STUDIES ON PRIMARY ATYPICAL PNEUMONIA. II. OBSERVATIONS CONCERNING THE RELATIONSHIP OF A NON-HEMOLYTIC STREPTOCOCCUS TO THE DISEASE

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Research Article

STUDIES ON PRIMARY ATYPICAL PNEUMONIA.

II. OBSERVATIONS CONCERNING THE RELATIONSHIP OF A NON–HEMOLYTIC STREPTOCOCCUS TO THE DISEASE [1,2]

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In a preliminary communication from this laboratory (1), the isolation of a non-hemolytic streptococcus from the lungs of 2 fatal cases of primary atypical pneumonia was reported. It was shown that convalescent sera of numerous patients with primary atypical pneumonia were capable of agglutinating this microorganism and some sera yielded precipitates with appropriate extracts of it. It was also shown that, except in rare instances, similar reactions were not demonstrable with acute-phase sera from the same patients, sera from patients with other acute infectious diseases, or sera from normal individuals.

Since the publication of these preliminary observations, additional strains of this non-hemolytic streptococcus have been isolated from the lungs and sputa of other patients with primary atypical pneumonia. A comprehensive study of the biological and immunological characteristics of this microorganism has been undertaken, and the results are described in detail in separate communications (2, 3). The serological reactions obtained with this streptococcus have been investigated, with additional specimens of serum from patients with primary atypical pneumonia, patients with other diseases, and normal persons, by means of a number of different immunological procedures. Of the patients with primary atypical pneumonia, 106 were studied in the Hospital

of The Rockefeller Institute, and an analysis of the clinical, roentgenological, and laboratory findings in these patients is presented in the accompanying paper (4).

It is the purpose of the present paper to summarize the information which has been obtained concerning certain properties of this streptococcus, the methods by which it was isolated from lung tissue, sputum, and throat swabs, and the serological reactions which were demonstrable with the convalescent serum of patients with primary atypical pneumonia. Evidence has been obtained which indicates that the strains of this microorganism which have been studied constitute a homogeneous group and belong to a single serological type, and may readily be differentiated from other varieties of non-hemolytic streptococci. It will be shown that the positive serological reactions obtained with this streptococcus and convalescent serum from patients with primary atypical pneumonia were due to specific antibodies which developed during the course of the illness. Moreover, evidence will be presented which indicates that these antibodies were independent of the properties of non-specific complement fixation (5) and cold hemagglutination (6, 7), which were also exhibited by certain of the convalescent sera studied.

For convenience, this streptococcus will be referred to in this report as "streptococcus MG." The problem of classification of streptococcus MG is discussed in a separate paper (2).

MATERIALS AND METHODS

Specimens

Specimens of sputum from patients with primary atypical pneumonia were frozen rapidly and stored at −70° C. Sputa from patients with other acute infections of the respiratory tract were treated in an identical manner.

Lung tissues from fatal cases of primary atypical pneumonia and from patients who died of other conditions were also stored at −70° C.[5] These various specimens were stored for periods ranging from a few days to 2 years before they were cultured. In addition to the frozen materials, fresh specimens of sputum and throat swabs from patients and normal persons were also cultured.

Culture media

For the isolation of streptococcus MG from specimens of lung tissue, Brewer's thioglycollate broth was employed (8). This medium was unsatisfactory for the culture of sputum specimens or throat swabs because of the rapid overgrowth of streptococcus MG by other microorganisms. For the isolation of streptococcus MG from such specimens, a semiselective medium was employed which permitted the growth of this microorganism and other varieties of non-hemolytic streptococci but inhibited the growth of most other bacterial species. This medium, which was similar in some respects to that recommended (9) for the selective cultivation of *Streptococcus salivarius*, was composed of the following constituents:

Proteose peptone Difco		5 grams
Yeast extract Bacto		5 grams
Beef extract Bacto		3 grams
Glucose C. P.		10 grams
Gentian violet		2 mgm.
Sodium azide		200 mgm.
Sulfapyridine		500 mgm.
Distilled water		1000 ml.

The pH of the completed medium was adjusted to 7.2 with 1N NaOH and the medium was then sterilized in the autoclave at 15 pounds pressure for 15 minutes.

For the maintenance and continued subculture of streptococcus MG in the laboratory, Todd-Hewitt broth was employed. When cultures were stored at 4° C. for periods of longer than 1 week, defibrinated rabbit blood was added to the broth in a concentration of 2 per cent. Blood broth cultures of streptococcus MG remained viable for as long as 2 months at 4° C.

Nutrient agar containing 5 per cent sucrose was employed for the differentiation of streptococcus MG from *Str. salivarius*. The latter organism has been shown to produce very large, mucoid colonies on agar containing 5 per cent sucrose (10). In contrast, streptococcus MG grew on this medium in the form of small fluorescent colonies.

Rabbit antisera

Antiserum was prepared by the intravenous immunization of rabbits with saline suspensions of heat-killed streptococcus MG. The details of the procedure used for

immunization are described elsewhere (3). The immune sera thus prepared usually possessed agglutination titers of 1:2500 or higher, and also caused definite capsular swelling of streptococcus MG.

Procedures for isolation and identification

A loopful of sputum was inoculated into approximately 4 ml. of the semiselective medium described above, and incubated at 37° C. for 24 to 48 hours. Throat swabs were placed directly in 4 ml. of this medium and incubated in a similar manner. Lung tissue suspensions were cultured in Brewer's thioglycollate medium.

It should be noted that streptococcus MG grew very poorly, or not at all, when suspensions of lung tissue containing it were streaked on the surface of blood agar plates, either under aerobic or anaerobic conditions. The reason for this is not clear, since broth cultures of streptococcus MG grew readily when inoculated on blood agar, and formed small, grey, coniform colonies. The failure to obtain growth with direct cultures of lung tissue on blood agar was apparently not due merely to the presence of small numbers of streptococci in the lung tissue suspension, since, as will be shown below, these suspensions even when diluted to 10^{-5} yielded growth in thioglycollate medium.

When visible growth in broth had occurred, a loopful of the culture was mixed with a loopful of rabbit antistreptococcus MG serum together with a loopful of 1 per cent methylene blue, and was then examined for capsular swelling. A positive quellung reaction was taken to indicate the presence of either streptococcus MG or *Str. salivarius*, type I. Evidence is presented elsewhere (3) which shows that the latter microorganism possesses certain antigenic components also present in streptococcus MG.

In order to differentiate streptococcus MG from *Str. salivarius*, type I, the cultures were then inoculated on the surface of 5 per cent sucrose agar plates. Single samples of the small, fluorescent colonies formed on this medium by streptococcus MG were inoculated in beef-infusion broth. Following growth in broth, the cultures were again tested for quellung in rabbit anti-streptococcus MG serum.

ISOLATION OF STREPTOCOCCUS MG FROM LUNG TISSUE

Lung tissues from 8 fatal cases of primary atypical pneumonia, and from 6 patients who died of other causes were cultured by the methods described above. The results are shown in Table I. Streptococcus MG was isolated from 6 of the 8 specimens of lung tissue from patients with primary atypical pneumonia. In contrast, this microorganism was not isolated from any of the 6 lung specimens from patients with other diseases, including 1 case of psittacosis, 1 of traumatic death, and 4 of pneumonia due

TABLE I

Isolation of streptococcus MG from the respiratory tract of human beings

Specimen	Diagnosis	Number patients	Positive cultures	
			Number	Per cent
Lung	Primary atypical pneumonia	8	6	75
	Other diseases*	6	0	0
Sputum or throat swab	Primary atypical pneumonia	97	53	55
	Acute respiratory infection without pneumonia	39	13	33
	Pneumococcal pneumonia	19	4	21
	Other bacterial pneumonias	15	3	20
	Influenza A	9	0	0
	Normal persons	57	7	12.5

* 1 psittacosis, 1 trauma, 4 bacterial pneumonia.

to other varieties of bacteria. The 6 patients with primary atypical pneumonia whose lung tissues yielded positive cultures died between the 8th and 22nd day after the onset of the disease, while the 2 negative specimens were from patients who died on the 25th and 34th days, respectively, after onset.

Str. salivarius was not encountered in any of the cultures of lung tissue from patients with primary atypical pneumonia. Four of the specimens contained small numbers of other bacteria which could be eliminated from the cultures by preparing higher dilutions of the suspensions of lung tissue before culturing in thioglycollate broth. It is noteworthy that pure cultures of streptococcus MG were obtained from 4 of the lung specimens when dilutions as high as 10^{-5} were cultured.

Occasional colonies of the strains of non-hemolytic streptococci isolated from lung tissue were encountered which did not exhibit quellung in the presence of rabbit anti-streptococcus MG serum. When grown in broth, subcultures from these particular colonies produced a flocculent type of growth, rather than the diffuse type of growth characteristic of streptococcus MG. Further comments on these strains will be found below under the heading: *Dissociation of streptococcus MG.*

ISOLATION OF STREPTOCOCCUS MG FROM SPUTUM

Specimens of sputum or throat swabs from patients with primary atypical pneumonia, patients with other infectious diseases, and normal persons were cultured in the semiselective medium by the methods described above. The results are included in Table I. Streptococcus MG was isolated from 53 of a total of 97 specimens from patients with primary atypical pneumonia. This microorganism was also recovered from 13 of 39 specimens from patients with acute upper respiratory infections without pneumonia, or so-called "catarrhal fever," from 4 of 19 specimens from patients with pneumococcal pneumonia, and from 3 of 15 specimens from patients with other types of bacterial pneumonia, but was not obtained from the sputum of 9 patients with influenza A. Streptococcus MG was isolated from the throat cultures of 7 of a total of 57 normal persons.

BIOLOGICAL CHARACTERISTICS OF
STREPTOCOCCUS MG

A total of 59 strains of streptococcus MG have been subjected to a detailed investigation. Each of these strains was selected on the basis of the following criteria: (1) quellung in rabbit anti-streptococcus MG serum, and (2) the formation of small fluorescent colonies on 5 per cent sucrose agar. Thirty-three of these strains were also tested by the agglutination technique with acute-phase and convalescent serum from selected patients with primary atypical pneumonia. Each of these strains was agglutinated by convalescent serum but not by acute-phase serum. The characteristics of the agglutination reactions with these strains were identical to those noted with the first strains of streptococcus MG isolated (1).

None of the 59 strains produced greening or hemolysis of rabbit blood agar when incubated for 24 hours. However, longer periods of incubation resulted in the production of distinct greening by some strains. Several strains produced more definite greening when grown on horse or sheep blood agar.

A detailed account of the biological properties

of streptococcus MG is given in a separate paper (2). It was found that there was a striking homogeneity in the properties of different strains of streptococcus MG, and that these properties made it possible to differentiate this microorganism from other non-hemolytic streptococci. It was also found that streptococcus MG was not pathogenic for any of the common laboratory animals. It was highly resistant to bile, and to the bacteriostatic action of sulfapyridine, as tested by the method of MacLeod and Mirick (11). It was however, susceptible to the effect of penicillin.

Effect of storage at −70° C.

Most of the specimens of lung tissue and sputum from patients with primary atypical pneumonia had been stored at −70° C. for periods varying from a few days to two years. It was therefore of interest to determine the effect of storage under these conditions upon freshly isolated cultures of streptococcus MG. Young broth cultures of 19 strains of this bacterium were stored at −70° C. for 3 months. At the end of this time, subcultures from these tubes were made in beef-infusion broth and in gentian violet-sodium azide-sulfapyridine broth. It was found that 3 of the strains failed to grow in either medium, and that 7 others grew in beef-infusion broth but failed to grow in the semi-selective medium. With 5 of the broth subcultures, capsular swelling in the presence of rabbit antiserum could no longer be demonstrated. The adverse effect of storage of streptococcus MG under these conditions suggests that this microorganism may be more readily isolated from freshly obtained specimens of sputum or lung tissue than from specimens which have undergone prolonged storage at −70° C.

Soluble specific substance of streptococcus MG

When sterile filtrates of broth cultures of streptococcus MG were mixed with homologous immune rabbit serum, definite precipitation occurred. Precipitation did not occur when such filtrates were mixed with normal rabbit serum or with the serum of rabbits immunized with pneumococci or beta-hemolytic streptococci. This finding together with the positive quellung reactions obtained with homologous immune rabbit serum suggested that streptococcus MG might possess a capsular polysaccharide analogous to those of type-specific pneumococci. Evidence has been obtained which indicates that streptococcus MG does possess a capsular polysaccharide and that the type-specific immunological reactions obtained with this microorganism are dependent upon the presence of this substance (3). The results of precipitation tests with the capsular polysaccharide and serum from patients with primary atypical pneumonia, intradermal tests with the substance in patients with this illness, and tests for the antigenicity of the polysaccharide in normal human beings will be given below.

Dissociation of streptococcus MG

By means of prolonged cultivation in broth containing homologous immune rabbit serum, dissociation of streptococcus MG was induced. The so-called R variants obtained by this procedure no longer were capable of synthesizing demonstrable capsular polysaccharide, and failed to give a positive quellung reaction with rabbit anti-streptococcus MG serum (3). The serum of rabbits immunized with R variants was capable in high dilution of agglutinating the R variants but either failed to agglutinate encapsulated strains of streptococcus MG or agglutinated them only at very low serum dilutions. In their biological and immunological properties, these R variants closely resembled certain single colony strains described above, which were obtained from the lung tissues of fatal cases of primary atypical pneumonia and which were negative in the quellung test. The available evidence suggests the possibility that these latter strains may represent similar non-encapsulated variants of streptococcus MG.

Antigenic relationship of streptococcus MG to Streptococcus salivarius, type I

The results obtained in studies on the biological properties of streptococcus MG (2), indicated clearly the marked differences between the characteristics of this microorganism and those of the *Str. salivarius* group. One striking and easily recognized differential property is their colonial morphology on nutrient agar containing 5 per cent sucrose. As was stated above, strep-

tococcus MG produced only small fluorescent colonies on this medium, whereas *Str. salivarius* produced very large mucoid colonies. In contrast with the significant differences in the biological properties of these microorganisms, evidence also has been obtained which indicates that streptococcus MG possesses certain antigenic components in common with *Str. salivarius*, type I (3). Various serological cross-tests revealed antigenic similarities between the capsular polysaccharides of these 2 different species of non-hemolytic streptococci. However, cross absorption tests with immune rabbit sera showed that although streptococcus MG and *Str. salivarius*, type I, were related antigenically they were not antigenically identical and could be distinguished from each other in immunological tests with appropriately absorbed antisera. It should be pointed out that streptococcus MG was not immunologically related to *Str. salivarius*, type II (3).

SEROLOGICAL REACTIONS WITH STREPTOCOCCUS MG

Agglutination tests with streptococcus MG were carried out with all available sera from 193 patients with primary atypical pneumonia. Of these patients, 106 were studied in the Hospital of The Rockefeller Institute, and the remaining 87 patients were studied in other hospitals.[6] As controls, similar tests were performed with sera from 321 normal persons and 120 patients with other diseases, including psittacosis, pneumococcal pneumonia, acute respiratory infections without pneumonia ("catarrhal fever"), scarlet fever, influenza A, rheumatic fever, and other streptococcal infections. Agglutination tests were also carried out with sera from 9 normal human beings who were vaccinated intradermally with the capsular polysaccharide of streptococcus MG, and from 2 normal human being who were vaccinated subcutaneously with a saline suspension of heat-killed streptococcus MG.

Selected specimens of serum were also tested by precipitation and complement fixation tech-

niques against the capsular polysaccharide of streptococcus MG, and by the quellung technique with the intact microorganism. Skin tests with this polysaccharide were performed in 22 patients with primary atypical pneumonia, 14 patients with other diseases, and 14 normal persons.

The methods employed in these studies, and the results obtained are presented in the following section.

AGGLUTINATION TESTS

Materials and methods

Bacterial suspensions for agglutination tests were prepared from 18-hour broth cultures of streptococcus MG. The bacterial cells were washed 3 times in 0.85 per cent NaCl and suspended in sufficient saline to give a turbidity approximating No. 5 in the McFarland scale. The streptococci were killed by heating at 65° C. for 1 hour. Merthiolate, in a final concentration of 1:10,000, was added as a preservative.

Serial 2-fold dilutions of unheated serum were made in saline. Each serum dilution was then mixed with an equal volume of streptococcal suspension. The final dilutions of serum tested ranged from 1:10 to 1:320. Dilutions lower than 1:10 were not used in routine tests because of the frequent occurrence of non-specific agglutination of streptococcus MG in 1:2 or 1:4 dilutions of either normal human or rabbit serum. The tubes containing the serum and the streptococcal suspension were placed in a water-bath at 37° C. for 2 hours, followed by 18 hours in the icebox at 4° C. They were then again placed in the water-bath at 37° C. for 2 hours, after which the tubes were shaken and readings of the degree of agglutination were made. The final period of 2 hours at 37° C. was found to be of importance since non-specific agglutination of streptococcus MG occasionally occurred at icebox temperatures. Such agglutination disappeared when the mixtures were brought to 37° C.

Sera were not heated at 56° C., because it was found that the agglutination titer of convalescent serum was sometimes reduced by heating at this temperature.

In estimating the degree of agglutination, the following standards were adopted. A designation of 4+ was assigned to tubes in which agglutination was complete, with a solid plaque or disc of bacteria and a clear supernatant fluid. Agglutination with large clumps and clear supernatant fluid, but without complete settling of the bacteria to the base of the tube, was designated as 3+. Agglutination with incomplete clearing of the supernatant fluid was designated as 2+. Agglutination with turbid fluid, but with particles visible to the unaided eye, was designated as 1+. Agglutination which required the use of a hand lens for visualization was designated as ±. The agglutination titer was taken as the highest dilution of serum in which reactions of 1+ or more were observed.

TABLE II

Results of agglutination tests against streptococcus MG with acute-phase and convalescent serum in primary atypical pneumonia

Case number	Day of disease	Dilution of serum					
		1:10	1:20	1:40	1:80	1:160	1:320
284	6	0*	0	0	0	0	0
	8	0	0	0	0	0	0
	14	4	4	4	4	4	3
	20	4	4	4	4	4	3
	29	4	4	4	4	3	0
	57	4	3	1	0	0	0
286	8	0	0	0	0	0	0
	10	0	0	0	0	0	0
	16	4	1	0	0	0	0
	32	4	4	4	4	2	0
	42	4	4	4	2	0	0
	54	4	2	0	0	0	0

* Figures refer to degree of agglutination
4 = complete agglutination with disc formation
1 = slight agglutination
0 = no agglutination

The results of agglutination tests against streptococcus MG with serum specimens obtained from 2 selected patients at various periods during the course of primary atypical pneumonia are shown in Table II. It will be noted that, in both instances, agglutination did not occur with sera obtained early in the course of the illness, whereas marked agglutination took place with sera obtained later. With sera from one patient, the agglutination titer was found to be 1:320 on both the 14th day and 20th day after onset,

1:160 on the 29th day, and 1:40 on the 57th day. With sera from the other patient, the agglutination titer was 1:20 on the 16th day, 1:160 on the 32nd day, 1:80 on the 42nd day, and 1:20 on the 54th day after onset. As is indicated by the results shown in Table II, the agglutination produced by the convalescent serum of these patients consisted of large plaque-like discs of bacteria with a clear supernatant fluid. Vigorous shaking of the tubes caused some breaking up of the larger clumps but obvious agglutination persisted even after prolonged shaking. The gross appearance of the agglutinated bacteria was indistinguishable from that which was observed with this microorganism in the presence of anti-streptococcus MG rabbit serum.

The results of agglutination tests against streptococcus MG with acute-phase and convalescent sera from 193 patients with primary atypical pneumonia are summarized in Table III. It will be seen that of 158 acute-phase sera which were obtained during the first week of the disease, only 5 were capable of agglutinating this microorganism. In contrast, 130 (67.4 per cent) of a total of 193 convalescent sera agglutinated streptococcus MG in serum dilutions ranging from 1:10 to 1:320. It will be noted that the convalescent sera from 54 patients (28.4 per cent) of the entire group, possessed an agglutination titer of only 1:10. Each of these sera was retested, with similar results. The significance of agglutination titers of 1:10 is obviously open to

TABLE III

Results of agglutination tests with streptococcus MG and the serum of human beings

Diagnosis	Serum	Number of patients	Agglutination titer							Total number positive	Percentage of positive
			<1:10	1:10	1:20	1:40	1:80	1:160	1:320		
Primary atypical pneumonia	Acute	158	153	3	1	1	0	0	0	5	3.2
	Conv.	193	63	54	35	19	16	4	2	130	67.4
Acute respiratory infection without pneumonia	Acute	36	36	0	0	0	0	0	0	0	0
	Conv.	36	31	5	0	0	0	0	0	5	13.9
Other acute infectious diseases	Acute	83	83	0	0	0	0	0	0	0	0
	Conv.	84	82	0	0	1	1	0	0	2	2.4
Normal persons		75	75	0	0	0	0	0	0	0	0
Normal persons (Training Station)		246	210	29	7	0	0	0	0	36	14.6
Vaccinated persons (Strep. MG)	Before	11	11	0	0	0	0	0	0	0	0
	After	11	0	0	3	3	2	3	0	11	100.0

question, particularly since serum dilutions lower than 1:10 were not employed as a routine. Convalescent sera from 76 patients (39 per cent) possessed agglutination titers of 1:20 or more, and convalescent sera from 41 patients (21 per cent) had agglutination titers of 1:40 or more. As is shown in the accompanying paper (4), there appeared to be a positive correlation between the severity of the illness and the agglutination titer of the convalescent serum for streptococcus MG.

In Table III, the results of similar agglutination tests with sera from patients with other diseases are also shown. It will be seen that the convalescent sera of 5 patients with acute respiratory infections without pneumonia, so-called "catarrhal fever," agglutinated streptococcus MG at serum dilutions of 1:10. None of the sera from 31 other patients with a similar illness agglutinated this microorganism. Negative results were obtained with both acute-phase and convalescent sera from 82 patients with other diseases, including psittacosis (11 patients), pneumococcal pneumonia (30 patients), influenza A (21 patients), rheumatic fever (6 patients), early pulmonary tuberculosis (4 patients), and scarlet fever (10 patients). The serum of one patient with subacute bacterial endocarditis and streptococcus viridans bacteremia possessed an agglutination titer of 1:80 and the serum of another patient with empyema due to a Group F beta-hemolytic streptococcus had an agglutination titer of 1:40. None of the sera of 75 normal persons, who had no history of recent respiratory infection, were capable of agglutinating streptococcus MG. In agglutination tests with sera from 246 persons living at close quarters in a naval training station, 36 sera (14.6 per cent) showed agglutination titers of 1:20 or less. The sera from this latter group were obtained at a time when an outbreak of acute upper respiratory infections was in progress, and it is probable that many persons in the group had either been exposed or were recovering from such infections.

The results of agglutination tests with sera from 11 human beings who were given injections of either the capsular polysaccharide or heat-killed suspensions of streptococcus MG are also shown in Table III. Agglutinins were not demonstrable in the serum of these 11 persons before vaccination. Following vaccination with a suspension of heat-killed streptococci, 2 persons developed agglutinins in titers of 1:20 and 1:40, respectively. Nine persons, who were injected with the capsular polysaccharide, developed agglutinins in titers ranging from 1:20 to 1:160. The agglutinins were demonstrable in serum obtained 8 days after vaccination, and were still present in undiminished titer 7 months later. It will be seen that the agglutination titers observed with sera from vaccinated persons were comparable to those obtained with the convalescent sera of patients with primary atypical pneumonia. The type of agglutination produced by the former sera was indistinguishable from that produced by the latter sera.

Agglutinins for streptococcus MG in the sera of patients with primary atypical pneumonia usually appeared between the second and fourth weeks of the disease and reached maximum titers during this period. In most cases, the agglutination titer began to diminish during the fifth and sixth weeks, and in some instances agglutinins were no longer detectable at this time. However, the sera of certain patients still possessed significant titers as late as nine weeks after onset. The variability in the time of appearance and disappearance of agglutinins for streptococcus MG was such that it was found advisable to obtain specimens of serum at weekly intervals from each patient, whenever this was possible.

The specificity of the positive agglutination reactions observed with convalescent serum was investigated by testing selected samples of strongly positive serum against suspensions prepared from cultures of other varieties of bacteria. These included *Hemophilus influenzae, Staphylococcus aureus*, pneumococci, and Group A hemolytic streptococci. In addition, 18 strains of other non-hemolytic streptococci which had been shown to be negative in the quellung test with anti-streptococcus MG rabbit serum were used. None of these various bacterial species was agglutinated by selected convalescent sera from patients with primary atypical pneumonia.

It has already been mentioned that an antigenic relationship exists between streptococcus MG and *Str. salivarius*, type I, which is ap-

parently due to antigenic similarities in the capsular polysaccharides of these two microorganisms (3). It was therefore of interest to determine whether suspensions of *Str. salivarius*, type I, were also agglutinated by convalescent sera. Acute-phase and convalescent sera from 99 patients with primary atypical pneumonia, each of whom was found to have developed agglutinins for streptococcus MG, were tested against suspensions prepared from cultures of *Str. salivarius*, type I, and *Str. salivarius*, type II.[7] It was found that the convalescent sera of 6 of these 99 patients also were capable of agglutinating *Str. salivarius*, type I, and that the convalescent sera of 3 other patients agglutinated *Str. salivarius*, type II. With sera from the remaining 90 patients, agglutination did not occur in tests against either type I or type II *Str. salivarius*. Acute-phase and convalescent sera from 45 patients with primary atypical pneumonia, no one of whom developed demonstrable agglutinins against streptococcus MG, were also tested against suspensions of *Str. salivarius*, type I, and *Str. salivarius*, type II. It was found that none of the sera from these 45 patients was capable of agglutinating either type of this microorganism.

An analysis of the increases in titer observed in agglutination tests against streptococcus MG with sera from patients with primary atypical pneumonia is shown in Table IV. In a group of 99 patients whose sera yielded positive results, and from whom 2 or more samples of serum were obtained during the course of the disease, a 2-fold increase in the titer of the convalescent serum was demonstrated in 47 patients, a 4-fold increase in 25, an 8-fold increase in 13, a 16-fold increase in 8, a 32-fold increase in 2, and a 64-fold increase in 1. In 3 patients, agglutinins were present in the acute-phase serum and no increase in the titer of the convalescent serum was demonstrable.

Analyses of the increases in titer observed in agglutination tests against either *Str. salivarius*, type I or type II, with sera from this same group of 99 patients, are also shown in Table IV. It will be seen that in tests with *Str. salivarius*, type I, no increase in the titer of the convalescent sera of 93 patients was observed, while a 2-fold increase in titer was demonstrated in 3 patients, a 4-fold increase in 1, an 8-fold increase in 1, and a 32-fold increase in 1. It will also be noted that in tests with *Str. salivarius*, type II, no increase in the titer of the convalescent sera of 96 patients was observed, while 2-fold, 4-fold, and 8-fold increases, respectively, were demonstrated in 3 patients.

It has already been mentioned that non-encapsulated, so-called R variants of streptococcus MG were obtained by inducing dissociation of this microorganism. These R variants possessed antigens which were immunologically distinct from the capsular polysaccharide of streptococcus MG (3). When acute-phase and convalescent sera from selected patients with primary atypical pneumonia were tested, by the agglutination technique, against such R variants, it was found that the convalescent sera agglutinated the R variants whereas the acute-phase sera did not. The agglutination of R variants produced by convalescent sera was distinctly different from the agglutination of encapsulated streptococcus MG produced by the same sera. Agglutination of R variants was characterized by the formation of very small bacterial clumps which were easily dispersed by gentle shaking. It is noteworthy that the agglutination of R variants produced by the serum of rabbits, immunized with either streptococcus MG or its R variant, was similar in all respects to that produced by the serum of patients convalescent from primary atypical pneumonia. These findings suggested that convalescent sera possessed

TABLE IV
Comparison of agglutination titers of acute-phase and convalescent sera of patients with primary atypical pneumonia

Streptococcal suspension	Number of patients	Increase in titer of convalescent serum						
		0×	2×	4×	8×	16×	32×	64×
Streptococcus MG	99	3*	47	25	13	8	2	1
Streptococcus salivarius, type I	99	93	3	1	1	0	1	0
Streptococcus salivarius, type II	99	96	1	1	1	0	0	0

* Figures refer to number of patients who showed designated increase in titer.

[7] The strains of *Str. salivarius*, type I and type II, were obtained through the courtesy of Dr. J. M. Sherman of Cornell University School of Agriculture, Ithaca, N. Y.

TABLE V

Results of agglutination tests with streptococcus MG and its R variant

Serum	Immunized with	Streptococcal suspension	Serum dilution (reciprocal)									
			10	20	40	80	160	320	640	1280	2560	5000
Rabbit	Normal	MG	0	0	0	0	0	0	—	—	—	—
		R	0	0	0	0	0	0	—	—	—	—
	Streptococcus MG	MG	4	4	4	4	4	4	4	3	1	0
		R	+	+·	+	+	+	+	±	0	0	0
	R variant of strepto-coccus MG	MG	+	0	0	0	0	0	0	0	0	0
		R	+	+	+	+	+	+	+	+	±	0
Human	Normal	MG	0	0	0	0	0	—	—	—	—	—
		R	0	0	0	0	0	—	—	—	—	—
	Streptococcus MG	MG	4	4	1	±	0	—	—	—	—	—
		R	+	+	+	±	0	—	—	—	—	—
	Capsular polysac-charide of strepto-coccus MG	MG	4	4	4	4	2	0	—	—	—	—
		R	0	0	0	0	0	0	—	—	—	—
Patient with primary atypical pneumonia	Acute phase serum	MG	0	0	0	0	0	0	—	—	—	—
		R	0	0	0	0	0	0	—	—	—	—
	Convalescent phase serum	MG	4	4	4	4	2	—	—	—	—	—
		R	+	+	+	+	+	—	—	—	—	—

MG = encapsulated streptococcus MG
R = R variant of streptococcus MG

antibodies specifically directed against at least 2 separate and distinct antigenic components of streptococcus MG. Further evidence supporting this interpretation was obtained from immunization experiments with streptococcus MG, its capsular polysaccharide, and an induced R variant in rabbits and in human beings. The results of certain experiments bearing upon this point are summarized in Table V. It will be seen that when either rabbits or human beings were immunized with encapsulated streptococcus MG, they developed agglutinins for both the R variant and the encapsulated form. When rabbits were immunized with the R variant, they developed agglutinins against the R variant but not against the encapsulated streptococcus. When human beings were immunized with the capsular polysaccharide, they developed agglutinins against the encapsulated streptococcus, but not against the R variant. It would therefore appear that the serum of patients convalescent from primary atypical pneumonia reacted in agglutination tests against streptococcus MG and its R variant in a manner analogous to

the serum of rabbits or human beings immunized with encapsulated streptococcus MG. It appears evident that such convalescent sera reacted differently in these tests from the sera of human beings, immunized only with the capsular polysaccharide, or the serum of rabbits, immunized only with the R variant.

PRECIPITATION TESTS

The capsular polysaccharide of streptococcus MG was employed in precipitation tests, using the capillary tube method (12), with the acute-phase and convalescent sera of 16 patients with primary atypical pneumonia. Similar tests were also made with the sera of 10 normal persons, before and after the intradermal injection of this polysaccharide.

The methods used for the extraction and purification of the capsular polysaccharide of streptococcus MG have been described in detail elsewhere (3). When the serum of rabbits immunized with streptococcus MG was tested against the capsular polysaccharide, visible precipitation occurred with the polysaccharide in

dilutions as high as 1:1,000,000. For tests with human sera, a 1:10,000 dilution of the polysaccharide was found to be satisfactory.

Convalescent sera from 11 of the 16 patients with primary atypical pneumonia produced visible precipitation with the capsular polysaccharide. None of the acute-phase sera from these patients yielded precipitates under identical conditions. Each of the sera from 10 normal persons was negative. Four weeks after the injection of the capsular polysaccharide, however, specific precipitins were demonstrable in the sera of 9 of these 10 persons.

Absorption tests were carried out with selected samples of convalescent serum, in order to determine whether the agglutination and precipitation reactions were produced by the same antibody. It was found that absorption of such serum with either a suspension of streptococcus MG or with its capsular polysaccharide resulted in the removal of both the agglutinins for the streptococcus and the precipitins reactive with the polysaccharide.

The precipitation test was found to be of limited usefulness with sera from patients with primary atypical pneumonia, since positive reactions were only obtained with sera which possessed relatively high agglutination titers. Moreover, positive precipitation tests were usually not obtained for as long a period during convalescence as were positive agglutination tests.

QUELLUNG TEST

Convalescent sera from 5 selected patients with primary atypical pneumonia, each of which had a high agglutination titer for streptococcus MG, were tested for their capacity to produce capsular swelling of this microorganism. A loopful of undiluted serum was mixed with a loopful of an 18-hour broth culture of streptococcus MG, and a loopful of 1 per cent methylene blue. The mixtures were then examined under the microscope for the presence of a positive quellung reaction. Definite quellung was produced by 2 different samples of convalescent serum, obtained from 1 patient during the second and third weeks of the disease. Each of these sera possessed an agglutination titer of 1:320. With the convalescent sera from 4 other patients,

which had lower agglutination titers, capsular swelling was not observed.

COMPLEMENT FIXATION TESTS

Complement fixation tests were performed with the acute-phase and convalescent sera of 12 selected patients with primary atypical pneumonia, using the capsular polysaccharide of streptococcus MG as an antigen. In other tests with this antigen, complement fixation had been shown to occur in high dilutions of anti-streptococcus MG rabbit serum. The method employed in these tests was the same as that used in tests with rabbit antiserum, and is described elsewhere (3).

It was found that the convalescent serum of 1 patient in a serum dilution of 1:32 produced complement fixation with the capsular polysaccharide of streptococcus MG. The convalescent sera of each of the other 11 patients were negative in this test, although some of these sera possessed relatively high agglutination titers and were positive in precipitation tests.

SKIN TESTS WITH THE CAPSULAR POLYSACCHARIDE OF STREPTOCOCCUS MG

The capsular polysaccharide of streptococcus MG was injected intradermally in 22 patients with primary atypical pneumonia, 14 patients with other acute infectious diseases, and 14 normal persons. Similar tests were also performed in 5 persons who had received an intradermal injection of the polysaccharide 2 weeks previously.

The skin test consisted of the intradermal injection in the anterior surface of the forearm, of 0.1 ml. of sterile saline containing 10 μg. of polysaccharide. A control injection of saline was made in a corresponding area on the other arm.

In 7 of the 22 patients with primary atypical pneumonia who were given an intradermal injection of polysaccharide during the second or third week after onset of the disease, a definite response occurred at the site of inoculation. This reaction, usually consisted of an itching wheal surrounded by a zone of erythema. It appeared within 2 hours after the injection and persisted for 24 hours or longer. In 15 of these 22 patients, the reaction was negative. Negative

results were also obtained in 14 patients with other infectious diseases and in 14 normal persons.

In each of the 5 normal persons who had previously received an injection of the polysaccharide, positive skin reactions occurred which were similar in all respects to those observed in the patients convalescent from primary atypical pneumonia.

There appeared to be no direct correlation between the presence of circulating antibodies and positive skin reactions in the patients with primary atypical pneumonia, and it was not possible by means of serological tests to determine at what period during convalescence the reaction might become positive. Repeated skin tests in the same individual during the course of the disease produced results which could not be interpreted, since a single injection of polysaccharide stimulated the production of circulating antibodies against streptococcus MG and produced sensitization of the skin to a second injection 1 week later. The skin test was considered to be of limited practical usefulness, although the results obtained with it provided further evidence for the presence of specific antibodies against streptococcus MG in patients convalescent from primary atypical pneumonia.

PROPERTIES OF THE AGGLUTININ IN CONVALESCENT SERUM

Certain properties of the agglutinin for streptococcus MG in the convalescent sera of 2 patients with primary atypical pneumonia were investigated. The agglutination titer of these sera was not affected by alterations in pH over a range extending from pH 4.5 to 9.0. It was found by fractional precipitation with ammonium sulfate that the agglutinin was present solely in the water-insoluble portion of the globulin fraction of the serum. These properties are among those which characterize specific antibodies (13).

The agglutinin was found to be relatively thermolabile, as compared with certain other antibodies. Four-fold or greater reductions in the agglutination titers of certain sera were produced by heating the undiluted serum for 30 minutes at 56° C. and the agglutinin was almost entirely destroyed by heating at 60° C. for a similar period. It is of interest that the

same degree of thermolability was encountered with the agglutinins which developed in the serum of monkeys which had been inoculated intratracheally with streptococcus MG. Moreover, heating for 30 minutes at 56° C. reduced the agglutination titers of the sera of human beings who had received intradermal injections of the capsular polysaccharide of this microorganism.

It has been shown that the acute-phase sera of patients with primary atypical pneumonia contain an abnormal protein which reacts in precipitation tests with the C-polysaccharide of pneumococcus (4). This C-reactive protein is also known to be present in serum during the early stages of many other varieties of acute infectious disease (14). It was of importance to determine whether any relationship existed between this protein and the agglutinin for streptococcus MG in the serum of patients with primary atypical pneumonia. In a series of 18 patients, C-reactive protein was found to be present almost invariably in acute-phase serum, while agglutinins for streptococcus MG were not demonstrable in any of these sera. On the other hand, C-reactive protein was not present in any of the convalescent sera at the time when agglutinins for streptococcus MG had reached their maximum titers. These findings indicate that the agglutinin for streptococcus MG was distinct from the C-reactive protein.

It has also been shown that the convalescent sera of certain patients with primary atypical pneumonia are positive in the cold-hemagglutination test (6, 7), and in complement fixation tests with a variety of unrelated tissue antigens (5). Each of these non-specific serological phenomena was encountered with the sera of certain patients in this series (4). However, many convalescent sera were found to possess agglutinins for streptococcus MG in high titer while cold-hemagglutinins were not demonstrable. Furthermore, the sera of a few patients were capable of causing cold-hemagglutination in high titer, although they did not possess agglutinins for streptococcus MG. Similarly, a number of sera which possessed agglutinins for streptococcus MG failed to exhibit the property of non-specific complement fixation. These observations suggested that the agglutinins for

TABLE VI

Results of cross-absorption tests with the convalescent serum of a patient with primary atypical pneumonia

Serum	Test	Serum dilution				
		1:10	1:20	1:40	1:80	1:160
Unabsorbed	1) Agglutination of streptococcus MG	4	4	4	4	4
	2) Cold hemagglutination	4	4	2	2	0
	3) Complement fixation with mouse lung	4	4	1	0	0
Absorbed with strep. MG	1) Agglutination of streptococcus MG	0	0	0	0	0
	2) Cold hemagglutination	4	4	2	2	0
	3) Complement fixation with mouse lung	4	4	0	0	0
Absorbed with human RBC	1) Agglutination of streptococcus MG	4	4	4	4	3
	2) Cold hemagglutination	0	0	0	0	0
	3) Complement fixation with mouse lung	4	4	1	0	0
Absorbed with mouse lung	1) Agglutination of streptococcus MG	4	4	4	4	4
	2) Cold hemagglutination	0	0	0	0	0
	3) Complement fixation with mouse lung	0	0	0	0	0

streptococcus MG were independent of the other 2 serological phenomena. In order to determine whether any direct relationship existed, cross-absorption tests were carried out with several samples of convalescent serum which were positive in each of these 3 different tests. The results of a typical cross-absorption experiment are illustrated in Table VI. It will be seen that, before absorption, this serum produced agglutination of streptococcus MG in a titer of 1:160, cold-agglutination of human group 0 erythrocytes in a titer of 1:80, and complement fixation with normal mouse-lung antigen in a titer of 1:40. Following absorption of this serum with streptococcus MG, the agglutinins for this microorganism were completely removed while the titer of both cold-hemagglutination and non-specific complement fixation remained almost unchanged. Similarly, when the serum was absorbed with human group 0 erythrocytes, or with a suspension of mouse-lung tissue, the cold-hemagglutinins or the property of non-specific complement fixation were removed, while the agglutinin titer for streptococcus MG remained the same as before absorption. These results indicated that the agglutinin for streptococcus MG in convalescent serum was independent of the 2 non-specific serological phenomena mentioned above.

DISCUSSION

Non-hemolytic streptococci are known to be present commonly in the upper respiratory tract of human beings, and are generally regarded as non-pathogenic saprophytes. Microorganisms belonging to this diverse group are encountered in cultures of the throats of many normal persons. The classification of non-hemolytic streptococci has not yet been completed although many workers have entered upon the problem. One distinct species of non-hemolytic streptococci, *Str. salivarius*, has been well characterized and can be distinguished readily from other non-hemolytic streptococci, both by biological and immunological tests (10). The so-called *Str. mitis* group appears to possess much less distinctive properties and probably cannot be considered to be a homogeneous species (10). The other varieties of non-hemolytic streptococci commonly present in the upper respiratory tract have not so far been classified satisfactorily.

The microorganism which has been referred to in this paper as streptococcus MG was readily differentiated from either *Str. salivarius* or the *Str. mitis* group. The strains of streptococcus MG which have been studied showed a sufficiently striking homogeneity as regards both their biological and immunological characteristics to warrant the conclusion that they constituted a distinct species and belonged to a single serological type.

Streptococcus MG was isolated from the lung tissues of 6 of a total of 8 patients who died of primary atypical pneumonia, and was not obtained from the lung tissues of 6 patients who

died of other causes. It was isolated from the sputum or throat swabs of 53 of a total of 97 patients with primary atypical pneumonia, and 20 of a total of 82 patients with other acute infectious diseases, as well as 7 of a total of 57 normal persons.

Evidence was obtained which indicated that in 4 of the lung tissues from fatal cases of primary atypical pneumonia, streptococcus MG was present in numbers of the order of 100,000 or more per gram of lung tissue.

It was found that 67 per cent of 193 patients with primary atypical pneumonia developed, during convalescence, antibodies directed against streptococcus MG. The available evidence indicates that the serological reactions obtained with this non-hemolytic streptococcus and the convalescent sera of patients with primary atypical pneumonia were due to the presence of specific antibodies and not to some non-specific property of these sera analogous to that responsible for cold-hemagglutination (6, 7) or complement fixation with various tissue antigens (5). With but relatively few exceptions, such antibodies were demonstrable only in the convalescent sera of these patients and not in the serum of patients with other infectious diseases. Positive reactions did not occur when convalescent sera possessing high agglutination titers for streptococcus MG were tested against a number of other bacterial species. *Str. salivarius*, type I, which has been shown to possess certain antigens in common with streptococcus MG, was agglutinated only by some 6 per cent of convalescent sera which were capable of agglutinating the latter microorganism. Moreover, it has been shown that the antibodies in the convalescent sera of patients with primary atypical pneumonia reacted with at least 2 separate and distinct antigenic components of streptococcus MG, namely, its capsular polysaccharide and one or more somatic antigens.

The significance of this non-hemolytic streptococcus in relation to primary atypical pneumonia is not yet clear. There appear to be a number of possible explanations which must be considered separately. First, it seems possible that the observed serological reactions might be the result of a coincidental antigenic relationship between this non-hemolytic streptococcus

and some other agent, perhaps a virus, which is itself the causative agent in primary atypical pneumonia. This possibility may be considered as analogous to the positive serological reactions obtained with members of the *B. proteus* group in various rickettsial diseases. There are, however, certain reasons for doubting the validity of this hypothesis. It would hardly account for the presence of this streptococcus in considerable numbers in the lungs of fatal cases of primary atypical pneumonia. Furthermore, it would be surprising if there were an antigenic relationship between 2 separate and antigenically distinct components of this bacterium and some other agent. In view of the presence in convalescent serum of antibodies directed against both the capsular polysaccharide and somatic antigens of streptococcus MG, such an assumption would be necessary.

Secondly, it seems possible that this non-hemolytic streptococcus might occupy the rôle of secondary invader in primary atypical pneumonia. In this regard, it is of interest that other investigators have called attention to the frequent occurrence of non-hemolytic streptococci in primary atypical pneumonia (15, 16). The possibility that these microorganisms may represent secondary invaders has been suggested (17, 18). An association between non-hemolytic streptococci and other clinical varieties of pneumonia has also been reported by a number of investigators (19 to 22). The possibility that streptococcus MG may be involved in primary atypical pneumonia as a secondary invader cannot be excluded. However, this term usually implies an accidental or random association between an infectious agent and a disease, rather than an association with such a relatively high proportion of cases as is indicated by the results of this study. It seems rather unlikely that an accidental association should occur with microorganisms which, in each instance, were members not only of a single species but also of one serological type.

Thirdly, it seems possible that this non-hemolytic streptococcus, either alone or in concert with some other infectious agent, might be primarily involved in the pathogenesis of primary atypical pneumonia. The available evidence is not sufficient to warrant the accep-

tance of this hypothesis at the present time, although the results of this study suggest paths for further exploration.

SUMMARY

Non-hemolytic streptococci, comprising a homogeneous group and belonging to a single immunological type, have been isolated from the lungs and sputa of patients with primary atypical pneumonia. Specific antibodies directed against this species of streptococcus have been demonstrated in the convalescent serum of patients with primary atypical pneumonia by means of a number of serological techniques. With few exceptions, such antibodies were not demonstrable in the serum of these patients during the acute phase of the disease, in the serum of patients with other diseases, nor in the serum of normal persons.

The significance of these results is not yet clear, but they suggest the possibility that this non-hemolytic streptococcus may, in some manner, be implicated in the pathogenesis of primary atypical pneumonia.

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