IMMUNITY REACTIONS OF HUMAN SUBJECTS TO STRAINS OF PNEUMOCOCCI OTHER THAN TYPES I, II AND III*

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Observations on immune reactions of human subjects to Types I, II and III pneumococci and their products have recently been reported from this laboratory (1). These observations confirm the previous demonstrations of the general presence among normal adults of bactericidal power for mouse-virulent type-specific pneumococci of 1 or more of these 3 types, the occasional presence of mouse-protective antibodies and the rare occurrence of type-specific agglutinins. They extended previous conceptions of natural immunity by showing that the type-specific actions of the whole blood and serum occur with differing frequencies, and in combinations that indicate their independence of one another. A marked variation in the incidence of the type-specific actions of whole blood and serum was noted with the age of the subjects tested.

The possibility of definitely identifying all but a small percentage of strains of pneumococci with the aid of sera recently developed by Cooper and her coworkers (2) has made feasible more detailed studies of the occurrence of the various strains in health and disease. Reports on the incidence of these newly classified types in the pneumonias of children (3) and in normal human subjects (4, 5) have already appeared and other studies of their occurrence in disease are in progress in this and other clinics. A more intelligent approach is, therefore,

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offered to the study of immunity to pneumococci other than those included in Types I, II and III.

It, therefore, seemed of interest to extend the above mentioned observations on natural immunity to include certain of these new types. The present communication is concerned, primarily, with immune reactions of the blood and serum of a group of human subjects, free from recent pneumococcic infections. Particular attention was given to those types, which have previously been described as immunologically related to, but not identical with, typical strains of Types II and III pneumococci (6, 7).

Materials and Methods

The Types I, II and III strains of pneumococci employed throughout the investigation were originally obtained from the Antitoxin and Vaccine Laboratory of the Massachusetts Department of Public Health and have been used as stock strains in this laboratory for the past 3 years. The following strains were obtained from Miss Georgia M. Cooper: Type V, Am., isolated in Feb., 1928, from the blood of a fatal case of lobar pneumonia; Type VI *a*, Fi., isolated in Feb., 1929, from a swab culture of a normal throat; Type VI *b*, Ro., isolated in Apr., 1931, from the sputum of a child suffering from an acute upper respiratory infection; Type VIII, He., isolated in Jan., 1928, from a mild case of lobar pneumonia; and Type IX, Bu., isolated in June, 1931, from the sputum of a case of influenza. These strains are hereafter referred to by their type designation only.[1]

The virulence of all of these strains was maintained by daily passage through mice and transfer of mouse heart's blood into rabbit's blood broth, in which they were transplanted at 8 hour intervals. In carrying out the experiments 10 to 16 hour rabbits' blood broth cultures were used. These cultures were found to contain 11 to 135 (rarely more) diplococci in 0.0000001 cc., as shown by the plate dilution method. With the exception of Type IX, they were regularly fatal for mice in one-tenth this amount and in most instances the organisms were of maximal virulence, so that doses containing 1 or more organisms proved fatal within 48 hours. For simplicity the greatest dilution (in multiples of 10) containing 30 or more colonies on plating was considered to contain 100 diplococci or 10 lethal doses.

The Type V and VIII organisms were agglutinated to a moderate degree in Type II and III antisera, respectively, but they were agglutinated in the homologous typing serum to a definitely higher titer. The agglutination reactions of these strains were as follows:

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[1] We are very grateful to Miss Cooper and to Dr. William H. Park of the New York City Department of Health for supplying these strains and the various typing sera.

Type of serum	Type of antigen	Agglutinin titer	Type of antigen	Agglutinin titer
II	II	1:80	V	1:80
V	II	0	V	1:160
III	III	1:160	VIII	1:40
VIII	III	1:10	VIII	1:160

The VI *a* and VI *b* strains gave identical agglutination reactions in all typing sera. No other significant cross-agglutinations were observed. All of the strains grew consistently in smooth colonies on the surface of rabbits' blood agar plates. With the exception of the Type IX strain, all the organisms retained their specific agglutinating characteristics, as well as their virulence for mice, throughout the time of this investigation. The Type IX strain regularly failed to agglutinate in the homologous antiserum or in any of the other typing sera available, it was only partly soluble in bile, grew in small smooth colonies, produced only a very small area of methemoglobin in rabbits' blood agar plates after 48 hours incubation and usually failed to change the color of defibrinated human blood after 24 hours' incubation, even when proliferation was demonstrable by transplants on the surface of blood agar plates. This strain was irregular in its virulence for mice, the lethal dose being 0.0000001 cc. or more on most tests.[2]

The pneumococcidal action was studied in the whole defibrinated blood by the method of Todd, as employed by Ward (8). Passive protection was tested by the simultaneous intraperitoneal injection of progressive dilutions of culture with 0.2 cc. amounts of serum, duplicate mice being used in most instances and for all culture controls. Pneumococcidal power was said to be present when 100 or more organisms were killed in 0.5 cc. of blood and protection was considered significant when duplicate mice were each protected against 100 or more lethal doses. Agglutinating antigens were prepared by resuspending the organisms from 8 hour plain broth cultures in a volume of normal saline equal to that of the original culture and adding 0.4 per cent formaldehyde. Agglutination tests were carried out by incubating 0.2 cc. amounts of antigen and serum dilution together for 2 hours at 37°C. and reading after overnight ice box storage.

The subjects for these tests were similar to those employed in the previous study (1), except that none of the infants or children were convalescent from infectious disease.

Results of Pneumococcidal Tests

The pneumococcidal power of the whole defibrinated blood was tested against Types I, II, III, V, VI *a*, VI *b*, VIII and IX organisms in 72 normal subjects and hospital patients having no recent infection or history of pneumonia. There were 6 infants from 2 weeks to 7

[2] See footnote 3.

months of age, 10 children from 1 to 9 years, 33 individuals from 16 to 38 years, 15 from 40 to 58 years, and 8 over 64 years of age. In several individuals, tests with Type II or III or VI *a* were omitted.

TABLE I

Incidence of Pneumococcidal Action against Different Types and among Different Age Groups

		Age group					
		2 wks. – 1 yr.	1–9 yrs.	16–38 yrs.	40–60 yrs.	Over 64 yrs.	All ages
Type I	Subjects tested	6	10	33	15	8	72
	No. pneumococcidal	0	0	16	4	1	21
	Pneumococcidal, *per cent*	0	0	48.5	26.7	12.5	29.2
Type II	Subjects tested	6	10	31	12	8	67
	No. pneumococcidal	0	9	28	11	7	54
	Pneumococcidal, *per cent*	0	90.0	90.3	91.7	87.5	80.6
Type III	Subjects tested	6	10	25	9	8	58
	No. pneumococcidal	0	6	14	4	4	28
	Pneumococcidal, *per cent*	0	60.0	56.0	44.4	50.0	48.4
Type V	Subjects tested	6	10	33	15	8	72
	No. pneumococcidal	0	8	27	10	3	48
	Pneumococcidal, *per cent*	0	80.0	81.2	66.7	37.5	66.7
Type VI *b*	Subjects tested	6	9	33	15	8	71
	No pneumococcidal	3	8	33	15	8	67
	Pneumococcidal, *per cent*	50.0	88.9	100.0	100.0	100.0	94.4
Type VIII	Subjects tested	6	10	33	15	8	72
	No. pneumococcidal	3	9	31	15	8	66
	Pneumococcidal, *per cent*	50.0	90.0	93.9	100.0	100.0	93.1
Type IX	Subjects tested	6	9	33	15	8	71
	No. pneumococcidal	6	9	32	15	8	70
	Pneumococcidal, *per cent*	100.0	100.0	97.0	100.0	100.0	98.6

The frequency with which killing power was demonstrated in each age group and against each organism is shown in Table I and represented graphically in Fig. 1. The curves for Types I, II and III pneumococci are similar to those constructed for the group of sub-

jects included in the earlier study with these strains (1). It may be pointed out again that the relative incidence of pneumococcidal

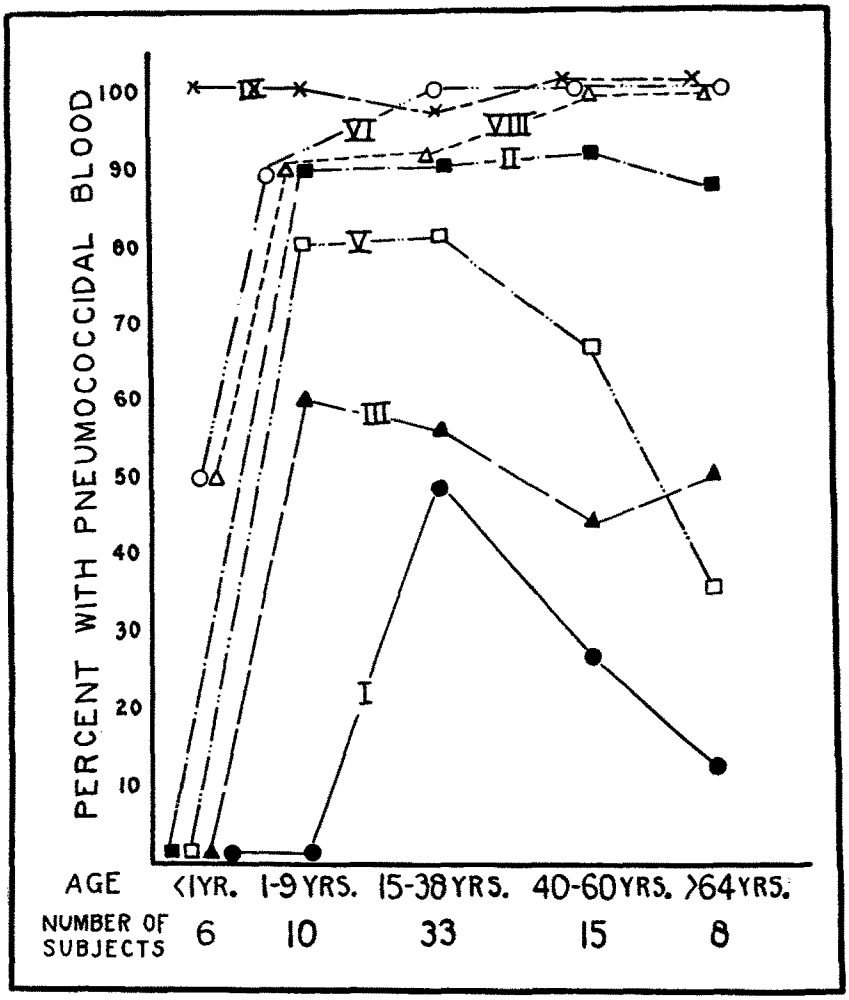

FIG. 1. Percentage of persons in different age groups whose blood is bactericidal for various types of pneumococci. The Roman numbers represent the types of pneumococci.

power for each of these 3 types is the same in every age group; that killing is least frequent for Type I, most frequent for Type II and

intermediate in frequency for Type III, and that killing power for these 3 types is absent during the 1st year of life, is highest in early adult life and becomes less frequent in old age. The anomalous position of the last point on the Type III curve in Fig. 1 probably depends upon the small number of observations in this age group.

The same relative incidence of pneumococcidal action in the blood of individuals of different ages is here demonstrated for Type V, which is seen to occupy a position intermediate between Type II and Type III. The remaining strains were all killed with greater frequency in the blood of individuals of all age groups. The Type IX strain was killed in practically every specimen of blood tested. The Types VI *b* and VIII strains occupied comparable positions between Types II and IX. Since the results of tests with the VI *a* and VI *b* strains in the individual bloods were the same, within the limits of error of the test, only the curve for Type VI *b* is included. The order of relative frequency with which different types of pneumococci were killed by this group of individuals is, therefore, from least to greatest frequency, as follows: I, III, V, II, VIII, VI and IX.[3]

For the purpose of evaluating the quantitative relationships of the killing power for the various strains, the number and percentage of persons who kill, respectively, 0 or 10, 100 to 10,000 and 100,000 or more pneumococci of each type are shown in Table II. In the column

[3] It is possible, in the case of the Type IX pneumococcus, that we were dealing originally with a variant that maintained its characteristics throughout our experiments. Since this paper was submitted, Miss Cooper was kind enough to send us a fresh transplant of the IX, Bu., strain. Subcultures of this strain were bile-soluble, agglutinated specifically in Type IX antiserum and had cultural characteristics, in whole human blood and on artificial media, similar to the other pneumococci. The whole defibrinated blood of 12 hospital subjects over 58 years of age was tested with this strain, with the stock Types I, II and III and with a fresh transplant of the V, Am., strains. The number of subjects killing, respectively, 0 or 10, 100 or 1000 and 10,000 or more pneumococci were as follows: Type I—11, 1 and 0; Type II—4, 5 and 3; Type III—9, 3 and 0; Type V—6, 1 and 5; and Type IX—3, 2 and 7. Except for Type IX the results appear very similar to those described above. Furthermore, the results of the Type III tests, when added to the above, tend to smooth out the latter part of the curve for that type. For the Type IX the killing was not quite as universal as those recorded above. It was, however, killed with greater frequency and in larger numbers than the other types used.

representing absence of killing power (0–10 diplococci), the types arrange themselves in the reverse order to that noted above for the presence of pneumococcidal action. It is also seen that the percentages of persons killing 100,000 or more pneumococci of the different types align themselves by types in the same order as in the case of the frequency of killing. This is shown graphically in Fig. 2.

The immunological relationships of the Types II and V and the Types III and VIII strains in artificially prepared immune sera, indicated in part by the cross-agglutination reactions shown above, suggest that similar relationships might be expected in the reactions of human blood with these strains. The bactericidal action of individual blood samples on the various strains failed to indicate such correlations. The action of any blood against Type V or against Type VIII pneumococci was apparently independent of the action of that blood on the Type II or the Type III organisms, respectively.

TABLE II

Frequency with Which Different Numbers of Pneumococci Are Killed

Type	No. of subjects tested	No. killing 0–10 diplococci	Per cent of subjects	No. killing 100–10,000 diplococci	Per cent of subjects	No. killing 100,000 or more diplococci	Per cent of subjects
I	72	51	70.8	19	26.4	2	2.8
II	67	12	17.9	39	58.2	16	23.9
III	58	30	51.7	26	44.8	2	3.4
V	72	24	33.3	42	58.3	6	8.3
VI *b*	71	4	5.6	26	36.6	41	57.7
VIII	72	6	8.3	46	63.9	20	27.7
IX	71	1	1.4	20	28.2	50	70.4

On the other hand, calculations of the frequency with which bactericidal power for each combination of 2 types was present for both, or absent for both, in the same blood samples showed a small, but constant, tendency favoring the simultaneous presence, as well as absence, of pneumococcidal action against each pair of type-specific strains. For every combination of 2 types, bactericidal action was present for both, or absent for both, in the same blood in from 3 to 13 per cent more subjects than was expected from calculations based upon the frequency with which each type separately was killed. Thus

there appears a slight, but constant, tendency for bactericidal action against each type to be correlated with the presence or absence of this action against all other types. Further investigation has shown that this correlation is quantitative; that is, killing of large numbers of organisms of one type tends to be associated with similar degrees of killing for other types. No greater correlation was, however, found

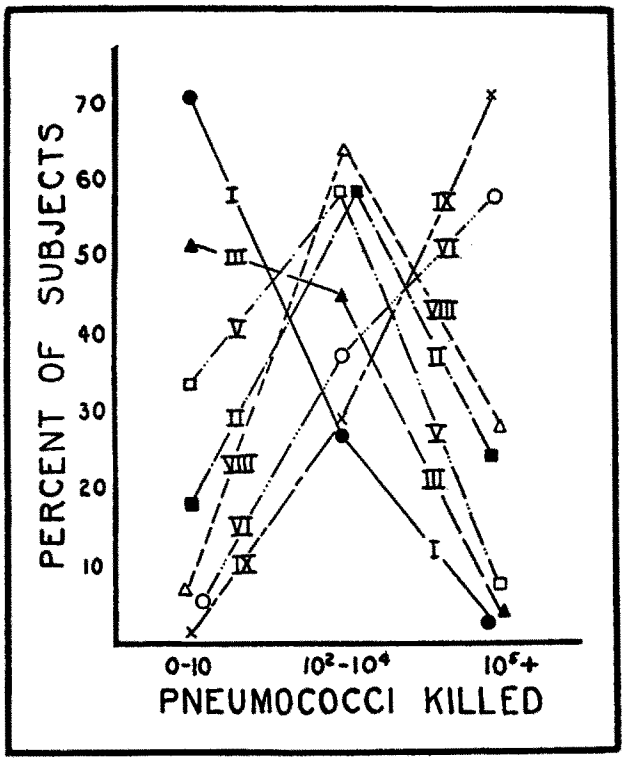

FIG. 2. Percentage of persons whose blood kills different numbers of pneumococci of various types. The Roman numerals represent the types of pneumococci.

to exist between Types II and V or between Types III and VIII than was observed for unrelated pairs of strains.

With the VI *a* and VI *b* strains, however, the relation was found to be closer. Their agglutination reactions with immune horse serum, as mentioned above, were practically identical. Both of these strains were used in the bloods of 32 persons, which were able to kill 100 or

more of each of these organisms. In 26, or 81 per cent, of these individuals the pneumococcidal titer of the blood for these 2 types was the same or showed a difference of only 10-fold, and in the remaining instances the difference was 100-fold in favor of one strain or the other. Such quantitative differences were interpreted as well within the limits of the method and only the results obtained with the Type VI *b* strain were used above, the bloods of all of the subjects having been tested with this organism.

TABLE III

Incidence of Mouse Protection by Age and Type

Type	1–9 yrs.			16–38 yrs.			40–60 yrs.			Over 64 yrs.			All ages		
	No. of subjects tested	Protection present	Per cent positive	No. of subjects tested	Protection present	Per cent positive	No. of subjects tested	Protection present	Per cent positive	No. of subjects tested	Protection present	Per cent positive	No. of subjects tested	Protection present	Per cent positive
II	5	0	0	23	5	21.7	11	0	0	8	1	12.5	47	6	12.8
III	2	0	0	16	3	18.8	10	3	30.0	8	2	25.0	36	8	22.2
V	5	0	0	26	4	15.4	12	1	8.3	8	0	0	51	5	9.8
VI *b*	4	1	25.0	22	9	40.9	13	8	61.5	8	2	25.0	47	20	42.6
VIII	2	0	0	23	7	30.4	13	1	7.7	8	0	0	46	8	17.4
All types*...	18	1	5.6	110	28	25.5	59	13	22.0	40	5	12.5	227	47	20.7

*The numbers refer to tests and not subjects, as above.

Results of Mouse Protection Tests

A total of 227 protection tests against the Types II, III, V, VI *b* and VIII pneumococci were performed with the sera of 53 persons all of whom were included in the studies for pneumococcidal properties, as reported above. All 5 types were tested against the sera of 30 individuals, and 4, 3 and 2 types used with the sera of 10, 11 and 2 subjects respectively. Sera of infants under 1 year of age were not studied. The numbers of persons whose sera protected mice against 100 or more lethal doses of the various types of pneumococci are shown, by age groups, in Table III.

From this table it appears that mouse protection against the types

of pneumococci studied is rare in the sera of children, is relatively frequent in adult life and is somewhat less frequent in old age. The order of frequency with which protection was found against different types of pneumococci is not quite the same as in the case of pneumococcidal action. Type VI protection was demonstrated in the sera of 43 per cent of subjects, Type III protection in 22 per cent and Types VIII, II and V in 17, 13 and 10 per cent, respectively. Except for the Type III organism, the order of types is, therefore, analogous to that for blood pneumococcidal power.

A comparison of the protective power of the same sera for the various combinations of pairs of organisms shows similar relationships between pairs of strains immunologically related and pairs not so related. Thus, of 47 sera tested for both Types II and V, 39 lacked protection against both, 2 had protection against both, 4 protected against Type II and not against Type V and the remaining 2 protected against Type V and not against Type II. With both Types III and VIII the sera of 35 subjects were tested. Of these, 21 protected against neither strain, 1 protected against both, 7 against the former only and 6 only against the latter. Similar results were obtained with the same types in unrelated combinations. There was, therefore, no discernible tendency for these sera to show any greater correlation in their protection against strains considered to be immunologically related than against those not so related.

Result of Tests for Agglutinins

All 8 strains of pneumococci used for pneumococcidal tests were also used to test for agglutinins in the sera of 22 of the young and middle aged adults. None of the sera showed any agglutinins for the Types I, II, III, V and IX strains. With Type VIII, agglutination was observed only in equal amounts of serum and antigen in 3 instances. The VI *a* and VI *b* antigens showed granular agglutination in most of the undiluted sera. Definite agglutination was obtained with the VI *a* organisms in 3 sera (in 1:1, 1:2 and 1:4 dilutions of serum, respectively), but none was obtained with the same sera and the VI *b* strain.

Correlations between the Tests

The correlation between the 3 tests employed was found to be about the same for all of the types used as has previously been described with respect to Types I, II and III (1). Protection or agglutination in any given serum was, in general, associated with homologous type pneumococcidal action in the corresponding defibrinated blood. In 3 sera, however, protection against Type III pneumococci was demonstrated (100 lethal doses in 2 instances and 1000 in the 3rd) when the corresponding whole blood failed to kill the homologous type organisms.

Of the 6 instances where agglutinins were found, 3 were associated with mouse protection against the homologous organism and 3 had no such protection. All had homologous pneumococcidal power.

DISCUSSION

Surprisingly little of specific significance can be obtained from the literature with respect to the natural immunity of man to the strains of pneumococci previously included in Group IV. According to the work of Cooper and her associates (2) and other investigators (3–5) we must consider these new types as fixed, their specificity being similar, immunologically, to that of Types I, II and III. It may thus be expected that the accuracy of deductions from previous work with unclassified Group IV strains would depend largely on whether these deductions were based on characteristics of the species rather than on type specificity.

It would be a tremendous task to carry out any large series of observations similar to those presented above and utilizing all of the 32 type strains identified and classified to date. The present study was, therefore, limited to a few types, strains of which had been isolated relatively frequently in this laboratory from human cases of pneumonia. The Type IX strain was included in this study because of its peculiar agglutinating and cultural characteristics which remained the same throughout the course of this study. It was assumed that the remaining strains were representative of their specific types since they were selected originally for their high and stable mouse virulence and were kept under uniform and favorable conditions.

Robertson and Cornwell (9), however, using recently isolated human strains of pathogenic pneumococci found that the reactions of a serum-leukocyte mixture against different strains within the type often varied considerably, but this difference was less, on the whole, than that between types. The results reported above in regard to relative virulence should not be applied in a literal mathematical sense to all strains of each type, as additional differences may exist between strains of the same type.

The comparative delicacy of the pneumococcidal test has been referred to here and elsewhere (1). It has previously been shown that the mouse protection test is a more delicate index of the presence of type-specific pneumococcic antibody, in patients recovering from lobar pneumonia, than is the agglutination test (10). In normal human subjects mouse protection against many of the virulent strains has been shown to be comparatively infrequent. Pneumococcidal power, however, is far more frequent for every type. It appears from the present observations that protection is comparatively rare in the first decade of life, although pneumococcidal power at this age is almost as frequent, for most types, as in early adult life. This discrepancy may depend on the comparative delicacy of these two tests and may thus indicate a greater degree of natural immunity in the latter period.

From the present observations certain deductions may be ventured in regard to the immunological reactions of normal human subjects to the newly classified types of pneumococci. These types have been shown to differ both in the frequency with which they are killed in different human bloods and in the number of organisms so killed. In general, the types most frequently susceptible to the action of human blood are also killed in the greatest numbers, the types aligning themselves in a definite sequence in this respect. Some types, such as Type VI or Type IX, are almost universally killed, usually in large numbers; others, like Type V, are killed less frequently. The age frequency curve for pneumococcidal power is probably similar for all types, showing a peak in adult life. Individual bloods apparently kill different types more or less independently.

Can these findings be interpreted in terms of the virulence of these pneumococcus types for man or in terms of the susceptibility of human subjects to the various types? These questions cannot be answered

separately, since virulence and resistance may be determined only in relation to each other. Robertson and Sia (11), however, in working with sera of different animals mixed with standard suspensions of rabbit's leukocytes, found differences in the growth inhibitory action of these mixtures, which paralleled the known susceptibility of the different animal species to experimental pneumococcic infections. In a similar manner, the frequency of pneumococcidal action in human blood may be considered, indirectly, as an index of the relative virulence of the different strains for man.

Two pairs of immunologically related strains, Types II and V and Types III and VIII, were here shown not to be related in their reactions with human blood or serum. From the present data, no greater correlation was observed in human subjects between these pairs of strains and other pairs not related by their reactions with artificially prepared immune sera. This lack of obvious correlation was found to hold both for pneumococcidal and mouse protection tests. Similar results may be deduced from the small number of earlier observations with normal human subjects against atypical Type II strains (probably Type V) in mouse protection tests done by Clough (12) and in the pneumococcidal tests carried out by Robertson and Cornwell (9). The correlation between the pneumococcidal action against Types VI *a* and VI *b*, however, indicates a closer relationship between these strains and thus tends to support the belief of Cooper (2) that they really belong to the same type.

It was inferred from previous work with Types I, II and III (1) that the frequency with which killing power was present or absent for any combination of types was a matter of chance depending only upon the frequency with which killing power was present for each type. A closer study of the previous data, as well as those presented here, has shown that, in every instance, killing power for both of any pair of types was either present or absent in a slightly larger per cent of subjects than was expected from calculations made on the basis of the per cent of subjects killing each type separately. The discrepancy was often slight, as low as 3 per cent for some combinations; but, in others it was 13 per cent higher than the calculated expectancy. No greater correlation, however, was observed with types related in their agglutination with immune sera and those not so related.

The constancy with which this correlation was observed in pneumococcidal tests with all the types suggests that it may have some significance. Its interpretation may depend, among other things, upon one or both of two factors. First, it is possible that any contact with a given type pneumococcus which is capable of stimulating specific immunity carries with it, in addition, a small degree of species immunity detectable, in some instances, and with some specific type strains, by a sensitive test for immunity, such as the pneumococcidal test. Such a spread of immunity would not be expected to manifest itself in specific mouse protection which is a far less delicate immune reaction. The second factor which may be responsible for this constant correlation goes back still further in its epidemiological source. It is possible that, in any group of individuals, the conditions which bring about immunizing contact with any one type of pneumococcus carry with them an increase of probability that contact with other pneumococci will also occur. The latter concept is, of course, applicable to organisms and viruses widely divergent in their occurrence and pathogenicity, but no accurate confirming data are available.

SUMMARY

1. A group of 72 human subjects were studied with respect to the immune reactions of their blood and sera to Types I, II and III pneumococci and to 4 other types (V, VI, VIII and IX) previously included in Group IV.

2. The same general relationships were observed for all of these types as were previously demonstrated for Types I, II and III. Each type was specific in relation to the bactericidal action of normal human blood and the protective action of normal human serum.

3. The frequency with which pneumococcidal action for any pair of types was present for both or absent for both in the same blood samples was slightly greater than that calculated from the frequencies with which each of the types was killed separately.

4. No closer correlation could be demonstrated between the reaction of the blood of these subjects to Types II and V or between Types III and VIII pneumococci, types related in their reaction with artificially prepared immune sera, than was observed between unrelated strains.

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