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The nature of the opaque colony variation in group A streptococci*

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INTRODUCTION

Griffith (1934) described the occurrence of opaque colony variants of streptococci from human sources and made extensive use of them in his serological studies. He found the selection of these variations to be useful in obtaining type-specific organisms for slide-agglutination tests. It has subsequently been suggested that the opaque colony character is a reflexion of a high content of type-specific M protein on the group A streptococcal cell-wall (Gooder & Maxted, 1961). However, selection of high M-producing variants by this procedure has given inconstant results (Wilson, 1959), and unpublished studies carried out in this laboratory some years ago indicated that there is no consistent relationship between the presence of M protein and the opaque colony form. In fact, several different opaque colony strains were isolated in which M protein was not detectable. The present investigation was undertaken to determine what property of the organism is responsible for this striking colonial variation. Although the initial working hypothesis was based on the assumption that some constituent of the bacterial surface was involved, no evidence was obtained for the presence of a new component or for a quantitative increase in a known component. Rather, it was concluded that the opaque colony results from an altered growth pattern of the organisms that depends on an unusually tenacious union between individual cocci following cell division.

MATERIALS AND METHODS

Streptococcal strains

Opaque colony lines were established with sixteen different group A streptococcal strains of the stock laboratory collection. These were of twelve different M types. In each case, the translucent (or blue) variant was isolated for comparison. Most of the detailed chemical and serological studies were carried out with opaque and blue variants of strain S 43 (type 6) and strain S 23 (type 14).

Selection of variants

The organisms were grown on a clear medium composed of nutrient agar to which 5% horse serum and 0·1 mg./ml. bovine testicular hyaluronidase† were added at 45–50° C. just prior to pouring the plates. The presence of hyaluronidase prevented occurrence of mucoid colonies which tend to obscure the other colonial

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† Worthington Biochemical Corporation, Freehold, New Jersey.

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characteristics. The further addition of 0·05 mg./ml. lyophilized beef liver catalase* is useful in enhancing growth and increasing colony size. Plates were streaked so as to give good distribution of colonies and grown at 37° C. overnight.

A colony microscope with obliquely transmitted light was used for examining colonies. The several stock strains varied in the degree to which opaque colonies or sectors were apparent on initial plating, but opaque variants were isolated from every strain in which the attempt was made. Repeated transfers of selected opaque and blue colonies were carried out until pure lines were established. The opaque colonies under these conditions appear white or yellowish and coarsely granular. The non-opaque colonies show some fine granulation in most cases, but are characteristically blue in appearance and translucent (Pl. 1, fig. 1).

Antisera

Agar-grown organisms were used for the preparation of immunizing vaccines. The suspensions of washed cells in physiological saline were heated at 56° C. for 30 min. Rabbits were injected intravenously on four successive days a week for 8–10 weeks. After a rest period of 1–2 months, a second course of injections was given.

Extraction and fractionation

Test antigenic extracts were prepared by a wide variety of techniques, for example, simple buffer extraction; heating at pH 2; treatment with trypsin or chymotrypsin; dissolution with *Streptomyces albus* enzymes and group C streptococcal phage lysin. Capillary precipitin tests, gel diffusion and immunoelectrophoresis were employed in tests of the extracts and antisera.

Cell-wall, cell membrane and mucopeptide fractions were prepared for serological and chemical analysis by the procedures previously described (Freimer, Krause & McCarty, 1959; Krause & McCarty, 1961).

Chemical analyses

Rhamnose, total hexosamine, glucosamine, muramic acid, nitrogen and phosphorus were determined quantitatively as in previous cell-wall studies. Paper chromatography was used in the analysis of the amino acid content of mucopeptide (Krause & McCarty, 1961).

RESULTS

Occurrence of known surface antigens

In the initial serological studies, the content of M protein of paired opaque and blue strains was estimated semiquantitatively by the capillary precipitin test. Serial dilutions of acid extracts were reacted with type-specific rabbit antisera. In certain instances the opaque colony variant showed a higher titre of M protein than either the blue variant or the original stock strain, although there was frequently no significant difference between them. Of more significance is the fact that four strains yielded opaque variants which showed no detectable M protein,

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in contrast to the parent strains from which the variants were isolated. These results led to the conclusion that this surface antigen could not be responsible for the opaque colonial characteristic.

The content of T antigen was assessed in the opaque and blue variants of a type 1 strain (T 1). No difference was demonstrable in precipitin tests with trypsin extracts. Similarly, the opaque variant of T 28 showed no increase in 28 R antigen. These explorations, therefore, yielded no evidence for a relationship between colony form and the known surface protein antigens.

Search for new serological components

The possibility was entertained that the opaque character is dependent on an unidentified antigenic component of the cell. In order to test this possibility, a systematic analysis was carried out employing sera obtained from rabbits at intervals during immunization with opaque and blue strains. The soluble antigens used in the precipitin analysis were extracted by widely varying techniques to increase the likelihood that the hypothetical antigen would be present in at least one preparation. Repeated double diffusion and immunoelectrophoretic tests produced no evidence for the occurrence of an antigen in opaque strains that was absent (or present in reduced amounts) in the blue variants. Exploratory agglutination and complement-fixation tests were equally unpromising.

Chemical analysis of cell fractions

The foregoing results would not have uncovered a non-antigenic component nor an antigen that was not readily detected by the methods employed. Since the chemical composition of the surface structures of group A streptococci is reasonably well established, it was feasible to use chemical analysis in an effort to detect the presence of an unidentified component. Thus, the presence of an appreciable quantity of an unknown substance in the cell-wall of opaque variants would depress the analytical values of the known components. However, there was no significant difference in the content of protein, rhamnose, glucosamine, nitrogen or phosphorus between the walls of paired opaque and blue strains. The mucopeptide fractions were alike in glucosamine and muramic acid content and had the same amino acid composition both qualitatively and quantitatively. In short, the chemical findings, like the serological studies, failed to suggest a basis for the colonial variations.

Morphological observations

The first clue to a possible explanation for the colonial differences came from the routine examination of Gram-stained bacterial smears. Suspensions of agar-grown opaque colony variants, even after repeated washing in saline, were found microscopically to consist of tangled masses of extremely long streptococcal chains (Pl. 1, fig. 3). In contrast, suspensions from blue colonies showed only clusters and short chains, the more common pattern in smears of streptococci from surface colonies (Pl. 1, fig. 4). These differences were consistent for all of the opaque and

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blue pairs examined. It must be emphasized that these findings relate specifically to organisms grown on agar surface, and the difference is much less apparent in broth-grown organisms because of the enhanced chaining of blue variants under these conditions.

The exaggerated chaining of opaque variants was sufficiently striking to warrant further study in an attempt to determine its basis. The chains are extraordinarily resistant to disruption. They are not affected by mechanical agitation. They survive extraction with lipid solvents and drying from acetone and ether. A variety of reagents, including 6 M urea, 50 % pyridine, 10 % trichloracetic acid, 90 % phenol, and glacial acetic acid leave them unchanged. Proteolytic enzymes (trypsin, chymotrypsin, pepsin and streptococcal proteinase) cause no appreciable shortening of the chains. Even cell disintegration by shaking with glass beads does not always result in complete disruption, so that preparations were obtained in which isolated cell-walls occurred in chains.

The most illuminating information came from formamide extraction, which has been shown to leave as an insoluble residue the mucopeptide fraction of the cell wall (Krause & McCarty, 1961). Acetone-dried cells from agar-grown opaque variants were subjected to two successive extractions with formamide at 180° C. for 20 min. The washed residue had the composition of typical mucopeptide, except for the retention of somewhat more cell-wall carbohydrate (3–5 % rhamnose) than in preparations previously described. Microscopy revealed that the mucopeptide, devoid of nearly all of the other cellular constituents, still retained the chain configuration of the original cells. This is illustrated in the electron-micrograph in Pl. 1, fig. 2. It appears, therefore, that the property of tenacious chaining of streptococci in opaque colonies resides in this rigid structural layer of the cell wall.

Relationship of chaining to genesis of the opaque colony character

It is proposed that the property of rugged chain formation, brought about by the elaboration of an exceptionally massive intercellular bridge of the structural mucopeptide, leads to spatial orientation of cells growing on an agar surface which results in the appearance of granularity and opacity. It is significant that the colonial differences are readily apparent only with obliquely transmitted light, suggesting that special optical properties in the dispersion of light are responsible for the effect. That chain formation exerts a direct effect on the pattern of surface growth and colony configuration can be readily demonstrated by the examination of microcolonies.

For the production of microcolonies, sterile microscope slides covered with a thin layer of nutrient agar were inoculated by streaking with cultures of opaque and blue variants and incubated at 37° C. for 3–4 hr. Colonies composed of a few dozen to a few thousand cells were readily visualized microscopically at a magnification of ×500. At this stage the opaque colonies (Pl. 2, figs. 5–7) give the appearance of being formed from a single convoluted chain of cocci, and the edges are scalloped by the peripheral occurrence of frequent open loops of the chain. The blue colonies, on the other hand, are more regular in contour and appear to be

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composed of closely packed spheres (Pl. 2, figs. 8, 9). These growth characteristics seem to be quite adequate to account for the differences in dispersion of transmitted light by the macrocolonies.

DISCUSSION

The observations reported in this paper suggest that the opaque variants of group A streptococci owe their colonial appearance to persistent chaining during growth on an agar surface. The rigid mucopeptide layer of the cell-wall is clearly involved in the formation of the exaggerated intercellular bridges, but there is no information on the nature of the process that leads to this result. One must assume that the architecture of cell-wall synthesis, at least at the site of the cross-wall, is modified so that greater continuity between cells is retained after division. The possibility that the overall thickness of the basic cell-wall is greater in the opaque colony variants is not eliminated in these studies, since the data do not permit a quantitative estimate of the weight of cell-wall or mucopeptide per coccus.

This proposed mechanism for the genesis of opaque colonies leaves unexplained the success that was encountered in the selection of type-specific strains on the basis of this character. Even though the results of this kind of selection were inconstant, as noted in the introduction, it is clear that M-containing strains are obtained more often by this procedure than could be due to chance alone. Thus, there may be some relationship, although not a consistent one, between the altered cell-wall synthesis resulting in exaggerated chaining and the synthesis of M protein. This is reminiscent of the association between the production of hyaluronate capsules (mucoid colonies) and M protein. As in the case of opaque colonies, the selection of mucoid colonies is frequently useful in the isolation of strains with enhanced M protein production, although mucoid M-negative strains are well known.

It is likely that the opaque variants employed in the present study are comparable to those which Griffith (1934) referred to as *very opaque* and which gave granular suspensions unsuitable for slide agglutination. However, the same properties are found in the somewhat less opaque and granular colonies which we studied, and they differed primarily in being composed of rather shorter and more fragile chains. The degree of opacity of a colony appears to depend on the durability of the intercellular bridges between the cocci.

SUMMARY

Opaque colonies of group A streptococci, detected on clear agar plates with obliquely transmitted light, are composed of extremely long chains of cocci. The mucopeptide layer of the cell-wall is involved in the formation of enhanced intercellular bridges. It is suggested that this exaggerated chaining results in an altered growth pattern which gives the appearance of granular opacity to surface colonies.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Appearance of the opaque and blue colonies in mixed culture from strain S43/137 (type 6). The granularity of the opaque colonies is apparent. Microphotograph taken by oblique transmitted light (approx. 15° from perpendicular). Magnification, × 10.

Fig. 2. Electronmicrograph of streptococcal chain extracted twice with formamide at 180° C. Magnification, × 6000. Micrograph taken by Dr Earl H. Freimer.

Fig. 3. Gram-stained smear of suspension of organisms from opaque colony of S43. Magnification, × 1600.

Fig. 4. Gram-stained smear of organisms from blue (translucent) colony of S43. Magnification, × 1600.

PLATE 2

Figs. 5–7. Microcolonies of opaque variant (S43) after 3–4 hr. growth. Magnification, × 1100.

Fig. 5. Small colony showing continuous chain.

Fig. 6. Larger, more compact colony still retaining loops of chains at periphery of colony.

Fig. 7. Moderately large colony illustrating the marked effect of chaining on colony form.

Figs. 8, 9. Microcolonies of blue variant (S43) after 3–4 hr. growth. Magnification, × 1100. The greater uniformity of these colonies and close packing of cocci is in contrast to the appearance of the opaque colonies.

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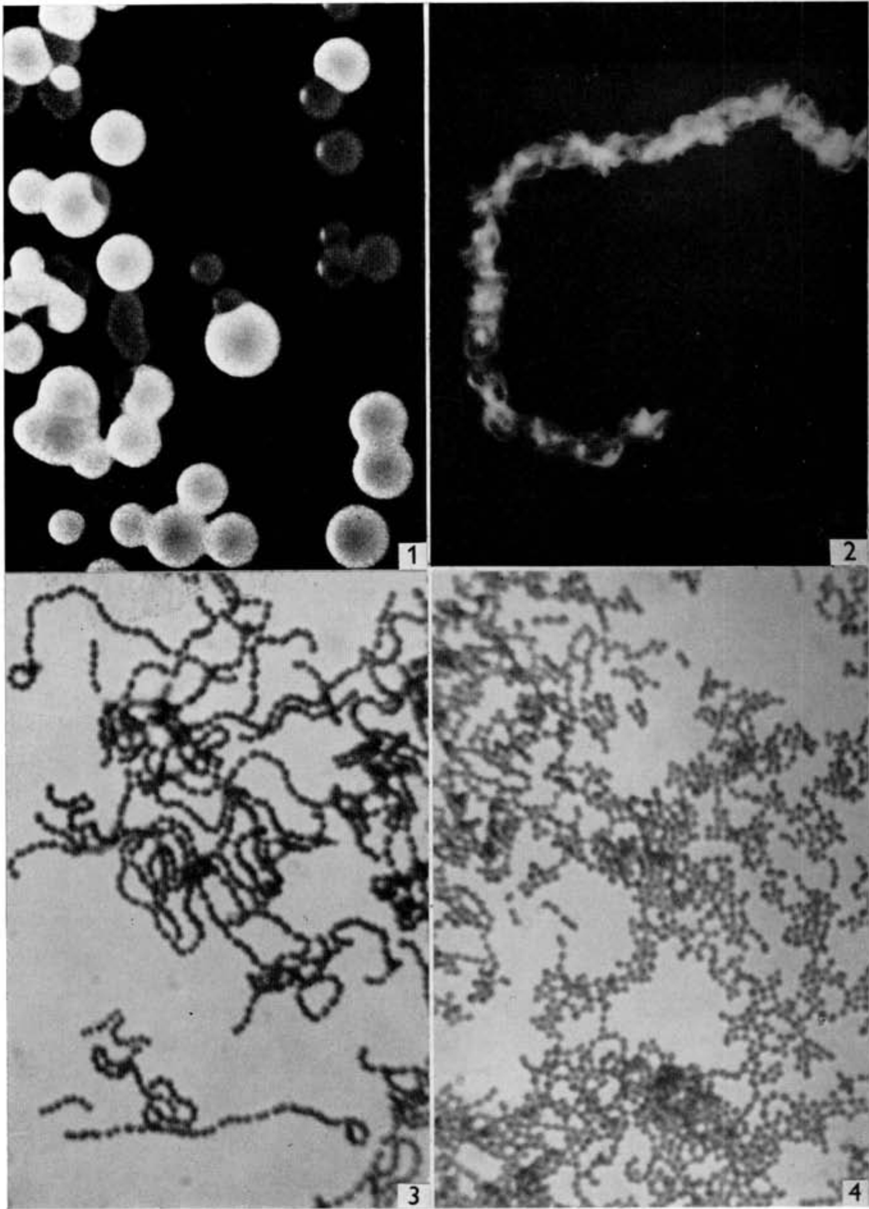

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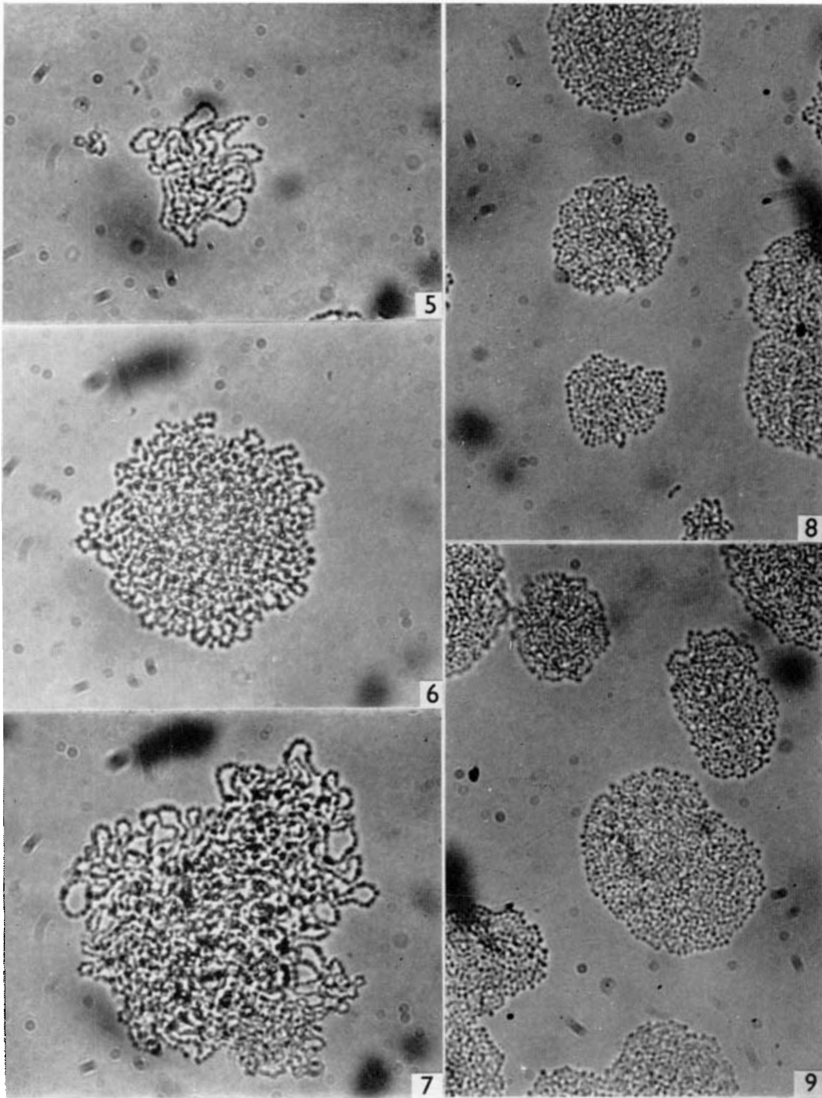

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