Isolation of L Forms from Group A Streptococci Exposed to Bacitracin

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Received for publication 3 February 1965

ABSTRACT

ROTTA, JIRI (Washington University School of Medicine, St. Louis, Mo.), WALTER W. KARAKAWA, AND RICHARD M. KRAUSE. Isolation of L forms from group A streptococci exposed to bacitracin. J. Bacteriol. 89:1581-1585. 1965.—L forms were obtained from group A streptococci by exposure to bacitracin on gradient plates, although novobiocin was ineffective in this respect. Subcultures of these L forms had morphological and bacteriological properties similar to those obtained with penicillin. M protein was detected in L-form colony smears by immunofluorescent staining with type-specific conjugate. The L forms were not stained with group-specific conjugate. Parallel precipitin tests performed with extracts from a heavy growth of L forms on agar confirmed these findings. Thus, the L forms obtained with bacitracin continue to produce M protein but are devoid of the group-specific carbohydrate which is a major component of cell-wall structure.

Cultivation of group A streptococci (Sharp, 1954) and many other bacteria (Klieneberger-Nobel, 1960) on solid medium containing penicillin has yielded L-form colonies. This influence of penicillin on bacteria is dependent in part upon the fact that penicillin inhibits certain steps of cell-wall synthesis. Although bacitracin has an antibiotic action similar to that of penicillin, the production of L forms with this antibiotic has not been documented. Two attempts to produce L forms from bacteria with bacitracin have met with failure (Ward, Madoff, and Dienes, 1958; Molander et al., 1964). This report is concerned with the isolation of L forms from group A streptococci exposed to bacitracin.

MATERIALS AND METHODS

Streptococci. The following types of group A streptococci were used: type 1 (T1/114/7A), type 2 (T2-30/58), type 3 (B930/24/5), type 4 (4/42/58), type 5 (T5B/90/3), type 6 (S6/100/14), type 8 (T8/28/50), type 9 (T9/101/1), type 11 (T11-81/2), type 12 (12/3/58), type 13 (13/8/50), type 14 (FY), type 15 (T15/32/15), type 18 (J17C), type 22 (22-36/58), type 27 (27/27), type 28 (28/105B/3), and type 30 (D24/94/4). These strains were obtained from R. C. Lancefield, The Rockefeller Institute, and from the Streptococcus Reference Laboratory at the Institute of Epidemiology and Microbiology, Prague, Czechoslovakia.

Medium. The solid medium for all L-form cultures consisted of Brain Heart Infusion (Difco) broth, 1.1% agar, 2% sodium chloride, 0.5% yeast extract, and 10% horse serum. This basal medium was supplemented in certain cases with antibiotics, as indicated.

Antibiotics. Bacitracin and novobiocin were the products of The Upjohn Co., Kalamazoo, Mich., and penicillin G potassium was the product of Chas. Pfizer & Co., Inc., Brooklyn, N.Y.

Serological identification. M protein was determined by the capillary precipitin method (Swift, Wilson, and Lancefield, 1943).

Fluorescein-labeled antibody. Fluorescein-labeled type-specific antibodies were prepared by the method previously described by Karakawa, Borman, and McFarland (1964). Group-specific fluorescein-labeled antibodies were prepared by the method of Moody, Ellis, and Updyke (1958). Specific antistreptococcal membrane conjugates prepared from rabbit antisera were obtained from E. H. Freimer and J. Zabriskie, The Rockefeller Institute.

Fluorescent-antibody (FA) staining. Direct FA staining was performed on smears of L-form colonies with group-specific, type-specific, and antimembrane conjugates by the method previously described (Karakawa, Rotta, and Krause, 1965). Immunofluorescence of whole streptococci and staphylococci was performed by previously described methods (Karakawa et al., 1964; Moody et al., 1958).
Results

Isolation of L forms with bacitracin. L forms were isolated from a streptococcal culture grown on a bacitracin gradient agar medium plate. The antibiotic gradient was established by adding 500 units of bacitracin to a groove in the agar. The details for the gradient plate technique were described by Freimer, Krause, and McCarty (1959). The same cultures were inoculated onto gradient plates to which 12 mg/ml of novobiocin had been added to the groove, and onto other plates to which 1,000 units per ml of penicillin had been added. After the plates were incubated for 4 days at 37 C, L-form colonies were identified on the border between the growth of streptococci and the inhibition zone. Bacitracin and penicillin produced L-form colonies in the same 7 of 18 strains tested. L forms were obtained from two additional strains with only penicillin. Stability of the L forms is indicated by the fact that, after 10 serial passages on plates without antibiotic, reverision to the bacterial forms had not occurred.

Production of L forms on gradient plates containing novobiocin was attempted because it has been suggested that the action of this antibiotic is similar to that of penicillin (Strominger and Threenn, 1959). However, despite repeated attempts, it has not been possible to recover L forms from streptococci grown on a medium containing novobiocin.

Serological identification of L forms. Detection of type-specific M protein production by L colonies of types 1, 5, and 14 was performed by the method of Freimer (1963). These experiments were performed only on the L-form colonies obtained with bacitracin. A generous block of agar containing numerous L colonies was frozen and thawed to liberate fluid. After dialysis to remove salt, the fluid was tested with grouping and typing serum by the capillary precipitin method.

Table 1. FA-staining reactions between typespecific and group-specific conjugates and streptococcal L forms

<table>
<thead>
<tr>
<th>L-form type</th>
<th>FA titer with specific conjugates*</th>
<th>Group-specific conjugate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type-specific conjugate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>1</td>
<td>80</td>
<td>&lt;10</td>
</tr>
<tr>
<td>5</td>
<td>&lt;10</td>
<td>80</td>
</tr>
<tr>
<td>6</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>54</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

* Reciprocal of highest dilution showing 3- or 4-plus staining reaction.

Table 2. FA-staining reactions between streptococcal antimembrane conjugate and streptococcal and staphylococcal L forms and bacterial forms

<table>
<thead>
<tr>
<th>Cells</th>
<th>FA titer</th>
<th>Conjugate adsorbed with cytoplasmic membrane*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unadsorbed conjugate</td>
<td></td>
</tr>
<tr>
<td>Type 1—Streptococci</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>L forms</td>
<td>320</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Type 5—Streptococci</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>L forms</td>
<td>160</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Type 14—Streptococci</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>L forms</td>
<td>160</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>L forms</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

* Conjugate adsorbed with cytoplasmic membrane (SS132) of group A streptococci for 1 hr at 37 C.

Although no reaction was obtained with grouping serum, the reaction with homologous typing sera indicates that the L-form colonies produce type-specific M protein. In each instance, the type of M protein produced by the L forms was identical to that of the parent strain.

The identification of L forms by immunofluorescent staining of L-form colony smears is depicted in Table 1. The smears of types 1, 5, 6, and 14 L forms were stained with group-specific and homologous and heterologous type-specific conjugates. The FA type-specific conjugates react in much higher titer with homologous L forms than with heterologous L forms. As was previously reported, the L forms are not stained with group-specific conjugate (Karaka (1965)). Thus, L forms isolated from streptococci grown on either bacitracin or penicillin continue to produce M protein but are devoid of the group-specific carbohydrate, which is a major component of cell-wall structure.

Zabriskie, Freimer, and Seegal (1964) described immunofluorescent staining of streptococcal protomembrane membranes with a conjugate prepared from rabbit antiserum developed against purified membranes. Presented in Table 2 are the FA-staining reactions between streptococcal antimembrane conjugate and the L forms and parent strains of streptococci. The antimembrane conjugate reacted in high dilution with L-colony smears of types 1, 5, and 14 L forms, but was relatively unreactive with whole streptococci. Specificity of the conjugate is indicated by the fact that it was unreactive with either L forms or bacterial forms of staphylococci. The reactivity of the conjugate was eliminated by adsorption of the streptococcal L forms.
with cytoplasmic membranes. The pattern of staining obtained with type-specific conjugate is unlike that obtained with antimembrane conjugate. This is illustrated in Fig. 1 and 2 for type 6 L-form smears. The L-form smear illustrated in Fig. 1, after exposure to antimembrane conjugate, exhibits discrete and granular staining, whereas the L-form smear depicted in Fig. 2, after exposure to type-specific conjugate, exhibits diffuse staining. It is to be expected that membrane antibody would be limited to the L forms and thus exhibit a granular appearance upon fluorescent staining, whereas the M conjugate would be diffusely distributed because the M protein, produced by the L forms, diffuses into the agar medium and is not confined only to the cellular elements.

**Antibiotic sensitivity of L forms.** The penicillin, bacitracin, and novobiocin sensitivity was determined for L forms obtained with either penicillin or bacitracin (Table 3). The L-form cultures of types 1, 5, and 14 employed in these experiments were first repeatedly subcultured on antibiotic-free medium. The final subcultures, 72 hr old, were inoculated onto plates which contained a range of concentrations of antibiotic. The L forms obtained by either bacitracin or penicillin grew in a concentration of 1.0 unit of bacitracin per ml, but growth was inhibited by 5 units per ml. All of the L forms were highly resistant to penicillin. Also presented in Table 3 is a comparison of the antibiotic sensitivity of the parent strains to that of the corresponding L forms. The L forms are resistant to more than 100,000 times the concentration of penicillin which inhibits growth of the parent strains, and, correspondingly, are resistant to 100 times the concentration of bacitracin. It is to be noted that L forms and the parent bacteria are equally sensitive to novobiocin. Failure to obtain L forms with novobiocin may be dependent upon this fact.

**Discussion**

Our results indicate that streptococcal L forms obtained with either bacitracin or penicillin have similar morphological and bacteriological characteristics. The most striking similarity is that colonies of L forms isolated with either antibiotic produce type-specific M protein. Absence of a bacterial cell wall is suggested by the fact that group A carbohydrate could not be detected in the L forms by the FA technique. Although the colonies were cultivated in series in hypertonic agar medium without bacitracin, reversion to the bacterial forms did not occur. Thus, the L forms isolated with bacitracin appear to fulfill acceptable criteria for L forms.

The isolation of L forms from streptococci

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**Fig. 1 and 2. Immunofluorescence of smears of a type 6 L-form colony stained with (1) anti-group A streptococcal membrane FA conjugate and (2) homologous anti-type-specific FA conjugate.**
exposed to bacitracin is undoubtedly dependent upon the fact that this antibiotic, like penicillin, inhibits certain steps in cell-wall synthesis. It has been shown, for example, that there is an accumulation of uridine diphosphate-N-acetyl-
muramic acid-peptide and uridine diphosphate-N-acetylglucosamine in a stable group A streptococcal L-form culture obtained with the aid of penicillin (Edwards and Panos, 1962). Accumulation of similar uridine nucleotides in the cytoplasm of staphylococci exposed to bacitracin indicates an antibiotic effect similar to that of penicillin (Park, 1968).

The failure to obtain L forms with novobiocin deserves comment. It has been reported that uridine diphosphate-N-acetylmuramic acid-peptide accumulates in staphylococci which have been treated with novobiocin (Strominger and Threml, 1959), suggesting an antibiotic action similar to that of penicillin. Although it would be anticipated that L forms might be produced from bacteria exposed to this antibiotic, such was not the case.

On the basis of these findings, it is likely that novobiocin may have an antibiotic action in addition to its inhibitory effect on cell-wall synthesis. Brock (1962) suggested that novobiocin binds magnesium, and thus induces an intracellular deficiency of this element.

**Acknowledgments**

This investigation was supported by The Hartford Foundation, by Public Health Service grants 08027 (National Heart Institute) and 5 T1 GM 807 (Research Training Branch), and by the Missouri-St. Louis Heart Association.

It was conducted under the sponsorship of the Commission on Streptococcal and Staphylococcal Diseases, Armed Forces Epidemiological Board, and was supported by the Office of the Surgeon General, Department of the Army, Washington, D.C.

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