

From the Institute of Epidemiology and Microbiology in Prague, from the Allergologic Department of the Faculty Policlinic in Prague and from the Ophthalmologic Department of the Faculty Policlinic in Prague, Czechoslovakia.

CUTANEOUS DELAYED-TYPE REACTIONS
TO DIFFERENT COMPONENTS OF
GROUP A STREPTOCOCCUS IN PATIENTS
WITH RECURRENT EYE
DISEASES AND IN NORMAL CONTROLS

By

I. HÁNA, M. KOLEČKÁŘOVÁ & L. KLENKA

INTRODUCTION

Strong evidence has been presented (5) in favour of the presumptive aetiological relationship of beta-haemolytic streptococci to recurrent uveitis. Several characteristic features were shown: multiple streptococcal history of these patients, a significantly higher degree of delayed-type hypersensitivity to streptococci than in controls (4, 8), frequent activating systemic reactions following the injection of streptococci (7), and a favourable and lasting therapeutic effect of intravenously administered streptococcal hyposensitizing vaccines. The pattern of positive reactions to streptococcal strains of various groups and of various types in group A was also analysed: it was shown that skin reactivity to strains of any other group, the differences in the degree of positivity to the individual types within group A being very small (5). We are also concerned with the question of the possible role of hypersensitivity to other agents in this disease; the results will be known in a short time.

Having obtained the answer to some basic questions relating to streptococci, we have tried to elucidate some further prob-

lems, the foremost of these being which of the components of the streptococcal cell participate in delayed type reactions in these patients. Attempts to investigate this particular problem by simultaneously performed skin tests with all the cell components obtained after disintegration of streptococci have not previously been described; that the results might be of interest even from the point of view of general validity for streptococci led us to extend our work to the study of this reactivity also in normal individuals. The results of this study concern group A streptococci.

The cell of group A streptococcus—like bacterial cells of other species—consists of several components, some of them antigenic, others nonantigenic. However, not all of these components are limited to streptococci of group A, many substances similar to or identical with some of them being also found in members of other streptococcal groups.

On the outer surface of the streptococcus cell, especially in young cultures, a capsule structure is found, composed of hyaluronic acid, which has not been proved to be antigenic. Hyaluronic acid is highly viscous, so that colonies of strains producing large amounts of hyaluronic acid have a very mucoid appearance. The function of this hyaluronate capsule is not quite clear; however, it seems to enhance the antiphagocytic effect under certain conditions. Furthermore, special capsule polysaccharides have been discovered in streptococci of group B and some other serological groups which make possible further serological differentiation of strains within these groups.

The cell proper consists of a rigid cell wall that gives the streptococcus its spheroidal shape, of a thin plasmatic membrane situated under the cell wall, and of an internal cytoplasmic membrane situated under the cell wall, and of internal cytoplasmic components. The cell wall of group A streptococcus contains the well known type-specific proteins, *viz.* the M-protein and the T-protein and, in some types, also a substance called the R-protein, the group-specific carbohydrate, and mucopeptide. The mucopeptide, in contrast to the other sub-

stances, is similar in all groups of streptococci and also in other bacteria and forms the rigid framework of the cell wall; it also forms the firm intercellular bridges joining streptococcal cells in the characteristic chains. Somewhere near the cell wall (but hitherto not yet exactly localized) are the glyceroteichoic acids whose biological significance is still unknown. The M-protein is the primary factor of virulence owing to its potent antiphagocytic activity. On the other hand, no data is available of a similar role of the T- or R-antigens are available. Mucopeptide exerts biological activity, but its role in the pathogenesis of streptococcal infections remains to be cleared up.

The plasmatic membrane or cell membrane, differs in composition from both cell wall and cytoplasmatic components. It has a lipoprotein structure and evidently plays an important part in the metabolism of some substances of the wall, capsule, and cytoplasm as shown particularly by studies on streptococcal protoplasts.

The intracellular components contain many substances of proteinic nature, nucleoproteins and nucleic acids. Actually, this rich mixture of very different compounds has been investigated very little. A nucleoprotein fraction, probably of a very heterogeneous nature, was investigated in 1928 by *Lancefield*, who attributed to it some sensitizing properties (9). Very little is known of the very numerous intracellular enzymes.

As this short outline indicates, our knowledge of streptococcal cell components, and particularly of the functions of this components in the interaction microbe-man, is still very incomplete. Hence, so far no cell component can be claimed free from a possible sensitizing activity: this question has not been elucidated up to the present, although several authors have investigated this field (3, 10, 12). Therefore, in an effort to locate the maximum of sensitizing activity in the cell, we separated the cell components into three fractions, each of them being a mixture defined by the methods used and its contents.

In routine testing of patients by simultaneous intradermal injections of 24 strains of streptococci, the number of injections was necessarily limited; therefore, only one strain was used for injections in the present study on cell components. We chose strain No. 65474, type 11, because this strain displayed in our previous studies positive reactions in the great majority of patients, and furthermore—as careful analysis in our previous paper showed (5)—because this positivity seemed restricted in a remarkable way to patients as contrasted with normal controls and the rare carriers encountered in the population.

MATERIAL AND METHODS

1. *Streptococcal Strains Used and the Preparation of Whole Cell Testing Suspensions*

Altogether 24 strains were used, 19 of them being members of different types within group A and 5 belonging to other groups, not so important in human pathology. Detailed description has been given in previous papers (4, 8).

2. *Technique of Testing, Reading of Reactions, and Evaluation of Results*

At four successive weekly intervals, 0.05 ml of each suspension of whole cells or individual cell fractions was injected intradermally, the concentration of the suspensions being every week 10 times higher than the preceding week. Evaluation of the resulting erythema or induration was performed by our own method taking into account both the size of the reaction and the concentration of the testing suspension: for the numerical expression (in points) of hypersensitivity to all the streptococcal strains used, the term streptococcal coefficient (SC) was devised. Reference to previous papers is recommended for detailed description (4, 8).

3. *Preparation of Streptococcal Cell Fractions*

Streptococcus strain No. 65474, group A, type 11, possessing M-protein was used; this strain was one of our standard testing set. Three fractions were prepared containing predominantly cell walls, cytoplasmatic components, or so-called cell granules. The procedure was based on the preparation of cell walls according to *Salton & Horne* (11). The lyophilized strain was inoculated onto blood agar, from it into 5 ml of Todd-Hewitt broth, after 4 hours the contents were transferred into 300 ml and after another 4 hours into 1000 ml of the same broth. Cultivation was performed at 37° C; purity was assessed, the culture was centrifuged at 2,500 R.P.M. for 60 minutes, the sediment resuspended in 100 ml of saline and inactivated at 56° C for 30 minutes. The suspension was washed three times with saline and centrifuged at 2,500 R.P.M. for 20 minutes. The sediment

was resuspended in 100 ml of saline and its density was measured in a Coleman spectrophotometer at wave length at $520\text{ m}\mu$ in order to make possible subsequent adjustment of the density to the same degree as in vaccines tested previously (8). The suspension was diluted so as to correspond to the transmission of 50 per cent and sterility was controlled. Then mechanical disruption of the cells in Mickle's disintegrator by means of glass beads was carried out until the microscope revealed that practically all cocci were disintegrated. The time necessary for this disintegration varied, according to different conditions, from 45 minutes to 3 hours. The disintegrated cells were centrifuged at 10,000 R.P.M. for 45 minutes at $+4^{\circ}\text{C}$. The supernatant contained intracellular substances (IS). The sediment was washed three times and centrifuged at 4,500 R.P.M. for 30 minutes. The supernatant was separated as a further fraction (granules-GR), and the sediment containing cell walls (CW) was resuspended in saline. The suspensions containing CW or GR were resuspended in such volumes of saline that the density of the fraction studied (CW or GR) corresponded to the density of the original vaccine containing undisrupted cells. As experience in our laboratory had shown (2), centrifugation resulted in an approximately 50 per cent loss of GR matter and 30 per cent loss of CW matter; the final volume of the vaccines containing these fractions therefore had to be reduced in this proportion. All fractions studied (IS, GR, CW) were checked for final sterility and the vaccines were stored at -20°C . All subsequent dilutions were made with addition of about 0.1 per cent chloroform.

4. Patients

The testing with the three streptococcal fractions in addition to the routinely performed testing with 24 streptococcal strains was performed in 54 patients affected by recurrent eye diseases (35 men, 19 women). The mean age was 43 years (21 to 64 years). The great majority (48 persons) had recurrent iritis; on the average, each of them had suffered 9.1 attacks (2 to 50); in 13 of them, ankylosing spondylitis had been diagnosed conclusively; 14 of the patients had experienced several years before the onset of their eye disease an acute inflammation of the joints which in some cases may have been postinfectious arthritis. Three patients had had recurrent episcleritis; the analysis of their history could not establish the precise number of attacks but approximately three cases 235 attacks, which meant an approximate average of 80 attacks per person. Two patients had had recurrent sclerikeratitis (25 attacks altogether) and one patient had suffered from recurrent perphlebitis (8 attacks). The history of these 54 patients revealed chronic and recurrent tonsillitis in 38 cases (tonsillectomy had been performed in 11 cases), nephritis in 3, otitis in 11, scarlet fever in 13, erysipelas in 1 case.

5. Normal Persons

Testing with streptococcal fractions and routine testing was performed in control individuals in the same manner as in patients. Twenty-six subjects (24

men and 2 women) were tested. The mean age was 49 years (27 to 71 years). These persons also had an ample streptococcal history, but no signs of hypersensitivity were discovered. Details may be found in the description of normal population control groups in our preceding paper (5).

RESULTS

Similarly to the results of our previous studies in more numerous groups, the mean delayed-type hypersensitivity to streptococci in our patients differed very markedly from control values. In our group of patients, this value expressed in terms of SC ranged from 83 (in a 40 year old man suffering from ankylosing spondylitis who had sustained 4 attacks of iritis) to 573 (in a 44 year old woman who had had 6 attacks of iritis and a history of nephritis, scarlet fever, and repeated tonsillitis), the mean being 284.0. In controls, on the other hand, the range of SC values was 18 to 141, the mean being 65.0. Evaluation by means of the 95 per cent confidence interval reveals a highly significant difference.

Positive skin reactions to the test-suspensions prepared from whole cells of strain No. 65474 (type 11, group A) were observed in all the 54 patients examined. The weakest reaction occurred in a patient with ankylosing spondylitis and the numerical expression of this reaction gave 6 points; the reaction to this strain was evaluated as maximum, *i.e.* giving 28 points per person, in 8 patients. The mean value per patient was 17.7 points. On the other hand, only 15 of the 26 control subjects developed positive reactions and the mean was very 100-1.73 point.

These data show that mean skin reactivity to any streptococcal strain, calculated in terms of SC, was about 4.4 times higher in the group of patients than in the control group: the mean reactivity per patient equalled 11.8 points, in controls 2.7 points. The ratio of mean reactivity to type 11 in patients (17.7 points) and in controls (1.7 point) was 10.2; the contrast between this ratio and the above-mentioned ratio is evident.

The results of skin reactions to the individual fractions of

this streptococcus—which were of the delayed type similar to the reactions to whole cells—are presented in Tables 1 and 2.

TABLE 1

Hypersensitivity to Individual Fractions Type II Streptococcus in Patients.

Fraction	Total scores of points in all controls	%
Intracellular substances (IS)	510	47.4
Granula (GR)	278	25.8
Cell walls (CW)	289	26.8
Total	1077	100.0

TABLE 2

Hypersensitivity to Individual Fractions of Type II Streptococcus in Controls.

Fraction	Total scores of points in all controls	%
Intracellular substances (IS)	36	72.0
Granula (GR)	4	8.0
Cell walls (CW)	10	20.0
Total	50	100.0

In the patients, the total score of points obtained in reactions to the three vaccines prepared from the corresponding fractions was 1,077, while the total score in reactions to the vaccine prepared from undisturbed cells was 958 points. Every patient who exhibited a positive reaction to whole cells also responded to their fraction(s). In general, individuals exhibiting a lower or higher degree of hypersensitivity to whole streptococcal cells also displayed a corresponding lower or higher degree of hypersensitivity to the individual fractions. In the 26 controls, the total score of points obtained in reactions to the three streptococcal fractions equalled 50, to undisturbed cells 45 points. A summary of the results in both groups is given in Table 3.

TABLE 3

Hypersensitivity to Undisrupted Cells and Cell Fractions of 11 Streptococcus in Patients and in Controls.

	No. of persons	Total scores of points in all subjects tested	
		with whole cell vaccine	with 3 vaccines each comprising individual fraction
Patients	54	958	1077
Controls	26	45	50

This table shows excellent agreement between the results of tests performed with whole cells and with the individual fractions, both in patients and in controls: the total score of points obtained in reactions to the three cell fractions closely corresponds with the score of points obtained in reactions to whole cells. This means that all or almost all the substances which react in the three fractions obtained after disintegration of whole cells play some role in eliciting the skin reaction to whole cells. It is of interest that the ratio of points in columns 3 and 4 of Table 3 is almost identical: in patients 1.124, in controls 1.111. This may be taken as a proof of the reliability of the results, despite the scores of points in controls being low.

As regards the evaluation of hypersensitivity reactions in normal controls, it should be noted that their reactivity both to whole cells and to fractions, was very low and the erythema or induration often, especially in higher dilutions, did not even reach a diameter of 7 mm; according to our methods, such weak reactions were not taken into account. Owing to this very low degree of hypersensitivity, some irregularities were observed: in 4 subjects a cell fraction produced a reaction, even though only a small one but no reaction to the whole cell vaccine was registered; on the other hand, in 3 subjects a reaction (also very weak) to the whole cell vaccine was observed, but no reaction to the fractions was registered. This means that reactions to fraction(s) were evaluated in 16 of the 26 subjects tested, whereas reactions to whole cells were evaluated in 15 individuals.

DISCUSSION

An experiment with cell fractions from only one strain obviously cannot give a sufficiently general answer to the question expressed in the introductory paragraph. Unfortunately, testing with fractions of all the 24 strains used would be quite impracticable since a patient would then receive simultaneously 96 intradermal injections. Therefore, we attempted to select a strain in our set which had previously displayed frequent positivity and consequently would be most likely to elicit positive reactions in the present experiment; if a strain of considerably weaker positivity were used, a sufficient quantity of data might not be obtained. We chose strain No. 65474, type 11, for the reasons already mentioned in the Material and Methods section. Our own experience with frequent positive reactions in patients to this type of streptococcus was, moreover, supported by the results obtained by *Woods* (13) and *Alvaro* (1). Our decision proved to be correct, since the frequent positive reactions obtained in our patients furnished us with valuable data pertaining to human reactivity to the individual fractions of the streptococcus. The relatively higher degree of positivity to this strain in patients as compared to controls suggests the necessity of further thorough investigation of the relation of this type to recurrent eye inflammations; the correlations described do not appear to be entirely accidental, even though our preceding paper had shown that the differences between the intensities of reactions to various types of streptococci in patients are rather slight (5).

The tests with individual fractions showed that the highest reactivity was obtained with IS in both groups studied. Owing to low scores of points in control persons, only a rough estimate of the percentage of reactivity to individual fractions was possible; however, the scores of points in patients with affections of the eye were high enough to give a more accurate picture. IS are clearly in the forefront with about 50 per cent of reactivity, whereas the share of the reactions to CW and GR equals approximately 25 per cent each.

It has already been mentioned in the Material and Methods section that the concentrations of individual fractions were chosen so as to preserve the quantitative relationship which exists in the whole streptococcal cell. The purpose of this was to enable us to compare the reactions to the individual cell fractions with the reactions to undisintegrated cells, and to test the activity of the different fractions in the natural quantitative relations present in the living microorganism. Only this form of approach can clarify the share of individual cell components in the interaction between streptococcal cell and the host in the course of infection.

Thus, the activity of the fractions was not studied in relation to equal units of weight. However, a check on fraction activity in relation to a weight unit would bring about no change: CW represent roughly 20 per cent dry cell weight, GR account for about the same or slightly greater portion, so that a maximum of 60 per cent of dry cell weight is left over for IS. Consequently, IS are twice or three times more concentrated than GR or CW, but testing with the individual fractions was performed successively four times, each concentration being ten times higher than the preceding one; therefore, the two-fold or three-fold quantity of dry substance in IS could not have played a significant role. It should be taken into account that under special circumstances the testing of activity related to a weight unit might be well-founded *e.g.* in the investigation of such substances as the M-protein which is intended to be used in defined quantities for the immunization of human beings. On the other hand, in investigating problems of pathogenesis such as the one dealt with in this paper such an approach would inevitably lead to false deductions.

Our findings support the hypothesis already expressed in our previous papers (5, 6), namely that the various components of the streptococcal cell, both type-specific and non-specific, each have a different share in the development of delayed-type hypersensitivity in patients. With both specific and non-specific substances, but especially with the non-specific ones, the quantity present in individual strains may also have to be

taken into account; we have no knowledge about this. The distribution of skin reactivity among the various streptococcal fractions corresponds with our findings that reactions to individual strains differ markedly in different subjects, but tend to equalize in large groups. The reactions to individual types do not, furthermore, correspond to the prevalence of these streptococcal types in our territory, the prevalence being very uneven (5). These facts imply that type-non-specific substances play an important part, which is actually confirmed by our results: the most frequently encountered activity is found in IS which do not include type-specific proteins.

There remains the task of discovering the sensitizing components proper in the individual fractions by means of finer fractionation and possibly using purified components; naturally the above-mentioned rule of the same proportional distribution of components as in the whole cell will have to be respected. The situation is still more difficult because it is not even known whether only one or more substances are active in the individual fractions which have yet to be determined exactly.

The composition of CW has been investigated most thoroughly: we know that especially fractions prepared in the manner described and designated as CW contain the M-, T-, and R-proteins, the group-specific carbohydrate, mucopeptide, and glyceroteichoic acids, besides parts of plasmatic membranes and cytoplasmatic substances adhering to CW. Soluble fractions, designated as IS, represent a complex mixture of nucleotides (nucleic acids) and nucleoproteins, in addition to other proteinic substances and occasional contamination by traces of solubilized CW components. The third fraction obtained after centrifugation of particles appearing in the cytoplasmatic fraction in the course of treatment has been given the working designation GR; from the morphologic point of view, these particles are artifacts, as no similar shapes are found in native cells; they probably consist of aggregated ribosomes, cytoplasmatic proteins, and certainly of parts of plasmatic membranes. The most important thing, however, is that group- and particularly type-specific substances are located

almost exclusively in the CW fraction, whereas IS contain substances which often do not display even species-specificity. The remark concerning our lack of knowledge of the quantity of the substances under study in individual strains extends to the cell fractions in the test as well.

At this point it is essential to realize that the list of streptococcal cell components given in the introductory section mentions only a very small part of the substances present in the cell; the number of functionally and/or chemically described substances—even very important ones in some cases—ranges to tens, while the number of enzymes only, essential for the metabolic systems of the cell, is estimated at thousands. The total sum of other substances cannot be estimated at all. The complexity of a bacterial cell, in particular a streptococcal one, is enormous, and an attribution of all the important functions of the cell to a few substances hitherto recognized would be very erroneous. If we now possess a relatively very large amount of data pertaining to CW components, it is only because on the one hand CW are the site of first contact between microorganism and macroorganism and the immune mechanisms of the host are directed against this part of the bacterial cell, and on the other hand adequate techniques for the isolation of these substances have already been elaborated: in this our knowledge is limited by methodological success. It is necessary to keep all these facts in mind whenever conclusions are to be deduced.

SUMMARY

Skin reactions of the delayed type in humans to different fractions of the streptococcal cell were studied, obtained after disruption of a strain of group A, type 11, streptococcus in Mikle's disintegrator. Tests were performed both in patients with recurrent eye diseases and in normal subjects. The comparison of reactions to the individual fractions of the type 11 strain and to undisrupted cells of the same strain, revealed that after injection of whole cells all or nearly all those substances which react in the fractions obtained by disintegration play some part in the reaction. Of all the cell fractions studied, intracellular

soluble substances elicited the greatest member of the positive reactions in both study groups; these substances do not contain type- or group-specific factors. Reactions to cell fractions containing cytoplasmatic particles and to cell walls were considerably weaker. The implications of these findings are discussed.

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Requests for reprints:
Milada Kolečkářová, M.D.,
 Prague 5., Lesnická 8,
 Czechoslovakia.

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