ANTIGENIC PROPERTIES OF HEMOLYTIC STREPTOCOCCI L-FORMS

(UDC 576.851.214.095.5.097.2)

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(Presented by Active Member AMN SSSR B. D. Timakov)
Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 59, No. 1,
pp. 78-81, January, 1965
Original article submitted October 10, 1963

In the present work a study of the antigenic structure of the L-forms of hemolytic streptococci and their revertants by means of serological analysis of the antigenic components extracted by various methods, of analysis of the distribution of antigens in the bacterial L-forms at the cellular level, and of the development of the antigenic complex of the L-forms and their revertants using the fluorescent antibody method in a direct Coons reaction was undertaken.

EXPERIMENTAL METHOD

A stable L-culture of β-hemolytic streptococci of group A No. 196, L, obtained experimentally [2] and having lost at the present time the ability to revert to the coccic form, a revertant strain of streptococci No. 196 r from the given L-culture [3], the parent culture of β-hemolytic streptococci No. 10-S, and also 2 strains of the L-form, No. 409 L and 406 L, which we isolated from the blood of a rheumatic carditis patient, and the cultures obtained from them—revertants No. 409 r and 406 r [4, 5] were used. Both L-cultures at the present time have lost the ability to revert to coccic form. Revertant strains No. 196 r and 409 r gave α-hemolysis in 5% blood agar, No. 406 r gave β-hemolysis.

To obtain suspensions suitable for immunization and antigens, L-cultures were grown on synthetic medium No. 199 with penicillin, without normal horse serum. After 7-10 days of incubation, the sediment consisting of L-colonies was washed 3 times in physiological solution with 20% Hanks solution, then resuspended, ground in a mortar and standardized with the GKI optical standard. Streptococcus cultures were grown in Puopp broth, washed in physiological solution and standardized. Immunization was carried out by the method described earlier [6]. All immune sera were labelled with fluorescin isothiocyanate by the standard method [1].

The antigens used for the serological research were obtained by the Lancefield hydrochloric acid extraction method, extracted by freezing at -70° and thawing at 37° ten times according to Grasse [6] and also by thermal extraction [7].

The antisera, antigens and cultures were examined as to ring precipitation, microprecipitation in gelatin and immunofluorescent reactions.

From the results of our work in which an increased fragility of the cell walls of the L-form revertants [5] was found, we assumed that, by treating them with lysozyme, it would be possible to rupture the cell wall and, using the free "wall-less form," to show the possible antigenic relationship of the revertants and the L-forms. For this purpose L-form revertants and the parent culture of β-hemolytic streptococci were treated with lysozyme. The sediments of broth cultures in the logarithmic growth phase were used, the supernatant was replaced by "tris" buffer at pH 7.2 20% sucrose and 0.1% lysozyme were added to the suspension and incubated at 37° for several hours. Protoplast formation was checked in the phase contrast microscope, using Naisy's staining method, by the determination of optical density and osmotic friability.

As a result of these experiments it was established that the parent streptococcus culture was not affected by lysozyme, while all 3 revertant strains of the L-form examined were converted under the influence of lysozyme into protoplast-free bodies.

Analysis of the Antigenic Structure of L-Forms and their Revertants Using Fluorescent Antigens

Fluorescent antiserum	Cultures										
	Before lysozyme treatment			After lysozyme treatment		Before lysozyme treatment		After lysozyme treatment	Before lysozyme treatment		After lysozyme treatment
	10-S	196 L	196 p	10-S	196 p	409 L	409 p	409 p	406 L	406 p	406 p
10-S	++	—	—	++	—	—	—	—	—	—	—
196 L	+	+++	—	—	++	+	—	—	+	—	—
196 p	+	—	+++	—	—	—	—	—	—	—	—
409 L	—	+	—	—	—	+++	—	+++	+	—	—
409 p	—	—	—	—	—	—	+++	—	—	—	—
406 L	—	+	—	—	—	+	—	—	+++	—	++
406 p	—	—	—	—	—	—	—	—	—	+++	—
Normal rabbit	—	—	—	—	—	—	—	—	—	—	—

Note. +++ fluorescence of maximal intensity in all cells of the population; ++ fluorescence of maximal and average intensity in all cells of the population; + fluorescence of average intensity in some of the cells of the population.

All the examined streptococcus cultures, before and after lysozyme treatment, were treated with homo- and heterologous antisera labelled with fluorescin isothiocyanate. The cultures were placed on slides, put in wet cells and stained at room temperature for 30 min, then washed with buffered physiological solution, covered with a cover slip and paraffin poured on.

The specificity of the fluorescence was verified in control experiments after preliminary treatment of the preparations with nonfluorescent immune serum, and also by treatment with fluorescent antibodies against other forms of bacteria and fluorescent normal rabbit serum. In these cases very weak natural fluorescence of the cultures was observed (—).

The preparations were examined in the ML-1 luminescent microscope illuminated by a DRSh-250 ultrahigh pressure spherical mercury-quartz lamp with filter combinations FS-1, S3S-7 and BS-8. Superfluous ultraviolet light was removed with a T-1N ocular light filter. The preparations were illuminated from above across an opaque-illuminator and objective. This permitted combining luminescent microscopy with phase contrast research.

EXPERIMENTAL RESULTS

The results of the ring precipitation reaction of the antigens and antisera examined showed a significant loss of the polysaccharide antigenic component (Lancefield) in the L-forms; antigens, extracted thermally, also did not appear. Using Grasse's extraction method, antigenic complexes, common to all 3 of the L-form strains studied, were extracted. The titer varied from 1:80 to 1:320 with homologous and from 1:20 to 1:80 with heterologous antisera. This complex had components related to L-form revertants which appeared in the precipitation reaction with sera against homologous revertants and hemolytic streptococci—undiluted.

The results of gelatin microprecipitation shows that serum against L-forms (No. 409 L) gave clear multiple lines of precipitation with the homologous antigen extracted by Grasse's method. This complex antigen contains components related to the antigens of heterologous L-form strains (Fig. 1, Exp. No. 2). We were not able to show related antigenic components of L-forms and their revertants in these experiments. Gelatin micropreicipitation of revertant antigens and the corresponding antisera indicate the complexity of all 3 of the examined antigens, according to the methods of Lancefield, Crawford and Grasse. Each of them consists of 2 components. From the arrangement and direction of the precipitate lines it is apparent that all 3 agents differ from each other. They were observed in all the examined revertants, showing their antigenic relationship (Fig. 2, Expt. No. 4, 13, 18).

Using immunofluorescence we were able to show the following (see table). Before treatment with lysozyme all the strains of streptococci examined which were treated with homologous antisera gave intensive luminescence. Staining of these cultures with homo- and heterologous L-form strains treated with homologous sera against L-forms gave intensive fluorescence and did not fluoresce when treated with sera against homo- and heterologous revertant strains.

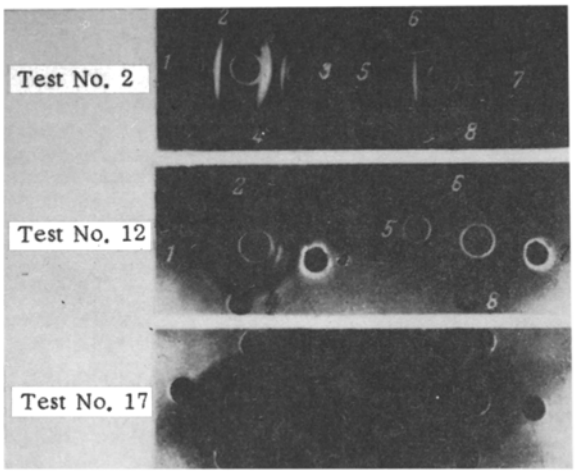

Fig. 1. Comparison of the characteristics of the antigens extracted according to Grasse (Exp. No. 2), Lancefield (Exp. No. 12) and Crawford (Expt. No. 17) from L-forms of hemolytic streptococci in gelatin microprecipitation reaction. In the center hole serum against L-form No. 409 L. Antigens: 1) strains No. 406 L; 2) No. 406 r; 3) No. 409 L; 4) No. 409 r; 5) No. 196 L; 6) No. 196 r; 7) No. 10-S; 8) control extract of wash medium.

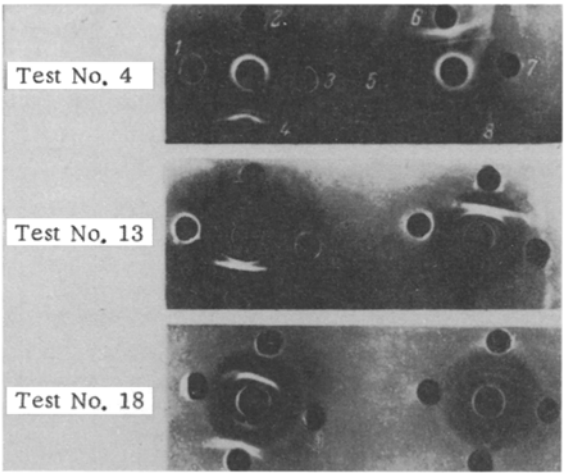

Fig. 2. Comparison of the characteristics of the antigens extracted according to Grasse (Exp. No. 4), Lancefield (Exp. No. 13) and Crawford (Exp. No. 18) from L-forms of hemolytic streptococci in gelatin microprecipitation reaction. In the center hole —serum against L-form revertant No. 409 r. Antigens: 1) strain No. 406 L; 2) No. 406 r; 3) No. 409 L; 4) No. 409 r; 5) No. 196 L; 6) No. 196 r; 7) No. 10-S; 8) control extract of wash medium.

Quite different results were obtained after treatment of the streptococci with lysozyme. Staining with labelled homologous sera of L-form revertants did not cause fluorescence and inversely, staining with labelled homologous sera against the L-forms caused intensive luminescence.

Treatment of the parent hemolytic streptococcus culture with lysozyme did not change the results of immunofluorescence. Treatment of these cultures with homologous antiserum before and after treatment with penicillin was accompanied by intensive fluorescence.

The L-cultures differed from the L-form revertants and the parent streptococcus strain in the character of the luminescence. Thus, all examined streptococcus strains before treatment with lysozyme were characterized by peripheral luminescence (Fig. 3 b) caused by localization of the antigens in the cell wall of the streptococci.

The L-forms devoid of cell walls were characterized by diffuse fluorescence (Fig. 3a), determined by components, in all probability, localized in the cytoplasmic membrane.

The fluorescent character of L-form revertants changed completely after their treatment with lysozyme. These "wall-less" forms of streptococci, labelled with L-antisera, were characterized by a bright, diffuse luminescence (Fig. 3c) due to antigenic components of cytoplasmic origin, common to the corresponding antigenic components in the homologous L-forms.

In the parent cultures of hemolytic streptocci the character of the luminescence before and after lysozyme treatment did not change.

Thus, a different antigenic localization in the bacterial form and in the L-form was shown. The first shows a surface arrangement of antigenic components of the cell wall, the 2nd, due to inhibition of the biosynthesis of the cells walls, shows antigenic components of cytoplasmic origin.

The antigenic complex of the L-forms and their revertants is determined by antigenic components of cytoplasmic origin.

SUMMARY

The results of studying the antigenic structure of the streptococci L-forms and their revertants indicate that

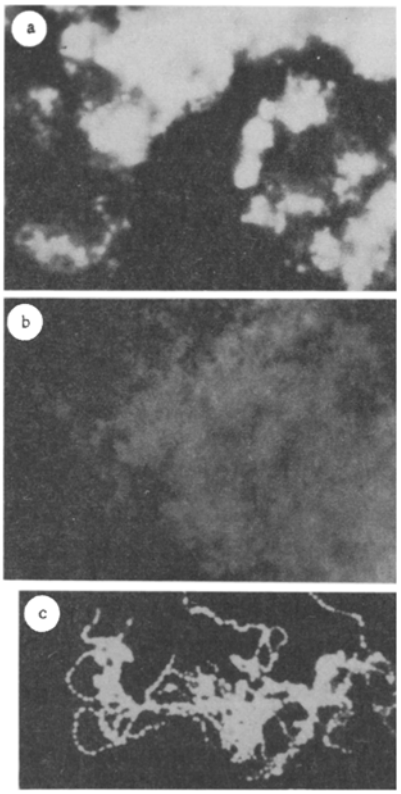

Fig. 3. Properties of the structural arrangement of antigens in L-form streptocci. a) L-form culture 196 L. Diffusing localization of antigens (treated with homologous antiserum); b) L-form revertant 196 r. Peripheral antigen localization (treated with homologous antiserum); c) L-form revertant 196 r after treatment with lysozyme. Diffusing localization of antigens (treated with serum to the L-form).

the antigenic component of the C-polysaccharide group (Lancefield) is largely lost during the process of L-transformation, this being due to biosynthesis block in cell walls. The component is restored in L-form reverts. L-form streptococci contain a complex antigen extractable after Grasse. This antigen occurs less frequently in the reverts. The efficacy of the antigen complex extraction after Grasse in the L-forms, less distinctly pronounced in the reverts, is evidently associated with the absence of the cell wall in L-forms and the presence of the indestructable wall in the coccal forms which resist freezing and thawing.

Employment of the fluorescent antibodies method in the direct Coons reaction demonstrated a different localization of the antigens in bacterial L-forms of streptocci. Superficially located antigenic components of the cell wall were detected in the former (L-form, including the L-form strains—reverts); in the latter (coccal form), due to the biosynthesis block of the cellular walls, antigenic components located in the cytoplastic membrane were revealed.

Proceeding from the formerly established fact of increased vulnerability of cell walls in the cultures reverted from L-forms, the cell walls were destructed by means of lysozyme. The use of such "wall-devoid" form for a serological analysis, aimed at establishing a possible antigenic affinity between L-forms and their reverts, has made it possible to ascertain the presence of common antigenic components of cytoplasmic origin.

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