L FORMS FROM PNEUMOCOCCI[1, 2]

SARABELLE MADOFF AND LOUIS DIENES

Departments of Medicine and Bacteriology, Massachusetts General Hospital and the Robert W. Lovett Memorial Foundation for the Study of Crippling Diseases, Harvard Medical School, Boston, Massachusetts

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The isolation of L forms from pneumococci is of interest because the properties of the pneumococcal L forms and the conditions under which they develop differ in many respects from those observed in other bacteria.

Attempts to obtain L forms from pneumococci have been made in this laboratory since 1940. All the accumulating information on the conditions influencing the development of L forms was investigated. An intensified effort was made when the need for high salt concentration for the isolation of certain L forms was recognized (Dienes and Sharp, 1956). About 70 freshly isolated strains of pneumococci were studied on different media with varying concentrations of NaCl, and Na and K phosphates. Successful growth of L forms from pneumococci occurred when media with high concentrations of sucrose were used following the observations of Lederberg (1956).

DEVELOPMENT AND PROPAGATION OF L FORMS

Transformation of pneumococci into L forms was observed only on agar media in the presence of penicillin. Of several agar bases tested, trypticase soy agar (BBL) was found to be the most satisfactory. L forms developed occasionally on a soft medium containing 10 per cent sucrose, 0.1 per cent $MgSO_4$, and 10 per cent horse serum, but the results were not uniform and the L forms could not be propagated in serial transfer. Since charcoal has been used by Lorkiewicz (1957) to enhance the growth of L forms of Proteus, the addition of 1 to 1.5 per cent charcoal to the medium was tested. It was found to improve the production of L forms considerably and to make possible their growth on transfer. A fairly transparent medium could be obtained by precipitating the charcoal in the agar by heating the medium with the addition of horse blood and allowing the precipitate to settle out. A medium which gave the best results with a great many strains was prepared as follows: 4 g of trypticase soy agar and 1 g activated charcoal in 100 ml distilled water was autoclaved. Immediately upon removal from the autoclave or after cooling of the agar, 3 ml of packed horse blood cells were added and the mixture reheated to the boiling point. After sedimentation in a 56 C water bath, the supernatant was decanted. To each 90 ml of supernatant, 60 ml of a sterile solution containing 40 per cent sucrose and 1 per cent $MgSO_4 \cdot 7H_2O$, 15 ml horse serum from defibrinated horse blood, and the required amounts of penicillin were added. This medium contains about 0.85 per cent agar; it is very soft and is easily contaminated. L forms developed on harder agar also but serial transfer was successful only with a soft medium prepared with charcoal.

It was essential that the concentration of sucrose be kept between 10 and 20 per cent. Media containing concentrations of 5 and 25 per cent sucrose gave negative results. Magnesium salts were found to be important. In the absence of Mg, the pneumococci grew to large bodies but these disintegrated without developing into L forms. The optimal concentration of $MgSO_4$ was between 0.1 and 0.3 per cent. Slight growth of L forms was obtained with 10 per cent glucose and 20 per cent maltose but none with raffinose, starch, or the pentose sugars. The addition of casamino acids, small amounts of lactate and acetate, biotin, and ascorbic acid were without effect. Ascitic fluid and human serum were less favorable than horse serum and L forms were not produced in the absence of serum.

Plates were sealed with paraffin but it was of no advantage to maintain anaerobiosis. L colonies did not develop initially in pour plates and growth

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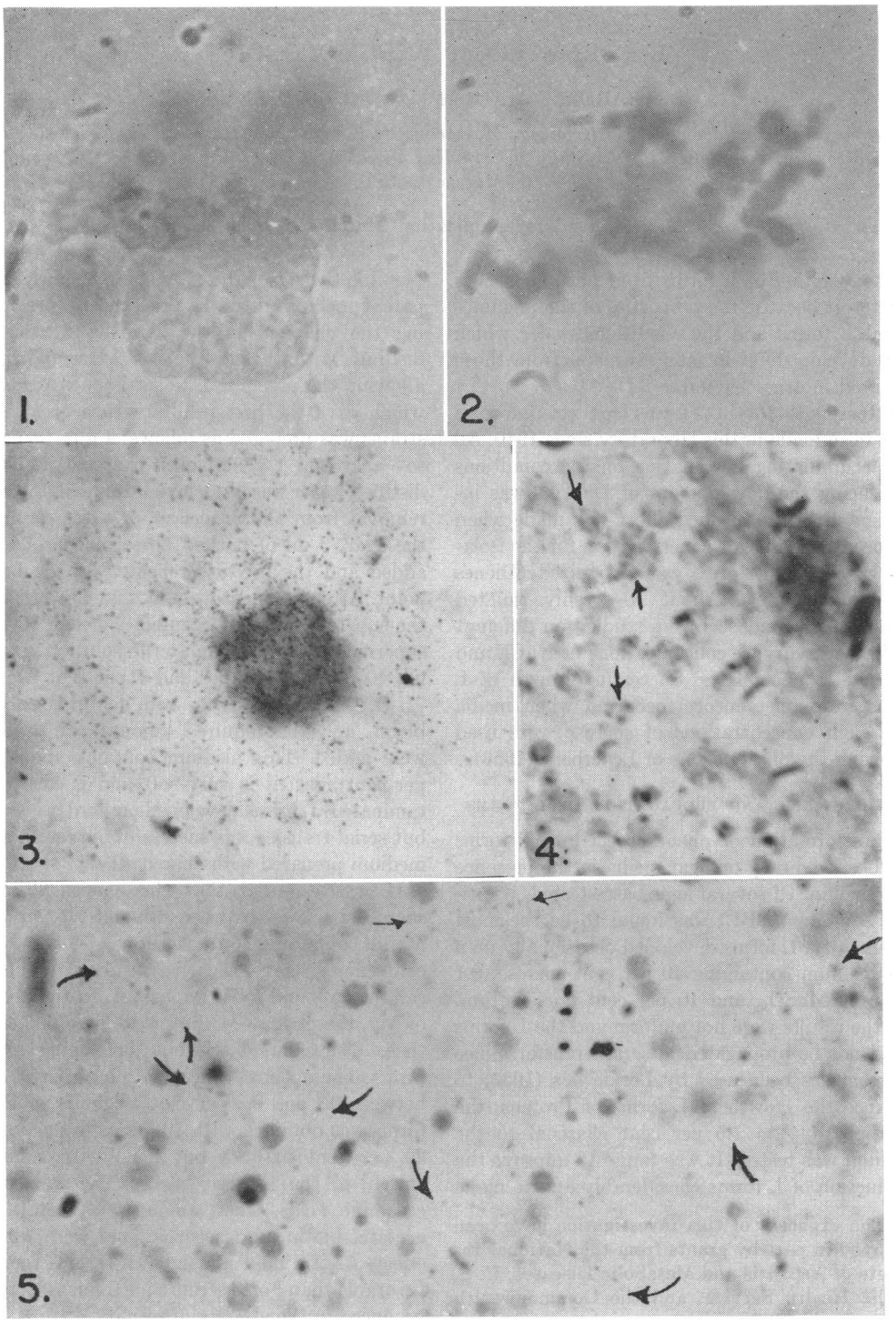

Figures 1–5

in transplant could be maintained in pour plates for one or two passages only. Attempts to grow the pneumococcal L forms in broth have been unsuccessful thus far, although colonies on agar blocks submerged in sucrose broth continue to grow.

The pneumococci were transformed into L forms by penicillin. No success was obtained with glycine which is effective in other species including streptococci. L colonies did not develop on plates containing high concentrations of penicillin (1000 units per ml). They were first observed when penicillin was deposited in a small trough cut into the agar and then only at the edge of the area of inhibition. This suggested that they develop only in a narrow range of penicillin concentration. Tests made by incorporating graded doses of penicillin in the media showed this range to be between 0.1 and 0.3 units of penicillin per ml and to vary within these concentrations with different media and with different strains of pneumococci. As small a difference as 0.02 units per ml often influenced the results markedly. In one experiment with strain 72, a type III strain most thoroughly studied, abundant L colonies were produced with 0.18 and 0.20 units of penicillin, respectively, and none with 0.16 or 0.22 units. Good results were also obtained with the commercial penicillin discs containing 2 and 10 units. L colonies developed in the areas of inhibition and were generally more abundant around one disc. When once the L colonies developed, their growth in subculture was equally good with high or low concentrations of penicillin as without it. Reversion to the coccal form was not observed.

Young broth cultures (4 to 6 hr old) from freshly isolated strains of pneumococci were used as inocula for L transformation. (We are indebted to Miss Marguerite E. Burke for the strains which were isolated from routine cultures examined in the bacteriology laboratory.) There was a marked variability in the production of L forms from different strains of pneumococci. Fifty-five strains were tested. Thirty-five produced a few to many L colonies. The L forms of two strains could be propagated on serial transfer. One was the type III strain mentioned above and the other a type V. These strains were the best of several which were consistently good L producers. The others did not survive in subcultures. The production of L forms by the strains in successive tests was about the same.

All strains identified as pneumococci were bile soluble and all of several tested gave *quellung* reactions with available typing or grouping sera. Further evidence for their identity as pneumococci was that the L forms of all strains were similar in appearance and differed from the L forms of streptococci in many respects. No relationship was observed between the serological type and the ability to produce L forms.

Since there is considerable variation in the isolation of L forms from different strains in response to varying concentrations of penicillin, agar and sucrose in the medium, a definite procedure for their isolation cannot be given. Some experimentation with the different factors involved and the examination of several strains may be necessary to get positive results.

A typical experiment was performed as follows: Four plates were prepared with the charcoal medium as described above and contained 0.16, 0.18, 0.20, and 0.22 units of penicillin per ml,

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Figures 1 and 2. Earliest development of an L colony. In figure 1, a large body containing faintly stained round granules and the superficial part of L growth are visible on the surface of the agar. In figure 2, the focus is set lower and part of the young L growth becomes apparent. The L growth may have started from the large body visible in the photograph or from another which has disintegrated and disappeared. Agar block stained with methylene blue and azur. ×2000.

Figure 3. L colony of pneumococcus with low magnification (×225). The center of the colony embedded in the agar is deeply stained. The periphery of the colony consisting of surface growth is indicated by the large bodies which appear as small dots. Their paucity and arrangement in rows can be seen. Specks of charcoal are present in the medium.

Figure 4. Periphery of an L colony at 24 hr. Several large bodies contain well stained granules not all of which are in sharp focus. Wet stained agar preparation. ×2000.

Figure 5. Periphery of a young colony extending on the surface. The different sizes and paucity of organisms are apparent. Long chains of small granules usually attached to a large body are indicated by arrows. Dried agar preparation stained with methylene blue and azur. ×2000.

respectively. Each plate was inoculated with three dilutions (10^{-0}, 10^{-1}, and 10^{-2}) of a 6 hr broth culture of strain 72. After incubation for 24 hr, the plate containing 0.16 units showed a confluent growth of pneumococci in each dilution. Discrete L-type colonies were seen on the plates containing 0.18 and 0.20 units, decreasing in numbers from many to a few colonies parallel with the concentration of the inoculum. Microscopic examination with stained agar preparations confirmed their identity as L colonies as illustrated in figure 3. No colonies of pneumococci were present in these concentrations of penicillin. No growth developed on the plate containing 0.22 units of penicillin per ml. Agar block transfers made from such cultures grew well on this medium containing high concentrations of penicillin as well as in the absence of penicillin. Pneumococci were never recovered from the L forms in serial transfers on penicillin-free agar or from transfer of agar cultures into broth.

DESCRIPTION AND PROPERTIES OF THE L FORMS

The development of the cultures has been followed in stained agar preparations. Transformation of pneumococci into L forms proceeds in the same way as in other bacteria. Under appropriate conditions some of the cocci grow on the surface of the agar to large round flat or polygonal bodies. Their size may be 10 μ or more (figure 1). Sometimes only a few, sometimes all, cocci develop to these forms. The large bodies at first stain deeply with methylene blue. Most of them become vacuolated after 2 or 3 days and then become transformed into empty blebs, or they may disintegrate. From a few large bodies a new growth starts which embeds itself into the agar. This growth consists of fairly large round or oval forms (about 1 to 2 μ) arranged in branching strands (figure 2). From this point on, the development of the L colony of pneumococcus is somewhat different from that of the previously studied bacteria including the streptococcus. The edge of the colony does not consist of small granules penetrating into the medium as in L colonies of other species, but it is fairly even inside the agar and consists mostly of large bodies. After some growth the colonies contain a large amount of transparent material which does not stain with methylene blue and in which organisms varying in size from small granules of a few tenths of a μ to large bodies of several μ are embedded. Many of the large bodies contain a few to many granules similar to those seen free in the cultures. The appearance of the colony is more like that of a pleomorphic coccus producing large amounts of extracellular material than that of the usual L colony, but in L colonies derived from the type III pneumococcus, this material did not give a *quellung* reaction. The special structure of the colony is probably the result of the prevailing mode of reproduction. The small granules grow to large forms most of which disintegrate into an amorphous mass, as noted above, but some of which reproduce the small granules. More will be said on this point in the Discussion.

It is characteristic of young L colonies when they begin to extend on the surface that tiny granules may be arranged in chains as long as 100 μ (figure 5). This has not been observed in L colonies from other species. The chains usually consist of small granules at the borderline of visibility but may include larger units similar to those in the large bodies and lying free in the cultures. These chains show some resemblance to streptococci degenerating under the influence of penicillin. However, the chains of granules seen in L forms from pneumococcus do not arise directly from the bacteria. The granules and connecting filaments are too delicate and chains of cocci are not present in the colonies. Furthermore, they are present in L cultures grown on successive transfer. These chains of granules are most abundant in the surface layer of young growing colonies but are also present in the center of the colony embedded in the agar. The chains are usually attached to a large body and are most probably produced by mechanical stretching of the content of the disintegrating large bodies

The extension of the colony on the surface, at least in part, is not the result of growth but of the extrusion of the material from the embedded center of the colony spreading on the surface. Even at the beginning of its development, the periphery of the colony at the surface does not consist of a layer of organisms but of a transparent material in which only a few organisms are present. Occasionally the flow of this material is indicated by the parallel strands of organisms embedded in it, by deformation of the large bodies and by the attachment of long filaments or of chains of granules to the large bodies. Disturbing the colonies with a cover slip transformed

many large bodies to long pleomorphic filaments indicating their plasticity but the chains of fine granules were not produced in this way. The extrusion of material onto the surface of the medium from L colonies growing just below the surface was observed in colon bacillus (Lederberg and St. Clair, 1958).

Whether the long chains of granules are growing elements could not be decided. They may include fairly large elements but such elements are visible inside the large bodies as well.

DISCUSSION

The pneumococcus is not the only species in which the superficial appearance of the L colonies differs from the L_1 of Klieneberger. The hard, dome-shaped colonies developing in 3A cultures of Proteus or the irregular masses extending mostly under the agar which are produced by some gram-negative spore-bearers show little resemblance to L_1. However, the basic morphology of the individual organisms in all these cultures is similar and unites the L forms of all species in a well-defined morphological group differing markedly from the usual bacterial forms. The way in which L type growth starts from bacteria, the fragility and plasticity of the elements and their pleomorphism including the presence of very small forms are common characteristics of all L forms. The lack of sensitivity to penicillin (Ward *et al.*, 1958) also indicates a common metabolic characteristic which may be reflected morphologically by the absence of the rigid bacterial cell wall. The special appearance of the L forms of the pneumococcus is not caused by the high concentration of sucrose necessary for growth. The L colonies of streptococci, diphtheroids, and Proteus are unchanged on sucrose media. The appearance of the L colony of the pneumococcus, as was noted above, is probably the result of the prevailing process of reproduction consisting in the growth of the granules into large bodies and the disintegration of these again into small granules. Such a reproductive process is not unexpected in the L forms. When these forms originate from bacteria, the bacteria always swell to large bodies and the granules of the L forms start to develop from the large bodies. Growth of the colony proceeds usually by multiplication of the granules some of which again develop into large bodies. This process is apparently changed in the pneumococcus L colony as well as in some others in that the small granules do not reproduce as such, but the cycle continues, from granules to large bodies to the disintegration of these again into granules. Amorphous material is produced in the colonies from disintegrating large bodies. Direct multiplication by division of the large bodies has not been observed in any L cultures and there is no indication for it in those of the pneumococcus. The fact that all transitory forms between small granules and large bodies are present in the cultures indicates that the small granules, as in other L cultures, are growing forms. The large bodies present similar reproductive processes not only in L cultures but occasionally in bacterial cultures as well. Although there is no consensus of opinion on the significance of the large bodies, and in some circumstances their production may be a purely degenerative process, it is well established that under certain conditions they participate in the reproductive process.

It has been noted that the production of L forms may be influenced by the concentration of penicillin used under certain conditions (Lederberg and St. Clair, 1958). Dr. Sharp (1957, *unpublished data*) observed that the production of L forms from group A streptococci was markedly enhanced when low concentrations of penicillin were used. However, with the pneumococcus it was observed that penicillin was effective only in low concentrations and within a narrow range. This is the first instance in our experience in which the optimal concentration of the antibiotic has proved to be so critical. Since growth and reproduction of the L forms, once produced, are unaffected by high concentrations of penicillin, it is evident that some process during the transitional stage is adversely influenced by high concentrations of penicillin. The growth of the pneumococci to large flat bodies occurs only at about the same concentrations of penicillin as are suitable for the production of L colonies. Very few of the large bodies, and not in all species, develop further.

Recently, attention was called to the similarities between the L forms and bacterial protoplasts (Weibull, 1956; Lederberg and St. Clair, 1958). The dependence of some L forms on high salt or sucrose concentrations is one of these similarities. Both forms seem to represent survival of the bacteria either in the absence of or with a markedly altered cell envelope. The idea

that this is the essential characteristic of the L forms was suggested to Ørskov (1936, *personal communications*), by morphological study of the L_1 of Kleineberger and the observations made later with Proteus seemed to give clear-cut evidence for this idea (Dienes and Weinberger, 1951). The large bodies produced in bacterial cultures either spontaneously or by exposure to penicillin, glycine, or various other chemical or physical influences, and the ensuing L growth, are different in many respects from protoplasts induced by lysozyme and cannot be regarded as corresponding entirely to such protoplasts. Expecially important is the fact that the large bodies and the L forms arise from bacteria by active growth and reproduction, and the primary influence in most cases is not the dissolution of the cell wall. The observation that L cultures with different properties and growth requirements can be obtained from a single bacterial strain (Dienes, 1953) indicates that the alteration of the cell produced by varying influences may differ in type and degree and that the continuation of life and reproduction may depend to a large extent upon the existing environment.

SUMMARY

L forms were obtained from pneumococci and propagated in transplants on soft horse serum agar media containing 10 to 20 per cent sucrose and 0.1 to 0.3 per cent $MgSO_4$. The pneumococci grew to L forms only in a narrow range of penicillin concentration (0.1 to 0.3 units per ml) but the L forms once produced were not sensitive to high concentrations of penicillin. There was a marked variability in the results obtained with different strains. Growth was not improved in pour plates and did not occur in broth.

The L colonies of the pneumococcus differ in appearance from most L colonies, especially from those of the closely related streptococcus. The colonies contain large amounts of extracellular material and long chains of granules which are probably produced by mechanical stretching of the large bodies that contain the granules.

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