A SEROLOGICAL STUDY OF SELECTED SPECIES OF CORYNEBACTERIA

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ABSTRACT

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A serological study of several corynebacteria species employing the techniques of intact cell agglutination and hemagglutination was carried out. The species used for the study were C. pseudodiphtheriticum, C. xerosis, C. diphtheriae type mitis, C. pyogenes, and C. fascians.

Using intact cell antigens for agglutination and acid extracts of whole cells as antigens for hemagglutination, antigenic relations between the various species were established. Adsorption of the antisera revealed the least number of antigens shared by the various species and indicate how closely these species are related.

INTRODUCTION

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The classification of corynebacteria at the present rests mainly on morphological characteristics, on differences in habitat, and on the biochemical reactions they elicit. Although the type species of the genus Corynebacterium has been clearly defined by the organism Corynebacterium diphtheriae, the boundaries of the genus (Gibson; 1952) and the inclusion of different species within the genus have become uncertain and controversial (Cummins & Harris; 1956).

Antigenic characteristics have proven to be valuable criteria for classification of the streptococci (Lancefield, 1933) and of the intestinal organisms (Kauffman, 1949) and more recently for a wide variety of other microorganisms. Used in conjunction with the standard methods of classification, the determination of antigenic structure has provided a method of evaluating relations between organisms (Cummins, 1962).

The introduction of more sensitive serological techniques such as immunofluorescent staining (Coons, Creech, Jones & Berliner, 1942) and passive hemagglutination (Keogh, North & Warburton, 1947) has revealed relations not detectable by the classical methods. Thus, Cummins (1954) was able to demonstrate, by cell wall agglutination, a common antigen to the mitis, gravis, and intermedius strains of C. diphtheriae that could not be detected by agglutination of intact cells.

In the work reported here, passive hemagglutination was used to study the relation between 5 species of corynebacteria.

HISTORICAL

HISTORICAL

The high specificity of serological reactions rests on the close complementarity between the chemical structures of the antigen and the antibody formed in response to its presence. It is therefore possible to detect differences between complex molecules that cannot be distinguished by normal chemical analysis. Serological typing can thus be used as a delicate tool for comparing and contrasting antigenic components of the microbial cell, providing information of use both in identification and classification (Shattock, 1955).

Bacteria are made up of a multiplicity of chemical constituents, many of which are capable of eliciting the production of specific antibodies. It is not surprising therefore that the injection into an animal of bacterial cells of one single type usually results in the production of several antibodies, each directed against one particular cellular component. Serological classification is thus based on the cross reactions elicited by similar antigenic structures present in different but related bacteria; it is assumed that the more closely related two different bacteria are, the greater the number of antigenic configurations they will share.

However several difficulties are encountered in the application of serological techniques to bacterial taxanomy. One of these is the loss of an antigen by a given species of bacteria as a result of mutation. Antigenic behaviour may also be conditioned by the "dissociative phase" in which the culture happens to be (Dubos, 1949). Another difficulty is engendered by any chance occurrence of the same determinant group in

widely different species; this gives rise to cross reactions of no direct taxanomic significance such as those observed between pneumococci, yeast and fungi (Neill, 1939) or between anthrax bacilli and type XIV pneumococci (Ivanovics, 1940).

Lancefield (1933) established serotyping as a valid tool for bacterial taxanomy through her classification of the genus Streptococcus. In her classical work, she isolated antigenic carbohydrates, collectively called carbohydrate c, from the cocci by hot acid and grouped the cocci by precipitin tests carried out on the carbohydrates. On this basis, she was able to divide the streptococci into 13 groups, several of which have been further sub-classified. More sensitive techniques such as the use of immunofluorescent stains (Moody, Ellis & Updike, 1958) and agglutination tests with cell wall suspensions (Cummins & Slade; 1961) have revealed more inter-relations between the members of this group.

A further important example of the use of serotyping in elucidating broad relations is provided by the large group of bacteria classified as the family Enterobacteriaceae (Bergey's Manual; 1957). Though the primary division into genera is made by physiological characters, serological analysis of flagellar and somatic antigens have been useful for differentiation of species and strains. Furthermore the sharing of some antigens between genera, e.g., Escherichia and Klebsiella (Kauffman, 1949) indicates that members of this family are related and form a continuous series.

The classification of the genus Salmonella (which also falls within this group) is firmly grounded on the antigenic structure of the species. Over 300 serotypes have been distinguished in the Kauffman-White scheme based on the pattern of the H (flagellar), O (somatic) and Vi antigens

that each organism possesses (cited in Topley & Wilson, 1955).

The genus Corynebacterium is characterized as a group of gram - positive rods, often swollen at the poles, containing irregular staining segments or granules. The principle features of the members of this group are the V or L forms and palisade arrangement that adjacent cells form. This may be due to the manner in which the smaller central cells proliferate (Bisset & Moore, 1952).

The type species of this genus is Corynebacterium diphtheriae which has been intensively studied because of its toxigenicity. The other species have not received much consideration and the term "diphtheroids" has been used to distinguish them from the type species. The classification of the species within this genus has been difficult because of the inadequate study of the diphtheroids, and consequently the identification of species often rests primarily upon the source from which the organisms were isolated and the biochemical reactions they perform. Corynebacterium diphtheriae is divided into 3 strains - gravis, intermedius and mitis. This division is made according to the virulence of the organism and their morphological appearance on blood tellurite agar medium.

The earlier workers studied the antigenic structure of the diphtheria bacillus by means of the agglutination technique. They reported that C. diphtheriae was antigenically heterogeneous and could be classified into various serotypes (Havens, 1920; Smith, 1923; Eagleton & Baxter, 1923). Ewing (1933) and Robinson & Peeney (1936) divided the gravis strains derived from various parts of the world into four and five antigenic groups respectively.

Hewitt (1947) extended this work and added 8 more serotypes to the gravis strains already classified. He also recognized 4 serotypes in the

intermedius strains and 40 in the more heterogeneous mitis strains. Some serotypes of different strains were found to be antigenically alike but could be differentiated by starch fermentation and hemolysis on blood agar medium as well as virulence and morphology on blood tellurite agar medium.

Although agglutination tests failed to reveal any antigenic relation between C. diphtheriae and other members of this genus (Bailey, 1925), the use of other serological techniques proved more successful. Krah & Witebsky (1930) showed that alcoholic extracts of C. diphtheriae, of certain diphtheroids, and of the tubercle bacilli, all fixed complement in the presence of an antiserum prepared against any one of these organisms. That some relation existed between species was supported by the work of Hoyle (1942) who described an antigen specific for C. diphtheriae type mitis and a group antigen common to the gravis, intermedius and mitis strains as well as C. hofmanni. Hoyle also utilized omplement fixation, with alcoholic extracts serving as antigens, to show the antigenic relation of these organisms.

Cummins (1954), in studying a mitis strain, found a protein surface antigen and a more deeply situated antigen, the latter being shared by the gravis and intermedius strains. The surface antigen was demonstrable by agglutination of intact cells and was specific for the mitis strain, whereas the common antigen could only be shown by agglutination of cell wall fragments. Cummins assumed that the common antigen was revealed only when the cell wall was broken, thus exposing the antigenic determinant. Because this antigen withstood heating at 100°C for 4 hours and was rapidly destroyed by periodate oxidation, it was believed to be polysaccharide in nature.

In an extensive survey, Cummins and his coworkers (1956, 1958, 1962) characterized the cell wall composition and studied the antigenic relations of corynebacteria and related organisms. They found that the characteristic sugars of corynebacteria, nocardia, and mycobacteria, were arabinose and galactose. The distinguishing amino acids of the species studied in these groups were alanine, glutamic acid and diaminopimelic acid. Two species, Corynebacterium pyogenes and Corynebacterium haemolyticum, did not fit the pattern since they lacked arabinose, galactose and diaminopimelic acid, but more closely resembled the streptococci in cell wall composition (Cummins & Harris; 1956).

Using cell wall agglutination Cummins (1962) showed cross reactions of cell wall fragments of 6 corynebacteria species to antisera prepared against C. diphtheriae type mitis. However, cell wall fragments of C. pyogenes did not agglutinate in this antisera. On the other hand, only the homologous reaction occurred in C. pyogenes antiserum. In conjunction with the facts revealed by chemical analysis of their cell walls, Cummins suggested that C. pyogenes should be reclassified.

Other methods such as immunodiffusion (Relyveld, Henocq & Raynaud; 1962) and fluorescent antitoxin elaborated in horses against the toxin of C. diphtheriae reacts with culture filtrates and surface antigens of other strains of the diphtheria bacillus as well as that of diphtheroids. Although such data arises from epidemological considerations, rather than taxonomical, a serological relation between these organisms is shown to exist.

With the introduction of more sensitive techniques, serological cross reactions were noted not only amongst the members of the genus Corynebacterium but also amongst those belonging to other genera.

Slack & Moore (1960) reported a relation between Actinomyces bovis and C. acnes which was revealed by using fluorescent antibodies prepared to A. bovis. However ther was no reaction between this antiserum and other species of corynebacteria, lactobacilli, propionibacteria, streptomycetes or nocardia. The sensitivity of the technique of immunofluorescent staining has been stated to be comparable to that of complement fixation (Beutner; 1961).

A hundred times more sensitive than agglutination is the serological technique of passive hemagglutination (Cummins; 1962b). This method was described Keogh North & Warburton (1947) who employed antigens from various Hemophilus species to sensitize erythrocytes rendered specifically agglutinable by the homologous antibodies. Middlebrook & Dubos (1949) gave a detailed account of the procedures involved for a similar system utilizing Mycobacterium tuberculosis. Since then, it has been found that various bacterial species possess antigens that are capable of modifying erythrocytes so that they agglutinate in the homologous antiserum (Neter; 1956).

The work by Keogh and his colleagues (1948) with Hemophilus, of Middlebrook & Dubos (1948) with Mycobacterium tuberculosis, of Fulthorpe (1954) with Salmonella, of Neter, Bertram & Arbesman (1952) with Escherichia coli, and of Roberts & Stewart (1961) with Streptococcus, indicates that all antigens participating in the passive hemagglutination test are polysaccharides or complexes of them. Indeed, Neter (1956) states that in all but two instances, chemical analysis of the modifying antigens has revealed that they are polysaccharides or contain polysaccharides as the serologically active determinant.

However the adsorption of protein antigens on erythrocytes followed

by specific agglutination in homologous antiserum can be accomplished by pretreating the red blood cells with dilute tannic acid (Boyden; 1951). Such treated erythrocytes are also able to adsorb polysaccharide antigens as well as protein ones, although differential adsorption of either can be facilitated. Chen & Meyer (1954) found that erythrocytes pretreated with tannic acid required sensitization for 1 hour at 37°C for polysaccharide adsorption whereas for protein adsorption a maximum of 15 minutes at room temperature was optimum.

That passive hemagglutination is one of the more sensitive techniques has been exemplified by the work of Gaines & Landy (1955) who were able to detect antibodies to Pseudomonas aeruginosa in the sera of normal individuals by this method. Hemagglutination titers as high as 1:960 were found while no titer could be detected by bacterial agglutination tests. Similarly, Neter, Westphal, Lüderitz, Gina & Goryznski (1955) have detected antibodies to enteropathogenic E. coli in healthy individuals when bacterial agglutination showed little or no titer.

One of the virtues of the method is that more than one antigen can be simultaneously adsorbed on the red blood cell. The red blood cell when sensitized with several antigens functions as a polyvalent antigen for the detection of any homologous antibody. Such systems have proven useful for diagnostic studies of enteropathogens (Neter et al; 1956) as well as for staphylococci (Florman, Scoma, Zepp & Ainbender; 1960).

Hemagglutination techniques have been primarily used for diagnostic purposes, but they also have been of value in elucidating serological relations between organisms. Rantz, Zuckerman & Randall (1952, 1956) reported a non-species specific substance common to species of staphylococci and streptococci that was capable of adsorbing to erythrocytes

and rendering them agglutinable in staphylocci antiserum. The work was extended by Neter et al (1959), Anzai et al (1960) and Goryznski and his colleagues (1960). These workers demonstrated the presence of this non-species specific antigen in various bacilli and gram-positive organisms, but showed it was completely lacking in gram-negative organisms.

McCarty (1958), using the capillary precipitin test, was able to demonstrate a polyglycerophosphate antigen that was randomly distributed among gram-positive organisms but entirely absent from gram-negative bacteria. Although the distribution of this antigen closely parallels that of Rantz's antigen, whether the antigens are one and the same is still in doubt. McCarty was unable to find any such antigen in corynebacteria species he tested.

Stewart, Steele, & Martin (1959) suggested that the technique of passive hemagglutination should be employed for the classification of the streptococci. Roberts & Stewart (1961) studied various streptococci by this method and showed a significant relation between the presence of rhamnose in the cell wall of the organisms and the hemagglutination pattern. They suggested that hemagglutination in conjunction with cell wall composition may be useful in classification. Chung & Hawirko (1962) using cell wall fragments to sensitize tanned erythrocytes were able to show a close relation between lactobacilli and streptococci. Indirect hemagglutination has also been used in the study of meningococci (Jyssum; 1958), pasteurella (Carter; 1959), Vibrio cholerae (Burrows & Schlub; 1958), and salmonella species (Sieburth; 1958) as well as for elucidating the classification of the streptomycetes (Douglas & Garrard; 1958).

Hemagglutination due to antigens from corynebacteria have been reported by few authors. Hayes (1951) demonstrated that the ethanol precip-

itated polysaccharide fraction of C. diphtheriae was able to sensitize sheep erythrocytes so that they took part in specific serum agglutination. Caille & Toucas (1960) used saline extracts of various corynebacteria to classify the organisms into two groups depending upon their possession of antigens capable of modifying red blood cells for agglutination. They found that the anaerobic or microaerophilic species possessed hemagglutinins whereas diphtheroids did not.

Serotyping has proven invaluable in bacterial classification in the past, and with the introduction of more sensitive techniques will be of greater importance in the future.

MATERIALS AND METHODS

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Reagents

Saline: Sodium chloride, 0.9% solution (w/v) in distilled deionized water was used for washing bacterial cells and in the preparation of buffer solutions.

Phosphate Buffered Saline: Buffered saline, (abbreviated PBS hereafter), pH 6.8 and 7.2, contained 0.15 M Na_2HPO_4 and 0.15 M KH_2PO_4 in an equal volume of saline.

Normal Rabbit Serum: Several young, male, albino rabbits were bled by cardiac puncture. The sera showing negative titers by agglutination with the test organisms were pooled and stored at -15°C. The serum did not require inactivation, but was absorbed 3 times with packed erythrocytes to remove any naturally occurring antibodies to the erythrocytes. The normal rabbit serum was used to prevent non-specific hemagglutination.

Alsever's Solution: The solution contained 2.05 g dextros, 0.80 g sodium citrate and 0.42 g sodium chloride in 100 ml distilled water. It was adjusted to pH 6.1 with 10% citric acid and autoclaved at 10 lbs/sq. in. for 15 minutes.

Sheep Erythrocytes: Sheep's blood was collected aseptically in 1.2 volumes of Alserver's solution, stored at 5°C, and kept at least 3 days before use. Thereafter it was used up to 5 weeks. Prior to use, the erythrocytes were washed 3 times in PBS (pH 6.8) and then centrifuged at 1000 rpm for 7 minutes in a Model H International centrifuge. The washed, packed erythrocytes were stored at 5°C and used the same day.

Cultures

The following organisms were used:

Corynebacterium diphtheriae type *mitis* (Laboratory culture)

Corynebacterium pseudodiphtheriticum (Winnipeg General Hospital)

Corynebacterium xerosis (ATCC 373)

Corynebacterium pyogenes (Laboratory culture)

Corynebacterium fascians (Laboratory culture)

Stock cultures were maintained on trypticase soy agar slants (BBL) at 28°C for periods up to 6 weeks; they were then transferred to fresh slants. Purity of cultures was checked routinely by gram stain and by plating the organisms on both trypticase soy agar (BBL) and trypticase soy agar containing whole blood and sodium tellurite.

Test cultures were grown in 500 ml volumes of trypticase soy broth on rotary shakers for 24 hours at 37°C except for *C. fascians* which was grown at 28°C.

The broths were pooled in 12 liter carboys and the cells were harvested on a Sharples centrifuge. In the case of *C. diphtheriae mitis* and *C. pyogenes* the cells were collected by centrifugation at 8,000 x *g* for 15 minutes in a Servall RC-2 centrifuge. After centrifugation, the bacteria were washed 3 times in saline, resuspended in saline to give a concentration of 0.2 to 0.5 g per ml and stored at -15°C for subsequent extraction of antigens or for absorption of sera.

Whole Cell Antigens

Twenty-four hour broth cultures, grown as described above, were treated with formalin to give a final concentration of 1 per cent and

were stored at 5°C until all bacteria had been killed (usually 1 to 2 days). The cells were then washed 3 times in PBS (pH 7.2) containing 1 per cent formalin and filtered through absorbent cotton to remove clumps. The cells were resuspended in PBS (pH 7.2), containing 1 per cent formalin, to give a density corresponding to a McFarland scale number of 3 (Kabat & Meyer; 1961) on the Beckman Model C colorimeter (43 per cent light transmission using Klett tubes and red filter).

The whole cell antigen was stored at 5°C and was employed (a) as vaccine for antibody production (b) as antigen for agglutination tests.

Preparation of Antiserum

Male albino rabbits were selected for antibody production. The sera prior to immunization were checked by agglutination tests to ensure that antibodies had not been produced by previous infection.

For immunization, Slavin's technique (1950) employing calcium alginate to hold the antigen in a depot was modified as follows. Whole cell antigen (200 ml) was centrifuged and 2 ml of 2 per cent (w/v) sodium alginate solution previously sterilized by autoclaving was added to the sediment. A homogeneous mixture, prepared aseptically by trituration, was injected intraperitoneally with a 20 gauge hypodermic needle. With the needle in situ, the first injection was followed by a further injection of 2 ml of sterile 0.5 per cent (w/v) calcium chloride solution. The alginate precipitated as calcium alginate and served to hold the antigen in depot.

After 2 weeks, weekly intravenous injections (2 ml of the stock vaccine concentrated tenfold) were given in the marginal ear vein. After the 4th injection, the rabbit was test bled and the titer checked by

agglutination. If the titer was sufficiently high, the blood was collected by cardiac puncture.

The blood was incubated at 37°C for 2 hours, stored at 5°C overnight and the serum separated. The serum was centrifuged at 2,200 x g for 10 minutes, divided into 2 ml portions and stored at -15°C.

Bacterial Antigen Extracts

Antigen extracts, for sensitization of erythrocytes in hemagglutination tests, were prepared from frozen stocks of bacteria. Fifteen ml of N/16 HCl was added to approximately 3 g cells, i.e., 10 ml of stock bacterial suspension. The cell suspension was placed in an ice water bath and macerated together with 3 g of Ballotini beads in a Gifford-Wood "MiniMill" (Hudson, N.Y.), at a micrometer setting of 3 for 15 minutes at 22,000 rpm.

The slurry was placed in a boiling water bath for 15 minutes and shaken at frequent intervals. After the mixture had been cooled in ice, the pH was adjusted to approximately 7.0 with 1.0 N NaOH. The gross supernatant was decanted to separate it from the bulk of the glass beads and finally centrifuged at 15,000 x g for 15 minutes to remove all debris. The pH of the clear yellow supernatant was adjusted to 6.8 with a pH meter and stored in glass vials at -15°C.

Such extracts were found to be stable for periods up to 6 weeks. The extracts when appropriately diluted in PBS (pH 6.8) served as the antigen for sensitizing the erythrocytes used in hemagglutination tests.

Dilution of Antigen Extracts

Each extract required dilution in an appropriate volume of PBS (pH 6.8) before sensitization of the red blood cells could be attempted.

Several dilutions of the extracts were empirically prepared and used to sensitize sheep erythrocytes as described in the next section. Each preparation was tested in serial dilutions of pre-immune and of the homologous antisera. The dilution of antigen that gave the lowest titer in the pre-immune serum and the highest titer in the homologous antiserum was used for subsequent sensitizations.

Sensitization of Sheep Erythrocytes

Appropriately diluted antigen extract (3 volumes) was added to one volume of packed erythrocytes which were suspended by gentle tritruation. Sensitization took place while the cells were incubated at 37°C for 2 hours with frequent stirring. The erythrocytes were then centrifuged, washed once with PBS (pH 6.8) and resuspended to a concentration of 2.5%. Sensitized cells were kept at 5°C and used on the same day.

Pre-treatment of Antiserum

Antiserum, diluted 1:2 in PBS (pH 7.2) was heated for 30 minutes at 56°C to inactivate the complement.

Absorption of the antiserum was required to remove Forsmann type antibodies. This was accomplished by adding 0.1 volume of packed erythrocytes to 1 volume of the antiserum and allowing the mixture to react at room temperature for 15 minutes with frequent shaking. The cells were centrifuged at 1000 rpm for 10 minutes and the antiserum removed. This procedure was repeated 3 times and the absorbed serum stored at -15°C.

Agglutinin Absorption

Absorption of the antiserum was carried on to remove homologous and cross-reacting antibodies. The cells were thawed and then centrifuged

at 5,000 x g for 10 minutes. One-tenth volume of the packed cells was mixed with 1 volume of antiserum and incubated at 37°C for 2 hours with frequent agitation. The reaction was allowed to continue overnight at 5°C. The procedure was repeated twice more on 2 successive days without removing the cells which had been previously added.

The antiserum separated from the cells by centrifugation was tested for the absence of antibodies. This was done by means of agglutination tests with the homologous intact cell antigen. If the homologous antibodies had not been completely removed, the procedure was repeated. The antiserum was stored at -15°C until used.

Hemagglutination Tests

Antiserum, which had been previously inactivated and absorbed, was serially diluted in 0.5 ml volumes. The two fold dilutions were made in 75 mm x 10 mm test tubes using PBS (pH 7.2) containing 1 per cent normal rabbit serum as a diluent. To each tube was added 1 drop of sensitized erythrocytes, after which the tubes were vigourously shaken and placed in a 37°C water bath. During the 2 hour incubation period, the tubes were shaken every 15 to 20 minutes. The tests were then placed at 5°C and read the following morning.

Each series of tests had two controls; (a) sensitized cells in PBS (pH 7.2) containing 1% normal rabbit serum, (b) unsensitized cells in a 1:40 dilution of the antiserum.

The tests were read by grading the hemagglutination patterns as seen at the bottom of each tube using the Fisher Kahn viewer. The grading scheme is given as follows:

4+ Mat of erythrocytes thrown up in folds.

3+ Smooth mat of cells covering most of the bottom of the tube.

2+ Small mat of cells with ragged and granular edges.

1+ Small, indistinct button ringed lightly by erythrocytes.

+- Small button with indistinct edges.

\- Discrete, sharply defined, small button.

Bacterial Agglutination Tests

Serial twofold dilutions of uninactivated and unabsorbed serum were made in 0.5 volumes in 75 mm x 10 mm test tubes using saline as a diluent. To each tube, 0.5 ml of intact cell antigen suspension was added. One control consisting of 0.5 ml antigen and 0.5 ml sanine was included in each series. The tests were incubated at 37°C for 2 hours after which they were placed at 5°C overnight. Reading of tests was done by gently tapping the tubes and observing the degree of clumping as seen under the Fisher Kahn viewer, and graded as 4+, 3+, 2+, 1+, and -, depending upon the degree of clumping.

Both hemagglutination and agglutination tests were further controlled by testing each antigen against a serial dilution of pre-immune serum.

RESULTS

RESULTS

Agglutination Tests

Whole cell antigens of each species showed titers of 2560 or higher with the homologous sera and cross reactions were not observed with the heterologous sera as shown in Table 1.

Hemagglutionation Tests

Acid extracts of each species showed titers of 1280 or higher with the homologous sera and titers of 160 or higher with the heterologous sera as shown in Table 2. In each case, absorption of the homologous serum with the cross-reacting species failed to remove the hemagglutination of the homologous strain indicating the presence of a species specific antigen or antigens. Absorption of the heterologous sera with the cross reacting species resulted in the removal of the antibodies, thus confirming the presence of common antigens as shown in Table 3.

Extracts of C. pseudodiphtheriticum reacted with each of the four heterologous antisera while reciprocal tests with the C. pseudodiphtheriticum antiserum showed hemagglutination with each of the cross reacting species.

Extracts of C. xerosis reacted with the antisera to each species with the exception of C. pyogenes. Reciprocal tests with the C. xerosis antiserum and the cross reacting species showed hemagglutination with C. pseudodiphtheriticum and C. fascians but not with C. diphtheriae.

Extracts of C. diphtheriae type mitis reacted with the antisera to each species with the exception of the antiserum to C. xerosis. However

reciprocal tests failed to show hemagglutination with C. fascians but gave significant titers with each of the other species, including non-cross reacting C. xerosis.

Extracts of C. pyogenes reacted with the antisera to each species with the exception of C. xerosis. Reciprocal tests failed to show hemagglutination to C. fascians as well as C. xerosis but gave significant titers with each of the other species.

Extracts of C. fascians reacted with the antisera of C. xerosis and C. pseudodiphtheriticum. However reciprocal tests with the C. fascians antiserum showed hemagglutination of each of the four other species.

Absorption Tests

Absorption of the homologous antiserum with C. pseudodiphtheriticum removed all hemagglutinins from the serum. The weak cross reaction between C. pseudodiphtheriticum extract and antiserum to C. pyogenes was eliminated by absorption with all organisms except C. fascians. The reaction between extracts of C. pseudodiphtheriticum and antiserum to C. diphtheriae type mitis was eliminated by absorption with C. xerosis and C. pseudodiphtheriticum; reduced by absorption with C. pyogenes and left unchanged by absorption with C. fascians. All organisms except C. pyogenes were able to absorb the hemagglutinins from C. fascians antiserum that were responsible for reacting with antigens of C. pseudodiphtheriticum.

Absorption C. xerosis antiserum with whole cells of C. xerosis removed all hemagglutinins from the serum. C. xerosis, C. pseudodiphtheriticum, C. diphtheriae and C. fascians were able to absorb hemagglutinins responsible for the reaction between extracts of C. xerosis and C. pseudodiphtheriticum antiserum. C. pyogenes was also able to lower the titer