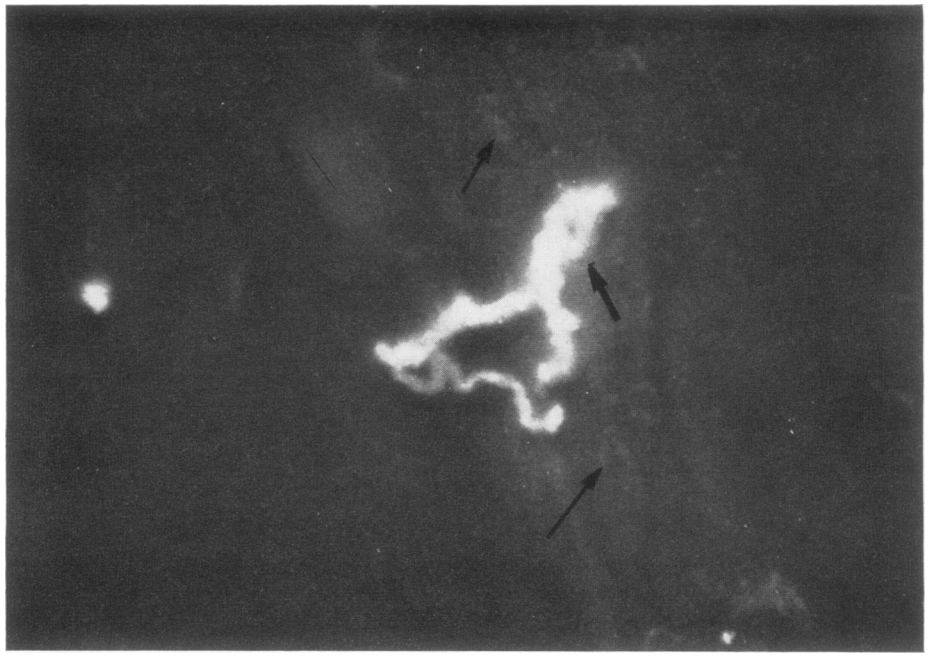

FIG. 4. *Human diploid cell line 48 hr after infection. Immunofluorescent staining with FITC-conjugated anti-group A streptococcal L-form immunoglobulins. Among unmodified streptococcal chains, very poorly fluorescent (thin arrows), a very bright and enlarged chain (thick arrow), showing the release of deep antigenic structures.* $\times$ *400.*

it was possible to observe in the supernatant fraction, by phase-contrast microscopy, globular elements larger than 10 μm in diameter among the numerous streptococci. The cultivation of these elements, however, did not lead to the growth of L-colonies on enriched TSA medium, whether or not it contained penicillin.

On the contrary, when the supernatant fraction was cultivated 24 or 48 hr after the inoculation of the cells, three types of colonies were noticed on enriched TSA minus antibiotics: typical streptococci, typical L-colonies, and intermediate colonies. The typical L-colonies (Fig. 5) were propagated on media suitable for L-phase growth with or without penicillin or vancomycin. They could be propagated for months on media containing penicillin and were able to revert to streptococci after several subcultures on TSA and TSB antibiotic-free media. The intermediate colonies had a granular center embedded in agar and an edge showing simultaneously streptococcal chains and large globules. These colonies, when propagated on TSA, also reverted to typical bacterial colonies more rapidly than the preceding type. Similar results were obtained by cultivation of the cell layers, but in this case the transfers and examination of the colonies were technically more difficult. However, it must be stated that positive results were obtained with the supernatant fractions of tissue cultures only when they contained numerous cellular fragments.

At this stage of the experiments, the presence of L-variants in cellular cultures inoculated with streptococci depended to a large extent upon the density of inoculum (see Table 4). With heavy inocula (10^6 streptococci per 2×10^6 cells), the production of L-variants was greater, but the isolation of L-colonies on media without antibiotics was made almost impossible by the overgrowth of streptococci. Medium inocula (10^3 to 10^5) gave the best results. With a small inoculum (10^2), the number of L-variants isolated was low, but these results were especially significant since, as pointed out in the preceding paragraph, the streptococci in these cultures frequently disappeared completely as such and no longer grew on standard media.

When the cultures for L-phase were performed after the division of the cell lines, it was no longer possible to isolate L-organisms until the 7th or 8th split, that is to say 25 or 30 days after inoculation, even though, during this period, a notable number of cells showed cytoplasmic inclusions reacting with fluorescent anti-L-phase globulins, as described in the preceding paragraph.

L-colonies isolated later, by use of media with

TABLE 3. *Percentage of tissue culture cells showing "L-like" elements after division*

Inoculum[a]	Days after inoculation	No. of cell line divisions	Percentage[b] of cells showing	
			Streptococci	L-like elements
10^2	1	0	10	0.1
	5	2	1	?
	11	4	—	—
	16	5	—	—
	20	6	—	—
	30	8	—	—
10^4	1	0	50	1
	5	2	40	1
	11	4	5	10
	16	5	1	10
	20	6	—	10
	30	8	—	15
10^6	1	0	90	5
	5	2	50	0.1
	11	4	10	5
	16	5	1	10
	20	6	1	10
	30	8	0.1	20

[a] Number of colony-forming units per milliliter inoculated into 2×10^6 tissue culture cells.

[b] The percentages are estimated, being the means of several microscopic examinations; — = none.

TABLE 4. *Isolation of microorganisms from tissue cultures infected with group A streptococci, 1 or 2 days after infection*

Inoculum[a]	No. of cultures infected	Tissue cultures giving positive results			
		Streptococci[b]		L-variants[c]	
		No.	Per cent	No.	Per cent
10^2	31	25	80	5	16
10^3 to 10^5	44	42	95	12	27
10^6 to 10^7	49	49	100	11	22
10^8	24	24	100	?[d]	

[a] Number of colony-forming units per milliliter inoculated into 2×10^6 tissue culture cells.

[b] Cultivated on Todd-Hewitt broth plus 1.2% agar and 5% defibrinated horse blood.

[c] Cultures considered as positive only when L-variants were detected on both TSA and TSA-P; results given concern both cultures of supernatant fractions and of the cells. On an average, five supernatant fractions were cultured for one tissue culture preparation.

[d] Undetectable owing to bacterial overgrowth.

and without antibiotics, were quite typical and gave a strong fluorescence with labeled anti-L globulins. However, we were unable to transfer them to fresh media, contrary to the results obtained with recently infected cells. Table 4 summarizes the results of the attempts at isolation of L-colonies. It shows that, in the conditions of the present experiment, this is an occasional phenomenon which occurs for one-fifth to one-third of the cultures infected with group A streptococci. This phenomen depends upon the size of the inoculum and, to a certain extent, on the conditions of the cultivation. As a matter of fact, we noticed that cell cultures in bottles or in petri dishes gave better results than those in tubes. Electron microscopy of L-colonies isolated from the tissue culture cells showed a typical structure which was strictly comparable to that of L-variants induced in vitro (28, 29).

The reversion of isolated L-organisms to typical bacteria was successful in only eight cases; in several other attempts, the reversion was incomplete. The growth forms showed typical chain morphology under microscopic examination, but we were not able to make them grow on ordinary media for streptococci (Todd-Hewitt broth or TSB). When reversion was possible, in each case the revertant was identifiable as a group A streptococcus by morphological and cultural characteristics, as well as by group precipitation with the anti-group A polysaccharide antiserum.

Controls. Uninoculated tissue cultures were always negative in regard to light and electron microscopy, as well as to culture on various media. No bacterial organisms (especially *Mycoplasma*) were noticed throughout these experiments. Also, the cells retained their normal original appearance throughout (Fig. 6).

Cultivation of L-colonies from controls infected with "stable" L-variants was easy only during the two first divisions. Thereafter, up to the 5th division, atypical colonies were isolated, which became typically L-like only after several transfers on TSA-P. After the 6th division, colonies were no longer observable. These observations are consistent with our previous results (33).

Results observed with streptococci other than group A. Table 5 shows that all of the cell cultures inoculated with streptococci belonging to groups other than A were rapidly destroyed. It also shows that most of these bacteria can divide actively in BME. This could explain the discrepancy with group A, but the experiments with other groups have been conducted on a too small scale to give conclusive evidence.

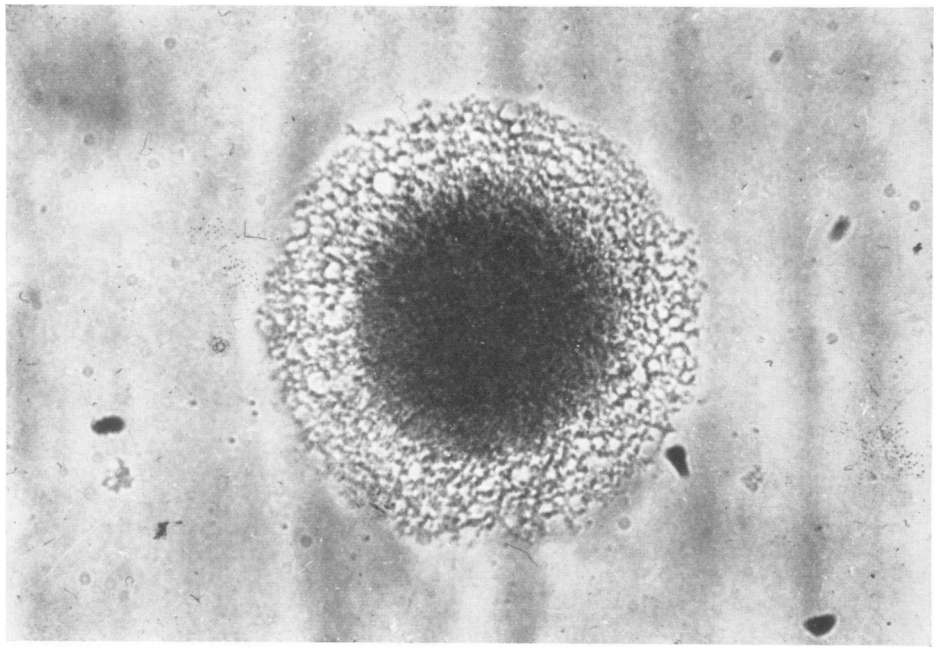

FIG. 5. *Typical L-colony isolated on medium without antibiotics by culture of the cell line 48 hr after infection Direct examination.* × *160.*

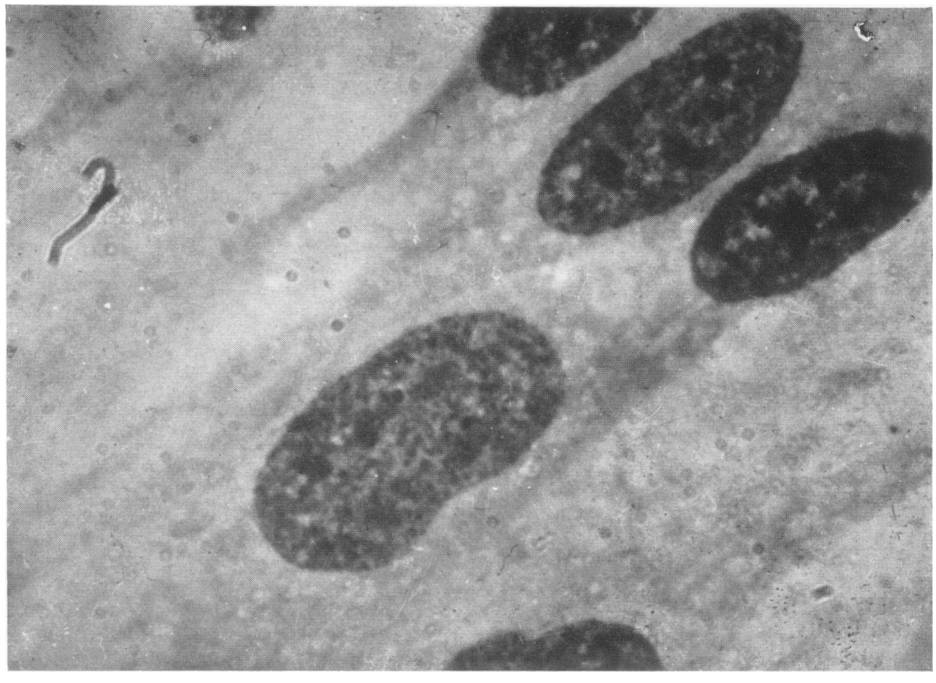

FIG. 6. *Appearance of the control cell line after seven divisions. No cytoplasmic inclusions are seen. Stain, hot Giemsa; differentiation, ethanol-xylene.* × *1,000.*

TABLE 5. *Results of inoculation of a human diploid cell line with streptococci belonging to groups other than A*

Streptococcal strain	Ability to grow in BME[a]		Inoculum[b]	Appearance of tissue culture cells after 24 hr[c]
	CFU/ml at inoculation	CFU/ml after 48 hr		
B 1	—[d]	—	4×10^3	Destroyed
C 22	1.9×10^3	1.7×10^7	10^4	Destroyed
D 2	6.8×10^2	$>10^{10}$	1.3×10^4	Very altered
F 4	2.4×10^3	5.4×10^7	2.1×10^4	Very altered
G 15	—	—	5×10^3	Destroyed
H 3	4.2×10^3	$>10^{10}$	10^4	Destroyed
K 2	1.2×10^3	6×10^5	6.5×10^3	Very altered

[a] The ability of the streptococci to grow in BME was determined by inoculating the indicated number of colony-forming units into BME with 2% serum without tissue culture cells.
[b] Number of colony-forming units per milliliter inoculated into 2×10^6 tissue culture cells.
[c] Division of the cells impossible in all instances.
[d] Not done.

DISCUSSION

The use of tissue culture cell lines for the study of bacterial infection meets with several difficulties, mainly owing to the nature and composition of the culture medium. From the results reported here, one may observe that, to achieve an equilibrium between the cells and the parasite, these must be in relative balance. When the number of living bacteria greatly exceeds that of tissue culture cells, whether this is due to a large inoculum or to fast multiplication, the latter are rapidly destroyed and the subsequent events are no longer observable. This has been the case, in our experiments, with the excessive amount of group A inoculum, and with streptococci of other groups which can multiply actively on the medium used. Thus, preliminary trials to determine the optimal size of inocula are a fundamental step in such experiments.

Another drawback to the use of tissue culture cell lines is the possible chronic contamination by *Mycoplasma*, which may be mistaken for L-variants. Such contamination is at present very frequent in the current cell lines (5, 10, 11, 16). Since it is well known now that the systematic use of antibiotics in culture media greatly encourages this contamination, we used a cell line established and propagated without any antibiotic. We have used and checked this line extensively, and it has always been found to be free from *Mycoplasma* contaminants, both by culture and by electron microscopy. We may, therefore, reasonably conclude that the atypical bacterial forms we isolated were really derived from the inoculated streptococci.

Our experiments are, in fact, very similar to those of Hatten and Sulkin (8, 9), but in the present study the cells were able to withstand an infection by the classical bacterial form which does not usually grow within the cell, as is the case with the brucellae. Quinn and Lowry (21, 30), having inoculated several human cell lines with group A streptococci, observed the complete destruction of the cells and noticed that these bacteria did not grow in the medium unless the cells were present. They concluded that the destruction of cells occurred only when they were in close contact with multiplying bacteria, even though the group A streptococcus was an extracellular parasite. The discrepancy between the facts reported here and Quinn and Lowry's conclusions probably lies in the fact that there is an optimal relationship between the size of inoculum and the number of cells. As a matter of fact, tissue culture cells of any kind are able to phagocytize bacteria in practice (to a variable extent, of course, depending on the nature of the cells), provided that they are not overcome by the growth of bacteria. For instance, by using antibiotics in the medium to prevent extracellular multiplication of the salmonellae, Smadel (37) was able to grow *Salmonella typhosa* within mouse fibroblasts until cultivable microorganisms disappeared completely.

After phagocytosis, the changes undergone by the group A streptococci consist mainly of the alteration of the cell wall. This seems obvious from the liberation of deep structure, proved by the bright fluorescence obtained with anti-L-form FITC-labeled immunoglobulins. The mechanism of this change has not yet been fully elucidated. However, there are some biochemical data which may open a way for its clarification. Once phagocytized, the streptococci may be eroded by a cellular enzyme(s) capable of destroying the cell

wall structure. A strong argument in favor of this hypothesis is the data reported by Ayoub and McCarty (1), who demonstrated that there is, in most human cells, a glucosaminidase able to attack the cell wall and to modify serologically the C polysaccharide of the group A streptococcus. There is a general tendency to locate this structure at the deepest part of the wall, close to the membrane (4), although there are some objections (R. Krause, 4th Int. Symp. on *S. pyogenes*, Iena, 1969). It is possible that the enzymatic release allows the rest of the cell wall to disappear and to be progressively destroyed by other enzymes. A confirmation of this possibility has been provided by Ayoub and White (2), who showed by electron microscopy that group A streptococci phagocytized by human polynuclear neutrophils undergo morphological changes similar to those caused by the action of the phage-associated lysin. The immunological studies revealed a degradation of the group carbohydrate by an *N*-acetylglucosaminidase.

It is therefore not out of the question that a part of the streptococci phagocytized by the tissue culture cells may have deteriorated to some intermediate "protoplastic" forms, able either to reverse quickly to the bacterial form when cultivated on normal media or to grow as L-colonies when plated on media containing penicillin. It is also possible that a part of these forms, when they are in a convenient environment, manage to survive and to multiply as a bacterial variant devoid of cell wall, that is to say, as an L-variant. Obviously, only a minority of the streptococci inoculated are able to follow this pathway; let us recall that isolation of L-colonies occurred, in our experiments, in only one of three to five cases. It seems to us that this mechanism could account for the isolation of L-variants after inoculation of mice with group A streptococci, as observed by Mortimer (25), Rotta et al. (31), Maxted (24), and ourselves (34), whatever may be the phagocytic cells involved in this process in vivo.

These data also tend to suggest that, besides the destruction of the cell by actively dividing bacteria or the destruction of the bacteria by an enzymatically active cell, there is a third possibility which consists of the survival within the cell of modified bacteria. This rejoins the problem, unsolved as yet, of the chronic contamination by bacterial forms of tissue cultures grown with antibiotics. Several authors have stated that the contamination of tissue cultures may derive from bacteria which evolved to L-variants under the influence of the antibiotics used in culture media (15, 16, 23). Barile et al. (3) showed that contamination by "*Mycoplasma*" is much more frequent in cell cultures kept under media containing antibiotics. Similar results were obtained by Holmgren and Campbell (15). They isolated L-variants from fibroblasts cultivated on medium containing polymyxin B and inoculated with gram-negative bacilli. However, similar experiments made by Carski and Shepard failed to produce L-variants from 13 different species of bacteria introduced into tissue cultures (5).

Attempts to reverse the contaminants of tissue cultures to the bacterial parental form have been generally unsuccessful, with the noticeable exception of the recovering of a *Corynebacterium* from a "PPLO" (39). Moreover, comparative studies of the nucleic acids of *Mycoplasma* and bacterial L-forms do not bear out his hypothesis, since it has been proved that the nucleic acids of an L-form are very similar to those of the parental bacteria (22, 38). Thus, no definitive answer can be brought to this question, although it seems probable that conversion of bacteria to L-variants in tissue cultures does exist in defined conditions.

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