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THE OPSONO-CYTOPHAGIC TEST IN CHILDREN WITH PERTUSSIS AND IN CHILDREN VACCINATED WITH H. PERTUSSIS ANTIGENS [1]

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The mechanism of immunity in pertussis is not known. It would be easy to say, but rather difficult to prove, that the immunity resides in the fixed tissue cells of the respiratory tract. There is no clinical test for immunity other than intimate exposure to the disease itself. Intimate exposure is reported to produce the disease in 70 to 80 per cent of children with negative histories (1). It still remains to be proven that it is possible to immunize actively against pertussis; the possibility seems likely, however, since the introduction of vaccines made from recently isolated, toxic strains having the characteristics of the " Phase 1 " strains described by Leslie and Gardner (2). In three field studies where such strains were used favorable results were reported (1, 3, 4). In another very thorough study (5) in which the method of preparation of vaccine was slightly different unequivocal evidence of immunization was not obtained. It is obvious that a convenient clinical test of immunity would greatly facilitate the trial of immunizing agents.

The complement fixation test and the agglutination reaction are primarily of academic interest though in Denmark the former is employed as a diagnostic procedure in cases of persistent bronchitis. These methods of detecting circulating antibody usually fail, three to five months after the antigenic stimulation, whether it be infection or vaccine. Curves of complement fixing antibody titers following vaccination (6, 7, 8, 9) are similar to those obtained after the disease (10, 11, 12, 13). Recently Mishulow and coworkers (14) have compared the curve of agglutinin titers after vaccination with that during and after an attack of the disease. Higher titers were obtained and persisted longer after an attack. These data are of great interest in that they prove that the vaccine employed stimulates the formation of *circulating* antibody. In the individual who has recovered from pertussis, we know, from clinical observation, that the failure to detect circulating antibody by these tests several months after recovery does not mean an absence of immunity. Do the immune bodies actually disappear from the blood and localize in the tissues, or are our methods of detecting them in the blood inadequate? When the complement fixation and agglutination reactions again fail us two or three months after vaccination, how can we test for immunity? There are two lines of approach left open. We can assume that antibody has left the blood and attempt to test for it in the tissues. The skin, at least, is available. Skin reactions due to the allergy of infection have been reported and also skin reactions of the Schick type.

The claims of the existence of specific skin hypersensitivity during and after an attack of pertussis (15 to 22) are undoubtedly plausible despite reports to the contrary (23, 24, 25). Specific allergic skin reactions occur in many bacterial infections. Because a positive allergic reaction occurs after an attack of pertussis, it is assumed to indicate immunity. A negative reaction is assumed to indicate susceptibility. Here one enters the controversy of the relation of allergy to immunity which is not well understood.

The description by Siebler and Okrent (26) of a Schick type of reaction, positive before the disease and negative afterwards, is difficult to credit. No circulating anti-endotoxin for *H. pertussis* has been demonstrated in man. *H. pertussis* vaccination of animals has failed to protect the skin from the necrotic action of *H. pertussis* endotoxin (27, 28).

A satisfactory skin test for immunity may, in the future, be developed using the allergic type of reactions mentioned above. However, it seems worth while to follow the other lead left open. Does the failure of detection of circulating anti-

[1] Aided by a grant from the Christine Breon Fund.

body by the complement fixation and agglutination reactions a few months after recovery prove its absence? The recent report of Kendrick, Gibbs and Sprick (29) utilizing the opsono-cytophagic test indicates that such is not the case. This test for circulating antibody is apparently more delicate. Kendrick and her associates have applied the opsono-cytophagic test of Veitch (30) to the study of antibody production after *H. pertussis* vaccination, and during attacks of pertussis. The technic employed was relatively simple. Equal parts (0.1 cc. each) of citrated blood and a suspension of killed *H. pertussis* organisms were incubated for 30 minutes at 37° C. Smears were then made, and the number of bacteria phagocytized by twenty-five polymorphonuclear leukocytes observed. They expressed the amount of phagocytosis noted, in arbitrarily devised grades.

In a group of 117 infants and children ranging in age from 8 months to five years these workers clearly demonstrated that the degree of phagocytosis increased during and after *H. pertussis* vaccination. The maximum usually was reached two months after completion of vaccination and thereafter declined very gradually. A rather high degree of phagocytosis was still present two years later.

Kendrick and her coworkers also observed a group of 119 individuals during or after an attack of pertussis. Some of these were adults who had had pertussis many years before. During the disease progressively increasing phagocytosis was usually encountered. The maximum degree occurred in general about two months after onset. No correlation between degree of phagocytosis and severity of attack was noted. " Weak " or " moderate " reactions were observed in individuals who had had the disease 3 to 40 years previously. As controls 154 non-injected individuals with negative histories for pertussis were studied. Phagocytosis was not nearly so marked though it tended to increase with age. The white blood cells of 21 newborn infants showed practically no phagocytic power regardless of the histories or opsono-cytophagic reactions of their mothers.

Kendrick, Gibbs and Sprick (29) report uniformity of results with different Phase I strains of *H. pertussis* (that is recently isolated, " smooth " strains). It was found that the presence of a high opsono-cytophagic titer for *H. pertussis* was not associated with high titers for nine other species of organisms except *Br. bronchisepticus*. It has long been known that this organism is antigenically closely related to *H. pertussis*. The report of Kendrick, Gibbs and Sprick, however, does not conclusively show that the increased phagocytosis following pertussis vaccination is specific. The effect of injecting other vaccines on the phagocytosis of *H. pertussis* was not determined. These authors conclude that the opsono-cytophagic reactions may " offer promise of help " in the investigations of immunity in pertussis. They suggest that it may possibly act as a useful guide for immunization but "as a means for determining whether an individual has had pertussis in the past, the test is not adequate."

Using a similar technic Bradford (31) has obtained data confirmatory to the above. He notes that the heparinized blood of children with histories of pertussis is more actively phagocytic toward *H. pertussis* than that of children with negative histories.

The following studies were undertaken to see if we could confirm the findings of Kendrick, Gibbs and Sprick and to test the value of the opsono-cytophagic reaction as an index of immunity.

PROCEDURE

Preparation of vaccine. A three day growth of Phase I (smooth) strains of *H. pertussis* grown on Bordet-Gengou media was washed once in Locke's solution, and then suspended in 1 : 10,000 dilution merthiolate in Locke's solution. The antigen suspension was standardized to contain 20 billion *H. pertussis* organisms per cubic centimeter. This antigen was put up in 5 cc. vials and stored in the ice box.

Technique of obtaining and preparing specimen. Chemically clean Kahn pipettes were used. Two hundredths cc. (0.02) of 5 per cent buffered sodium citrate solution [2]

[2] The 5 per cent sodium citrate solution was buffered at pH 7.2 with Sorensen's phosphates. Evans (32) has shown that in weakly acid solution leukocytes take up H-ions and become less active. The buffered citrate solution was prepared every month and kept tightly stoppered. The final concentration of sodium citrate in the blood was 1 per cent.

It is known (33) that citrate decreases phagocytosis by binding calcium. For this reason heparin may be a better anticoagulant. The latter is advocated by Veazie and Meyer (34). Huddleson et al. (35) prefer sodium citrate for " it inhibits the action of those brucella op-

was first drawn into the pipette and immediately followed by 0.08 cc. of freely flowing blood obtained by puncture of ear lobe or heel. The specimen of citrated whole blood was then blown into small agglutination tubes (diameter 8 mm.). Within 30 minutes to 1½ hours after obtaining the specimen, 0.025 cc. of the standardized *H. pertussis* antigen was added. This blood cell antigen suspension was thoroughly mixed by very gentle rotation for one minute and then placed in the incubator at 37 degrees for 30 minutes. Without further shaking,[3] the blood from the bottom of the tube was drawn into capillary pipettes. A drop of this blood was placed on a thoroughly clean glass slide and smeared in the usual manner. The smears were dried in the air quickly and fixed immediately with methyl alcohol. Three to four smears of each sample were made.

Staining of slides. Freshly diluted Giemsa stain was found to be more satisfactory than Hastings stain or methylene blue. Giemsa stain (G. Gruber and Co.) was diluted one drop of stain per one cc. of distilled water which contained 0.001 per cent NaH_2CO_3. The slides were flooded with diluted stain for 20 minutes after which they were washed gently with distilled water and dried quickly in the air.

Microscopic examination of slides. The smears were examined under oil immersion. The number of organisms or absence of organisms in each of 25 polymorphonuclear neutrophiles were recorded. Rarely an eosinophile containing bacteria was encountered. Frequently large mononuclears engorged with bacteria were seen. Notation of these were made; however, only the number of bacteria in the polymorphonuclear neutrophiles were used in determining the opsono-cytophagic titer. Areas of the smear where clumping of either cells or bacteria occurred were avoided. Smears prepared in the manner described usually gave good preparations with an even distribution of white cells and bacteria. It was customary to examine 25 cells on each of two to three slides, thus obtaining a count of from 50 to 75 cells of each specimen of blood.

The slides were filed for examination according to number rather than name of patient. Specimens were obtained from both control and test children on the same day. All of the microscopic examinations were made by one observer who did not know the history of the patient from whom the specimens were obtained. About ⅛ of the specimens were examined by an additional observer and the independent results compared.

Clinical tests. The technique described was used to test 150 children who were divided into the following groups.

sonins which are present in the serum of normal individuals."

[3] Shaking the specimen after incubation tended to increase phagocytosis, and clumping of bacteria and cells occurred frequently. Smears were more easily read if the mixture was not agitated after incubation.

Group I. Forty children were tested before and after injection of Phase I *H. pertussis* vaccine.[4]

Group II. Twenty-four children were tested at varying intervals after injection of Phase I *H. pertussis* vaccine. Initial or control counts were not obtained on these children.

Group III. Twenty-seven children injected with 10 cc. of *H. pertussis* "Undenatured Bacterial Antigen"[5] were subjected to these tests.

Group IV. Thirty-two control children were tested. Fifteen of these received three injections of 1 cc. normal saline and seventeen received no injections. Many of these children had more than one test after intervals of one to four months.

Group V. Twenty-eight children were tested during the course of pertussis.

RESULTS

Group I

The forty children tested before and after injection of Phase I vaccine fell into the following age groups:

4 months to 6 months	4 children
6 months to 12 months	14 children
12 months to 18 months	10 children
18 months to 24 months	2 children
2 years to 5 years	7 children
5 years and over	3 children

The initial test was done on the day of the first injection of vaccine. This total count per 25 cells ranged between 16 to 440 bacteria with a mean of 114. The per cent of cells participating in phagocytosis was between 30 and 100 per cent with a mean of 60 per cent. In all instances in which the initial total count per 25 cells was over 200, it was noted that the children fell into groups over 18 months of age. It should not be inferred from that that *all* older children give high titers since very low titers were not infrequently observed in older children.

The second test was done on the day of the third injection of vaccine (two weeks after the first injection). Thirty-nine of the 40 children were tested. The total counts of bacteria ranged between 172 to 1385 organisms per 25 cells with a mean of 637. Following injection of vaccine, successive tests showed practically 100 per cent of the cells participated in phagocytosis in all cases.

Twenty-two of the children were given a third

[4] Kindly supplied by Cutter Laboratories from strains isolated by the authors.

[5] Kindly supplied by Eli Lilly Co.

test about two months after their last dose of vaccine. The total counts of this test ranged between 116 and 1830 bacteria per 25 cells with a mean of 916 bacteria.

The fourth and last test was taken on fourteen of the children at intervals varying from three to six months after their last dose of vaccine. The total counts of this test ranged between 758 and 2985 organisms per 25 cells with a mean of 1608 organisms.

These studies were continued over a six month period using the same lot of *H. pertussis* bacterial suspension. During the last two months of the study a higher initial or control count was observed frequently. It was thought that ageing may have been a factor altering the antigen suspension and producing this effect. Three new lots of antigen were prepared simultaneously using three different strains of freshly isolated Phase I *H. pertussis*. These were checked with the first antigen used throughout the six month period. It was found that the new lots of antigen varied considerably with each other as well as with the original old antigen when tested on the same individual's blood. Obviously, subsequent tests on any of the forty children of this group using the new antigen would not be comparable. These blood studies were discontinued and the data on hand obtained from the one lot of antigen were subjected to the following statistical analysis.

To determine whether an alteration of the antigen suspension occurred with ageing, the data obtained on the 1st, 2d, 3d, and 4th test were divided into the tests done during the first four months and the tests done during the last two months. The mean, standard deviation, standard error of the means, standard error of the standard deviation and standard error of the difference of the means of the total counts of organisms per 25 cells were calculated for each of these two periods.

The difference of the means and standard error of the difference of the means between the results obtained during the first four months and those obtained during the last two months for the 1st, 2d, 3d, and 4th test show that while apparently some alteration of the antigen had occurred with age (indicated by the increase in the means of the second series over the first) (Table I), these differences were not statistically significant, a fact probably dependent on the smallness of the samples.

TABLE I

Comparison of counts of the number of bacteria ingested by 25 cells in successive tests during the first 4 months and last 2 months' periods

	First 4 months of use of antigen. Mean counts with standard errors	Last 2 months of use of antigen. Mean counts with standard errors	Difference between means with standard error of differences
Initial or control test (1st test)	$N^*=29$ 83±15	$N=11$ 189±52	106±70
14 days after first injection (2d test)	$N=23$ 514±43	$N=16$ 813±77	298±77
2 months after last injection (3d test)	$N=14$ 703±53	$N=8$ 1289±212	587±219
3 to 6 months after last injection (4th test)	$N=3$ 1172±139	$N=11$ 1727±211	655±253

* N = Number of cases upon which the mean is based.

Calculations are shown in Table I.

Therefore, because no significance could be attached to the differences in means, although consistently in favor of the older antigen, it was decided to determine the means and standard deviations of the total series for each successive testing by counts. Summary of results of these calculations is shown in Table II. The difference of means, between the first and second, second and third, and third and fourth determinations (indicated by figures in italics in Table II), are

TABLE II

Differences in titers on successive tests of all cases during a six month period of study

	Number of cases	Means (M) of total number of bacteria per 25 cells with standard errors	Standard deviation of total count per 25 cells with standard errors	Differences of means—with standard errors of differences
Initial or control (1st test)	40	$M_1=114\pm19$	118±13	M_2-M_1 *523±44*
14 days after 1st injection of vaccine (2d test)	39	$M_2=637\pm40$	250±28	M_3-M_2 *279±92*
2 months after last injection of vaccine (3d test)	22	$M_3=916\pm83$	390±59	M_4-M_3 *692±185*
3 to 6 months after last injection of vaccine (4th test)	14	$M_4=1608\pm166$	612±116	

found to be statistically significant. Each difference is greater than three times its standard error.

Correlations between the first and second, second and third, first and third, and first and fourth tests were calculated using the Pearson coefficient $r_{xy} = \frac{\Sigma xy}{n\sigma x\sigma y}$. Since all of the coefficients were low and not statistically significant, the calculations show that a child with an initial high titer will not necessarily give a proportional higher titer on successive tests following injection of Phase I *H. pertussis* vaccine.

Group II

Twenty-four children who had received injections of *H. pertussis* vaccine before this study was undertaken were brought into the clinic for testing. Fifteen of the twenty-four children were under 18 months of age. Tests were repeated at intervals of several months on most of the children. While results of tests on children without initial or control counts would not be of significance by themselves, it was felt that they might prove to be of value when compared with the tests of control children in a similar age group. The results are summarized in Table III.

TABLE III

Titer of children in Group II

	Interval between injection of vaccine and tests			
	0 to 2 months	3 to 6 months	6 to 9 months	Total
Number of children tested...	15	19	8	42
Average total number of bacteria per 25 cells..........	617	714	942	760

There were several children in Groups I and II who persisted in showing low total counts on successive tests following vaccine injection. The significance of this is not known. These children will be followed closely in the future to determine whether there is any correlation between opsono-cytophagic titer and susceptibility to pertussis.

Group III. Undenatured bacterial antigen

Twenty-eight children who received a total of 10 cc. of *H. pertussis* undenatured bacterial antigen (Krueger) were tested. Fifteen of the twenty-eight children were under 18 months of age. Seven of these children had initial or control counts. The rest of the children had one or more tests at varying intervals. The resulting total counts of these children did not vary perceptibly with the time lapsing between the injections of undenatured bacterial antigen and the test. The results are summarized by age groupings in Table IV. The variation of total count following injection of undenatured bacterial antigen in relation to time interval after injection is shown in Figure 2 (composite scatter diagram of all groups).

TABLE IV

Titers of children in Group III

	Under 6 months of age	Between 6 and 12 months of age	Between 12 and 18 months of age	Between 18 and 24 months of age	Over 2 years of age	Total
Number of children....	3	10	5	2	8	28
Number of tests done...	11	26	11	2	14	64
Average total number of bacteria per 25 cells.....	58	53	141	92	148	93

Control Group IV

Thirty-two children were used as controls. Seventeen of these had no injections and 15 of these received three injections of 1 cc. each of normal saline. Nineteen of these children were under 18 months of age. Tests were repeated at varying intervals. Results are summarized according to the age of the child in Table V.

TABLE V

Titer of children in Group IV

	Age of child tested					
	Under 6 months of age	Between 6 and 12 months of age	Between 12 and 18 months of age	Between 18 and 24 months of age	Over 2 years of age	Total
Number of children tested......	13	2	4	3	10	32
Number of tests done...	26	3	5	9	12	55
Average total number of bacteria per 25 cells.....	44	195	87	121	152	91

In Groups II, III, and IV, the number of children tested and the number of children in each group under eighteen months of age is very similar. Comparison of the total results of each group (II—average 760 bacteria per 25 cells; III —average 93 bacteria per 25 cells; IV—average 91 bacteria per 25 cells), clearly shows that the opsono-cytophagic titer for *H. pertussis* is increased following injection of vaccine. Krueger's undenatured bacterial antigen does not produce an increase in titer. Titers of children injected with the undenatured bacterial antigen are almost identical to titers of the control children.

Group V. Test for specificity

We wished to determine whether *H. pertussis* Phase I vaccine was the only agent which would increase the opsono-cytophagic titer for *H. pertussis*. In order to do this seven children were injected with a mixed respiratory vaccine containing *Staphylococcus aureus* and *albus*, streptococcus, *H. influenza, M. catarrhalis*, pneumococcus and Friedlander bacillus in a total count of one billion per cc.

Because of the tendency to produce local reactions, amounts of 0.1 to 0.3 cc. of the respiratory vaccine were injected. The ages of these children ranged between 6 months and 6 years. Results of the tests before and after injection are summarized in Table VI.

It will be noted that in five children a definite increase in titer occurred soon after injection of mixed respiratory vaccine. This will be discussed later.

TABLE VI

Titers of children in Group V

Case	Age of child	Number of *H. pertussis* bacteria per 25 cells		
		Control test before injection	2d test 2 weeks after 1st dose vaccine	3d test 2 to 5 months after injection of vaccine
(1) R. D.	2 years	88	382	200
(2) S. P.	17 months	15	8	
(3) S. F.	4 years	25	123	484
(4) E. H.	8 months	46	1231	422
(5) R. U.	6 years		422	484
(6) S. T.	6 months	86	871	
(7) J. M.	6 years	119	814	

Group VI. Studies during the course of pertussis

Twenty-eight children and one adult were tested during their course of pertussis. One to seven tests were done on each individual. Two children of this group had tests 3 months prior to the onset of pertussis. One of these was injected with 10 cc. undenatured bacterial antigen eight months before the onset of pertussis. The other child received injections of saline and served as a control for the undenatured bacterial antigen. One adult, mother of children with pertussis, was tested 7 days before she developed the disease. It would have been desirable to have tested all of these twenty-eight children before the onset of pertussis as well as during the course of the disease. Unfortunately, we were not able to predict the onset of pertussis in advance.

Results of the three patients mentioned are shown in Table VII.

TABLE VII

Titers of three patients with pertussis

Patient	Age	Total bacterial count per 25 cells
	years	
V. K.	1	40—3 months before onset of pertussis
Received 10 cc. of undenatured bacterial antigen 8 months prior to onset	1 4/12	327—7 days after onset of pertussis
	1 5/12	578—35 days after onset of pertussis (5 days after cessation of cough)
M. L. K.	2	109—3 months before onset of pertussis
Saline Control	2 4/12	203—7 days after onset of pertussis
	2 5/12	520—35 days after onset of pertussis (7 days after cessation of cough)
Mc. C. B.	45	223—7 days before onset of pertussis
		301—12 days after onset of pertussis
		600—27 days after cessation of cough

These three patients had mild cases of pertussis. The results in Table VII are probably a fair sample of the change in titer during the course of the disease. The results of all of the tests done on the children during the course of pertussis are shown in Figure 1, a scatter diagram in which the opsono-cytophagic titer is plotted against days from onset and days after cessation of cough. The marked increase during the course of the disease and persisting for six weeks after cessation of cough is clearly shown in this scatter diagram. For comparison, the average of all titers obtained from children before treatment with pertussis antigen are shown in space at the left.

In order to compare more easily the titers re-

sulting from tests on children in Groups I, II, III, IV, and V, a composite of the results is shown in a scatter diagram (Figure 2). On this figure the titers are plotted according to interval lapsing between treatment and test.

Observing the total of 94 control tests (which includes initial or control titer of Groups I, II, III, and V as well as all the titers of control Group IV) the wide individual variation of titer is brought out. Eighty-seven per cent of these fall below 200 organisms per 25 cells. The 12.8 per cent of control titers above 200 bacteria per 25 cells were obtained from children over 18 months of age. Likewise, it will be noted that 41 of the 46 tests (89 per cent) made on children injected with undenatured bacterial antigen (indicated by a bar) also fall below a titer of 200, regardless of the interval lapsing between treatment and test. *H. pertussis* undenatured bacterial antigen apparently does not increase the opsono-cytophagic titer for *H. pertussis*. Titers of this group are very similar to the controls. The high range of titer indicated by the dots, circles and " R " connecting lines clearly demonstrates the marked rise of titer following injection of Phase I *H. pertussis* vaccine and mixed respiratory vaccine.

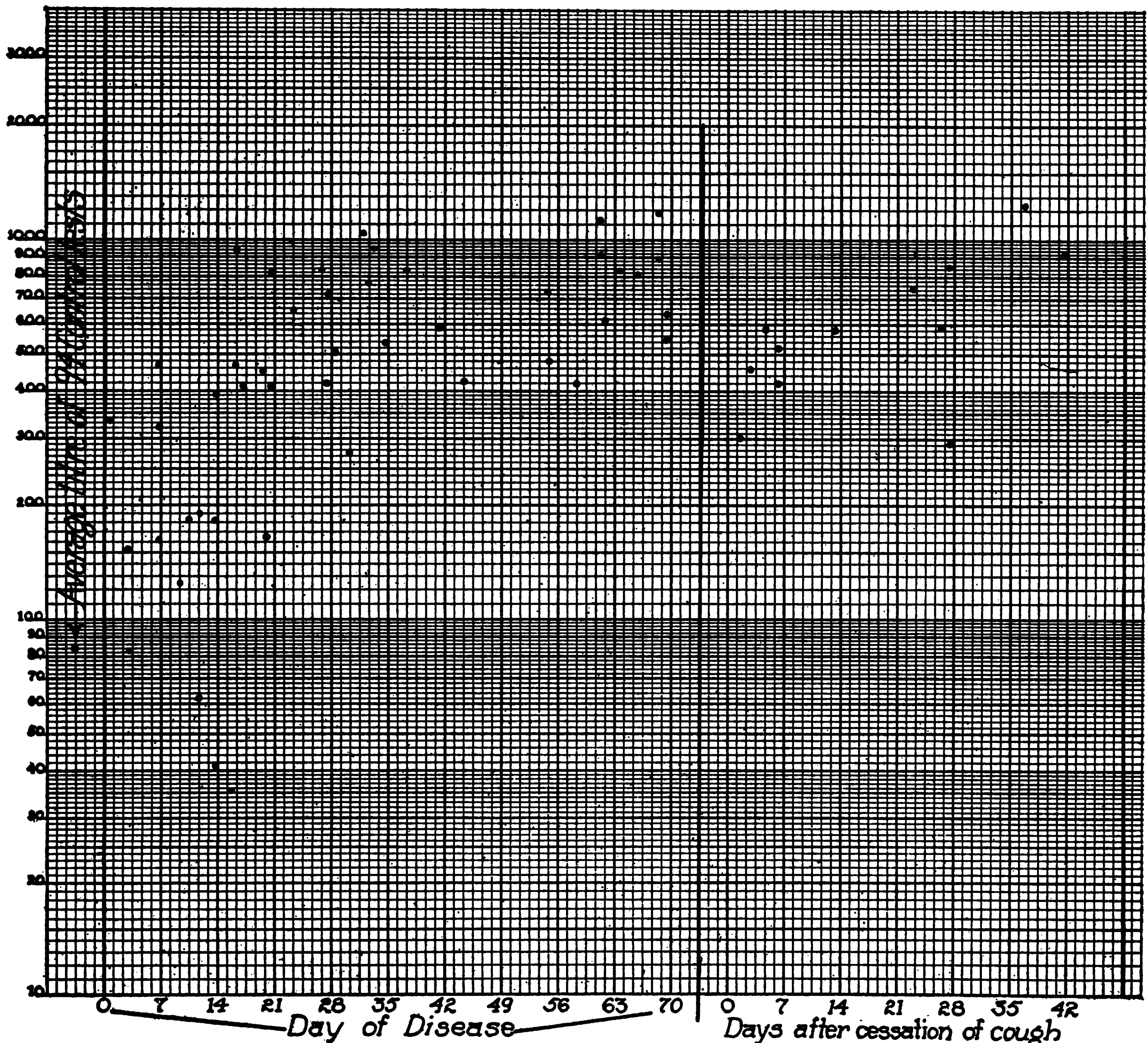

FIG. 1. ORDINATE—PHAGOCYTIC TITER (TOTAL NUMBER OF *H. pertussis* PHAGOCYTIZED BY 25 LEUKOCYTES) PLOTTED LOGARITHMICALLY. ABSCISSA—DAYS OF THE DISEASE FROM ONSET AND DAYS AFTER CESSATION OF COUGH

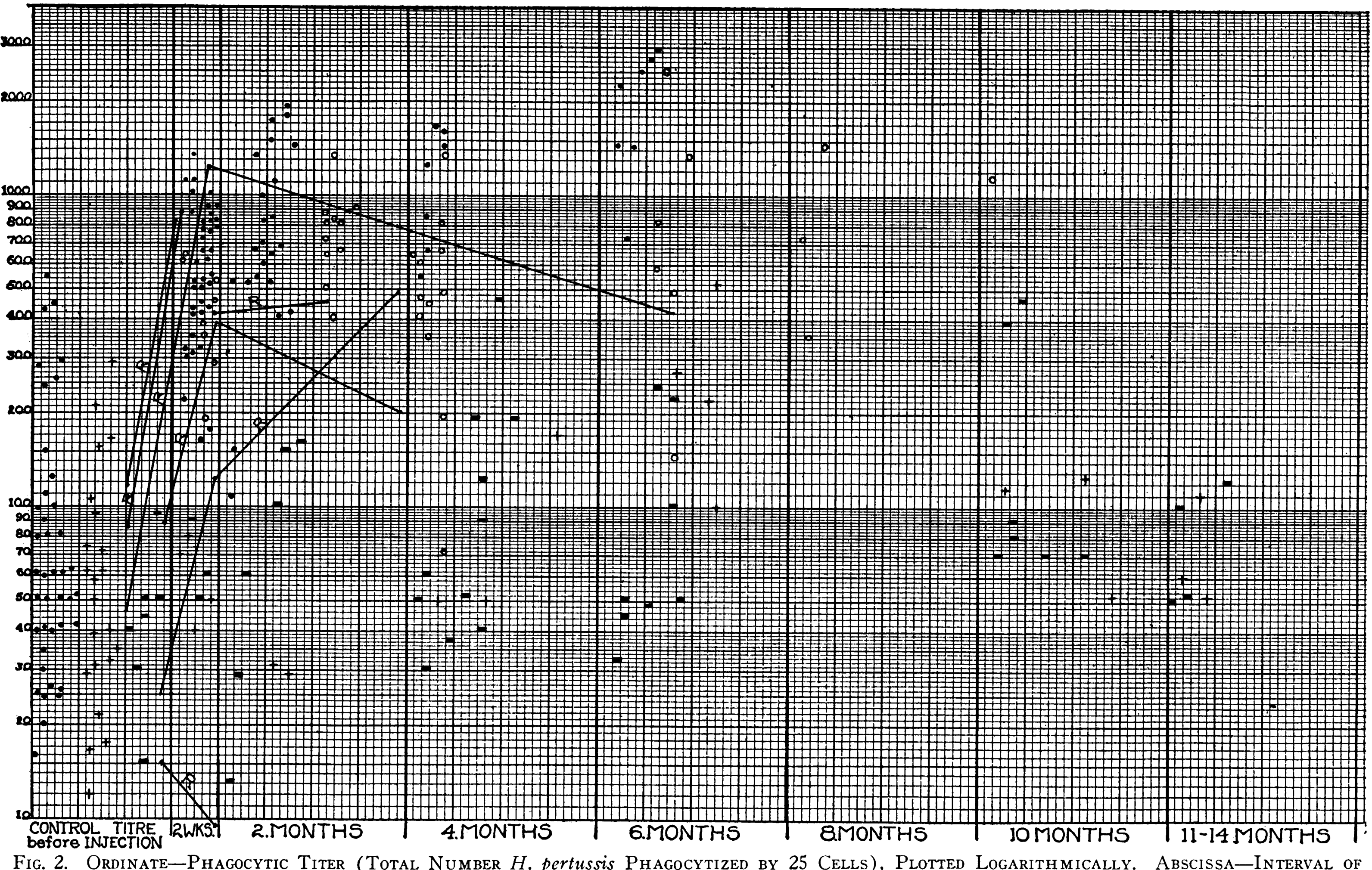

FIG. 2. ORDINATE—PHAGOCYTIC TITER (TOTAL NUMBER *H. pertussis* PHAGOCYTIZED BY 25 CELLS), PLOTTED LOGARITHMICALLY. ABSCISSA—INTERVAL OF TIME LAPSING BETWEEN THE FIRST (PRE-TREATMENT) TEST AND SUCCESSIVE TESTS

The dots indicate titer of children in Group I (tested before and after injection of *H. pertussis* Phase I vaccine). The circles indicate titers of children in Group II (tested subsequent to injection of Phase I *H. pertussis* vaccine: no initial or control count). Bars indicate titers of children in Group III (injected with *H. pertussis* undenatured bacterial antigen). Crosses indicate titers of control children in Group IV. Since Group V (injected with mixed respiratory vaccine) was small, the titers are indicated by "R" connecting lines to show individual increases or decreases. Note that after injection the dots are found in the high range; circles show a similar distribution, whereas the crosses and bars remain in the range similar to all initial titers.

DISCUSSION [6]

The variables in this test may be listed and discussed as follows:

A. Immunological variables

1. Specific opsonin. The immune body for which we are testing sensitizes *H. pertussis* rendering the latter more susceptible to phagocytosis, not only by circulating polymorphonuclear leukocytes but by sessile phagocytes. If specific opsonin was the only variable involved the opsono-cytophagic test would be a direct measure of circulating immune body, which manifests itself more readily by sensitizing the bacterium for phagocytosis, than by agglutinating the bacterium, or fixing complement in the bacterium's presence.

2. Normal opsonin. Wells (38) noted a rapid decrease in normal opsonin in the sera of infants during the first four weeks of life. Tunnicliff (39) confirmed this observation and found that not until the end of the second year does the titer regain the average level of that for adult serum. It is obvious that this knowledge of the variation of normal opsonin with age should be considered in discussing our findings. Kendrick, Gibbs and Sprick (29) noted increased phagocytosis of *H. pertussis* as age advanced, and our findings are confirmatory. Furthermore, our observations of increased phagocytosis of *H. pertussis* after injections of mixed respiratory vaccine can be interpreted only on the basis of an increase in normal opsonin. The question then arises, is the increased phagocytosis of *H. pertussis* after *H. pertussis* vaccination due to an increase in normal opsonin with probably little real increase in resistance, or, is the observed increased phagocytosis due to formation of specific opsonin which is an indicator of immunity? This question might be answered by making determinations of the opsonizing power of inactivated serum on the washed leukocytes of infants. The use of whole citrated blood in the opsono-cytophagic test leaves the question unanswered.

3. Variations in the inherent phagocytic activity of leukocytes. Glynn and Cox (37) showed that the phagocytic power of leukocytes is subject to variations in the same individual. There was no evidence that the variations were specific in the uninfected. Tunnicliff (39) found that "leukocytes at birth are a little less active than those of adults. Their activity diminishes considerably during the first months of life and does not regain that of leukocytes of adults until about the third year." Here again, as with normal opsonins, one must consider a gradually rising base line under the age of three years when one measures degrees of phagocytosis. However, the increase in phagocytosis of *H. pertussis* which we observed after vaccination and during the disease is far too great to be attributed to an increasing inherent activity of leukocytes. Our control data indicate this.

Since the test as here described involves these three variables, the degree of phagocytosis in each test is the product of the variables. At the same time it seems more rational to test for the product of these three factors than for any one of them individually. This product is what Tunnicliff (39) has termed "the anti-infectious power of the blood."

B. Technical variables

The test is not rendered any simpler by the following technical variables and difficulties.

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[6] A review of the following definitions may be of interest.

1. The *phagocytic index* (36)—the average number of bacteria phagocyted per leukocyte (50 or 100 leukocytes are usually counted).

2. The *opsonic index* (36)

$$\frac{\text{Phagocytic index of leukocytes in the test serum}}{\text{Phagocytic index of same leukocytes in a ``normal'' serum}}.$$

It is a test for antibody.

3. The *cytophagic index* (37)

$$\frac{\text{Phagocytic index of leukocytes to be tested in a given serum}}{\text{Phagocytic index of control leukocytes in the same serum}}.$$

It is a test for the inherent phagocytosing power of leukocytes.

4. The *opsono-cytophagic index* (37)

$$\frac{\text{Phagocytic index of leukocytes to be tested in serum to be tested}}{\text{Phagocytic index of control leukocytes in control serum}}.$$

Leukocytes and sera need not be added separately. Whole blood may be used. It is a test for antibody plus phagocytosing power.

1. *Variations in the number of leukocytes available in the blood antigen mixture.* Abnormal or even wide variations in the total white blood counts of the patients tested will influence the degree of phagocytosis observed. Fleming (40) has pointed out that the fewer the available leukocytes in the blood sample, the greater will be the number of bacteria ingested by an arbitrary number of leukocytes.

2. *Interference by agglutination.* With the present technique of the test, allowing incubation of the blood and bacterial suspension for 30 minutes, agglutination of the organisms is sometimes observed. Clumps of agglutinated bacterial cells are not seen within the leukocytes, though they are noted sticking to the outer surface of the leukocytes. If agglutination is marked, the number of bacteria observed within 25 leukocytes will be low. Conceivably, if a longer incubation period were used, time for phagocytosis of these clumps might be available. (It seems probable that *in vivo* the macrophages are concerned with the phagocytosis of agglutinated organisms.) This interference of phagocytosis by agglutination is an important drawback to the test. In the above experiments it certainly accounts for some of the wide individual variations.

3. Kendrick et al. (29) state that all Phase I *H. pertussis* strains used in their tests gave consistent comparable results. In our experience, however, different smooth *H. pertussis* strains gave considerable variation in titer when used to test the same individual's blood. This factor is a distinct handicap in continuing these studies over long periods of time.

4. *The age of the bacterial suspension used.* As is mentioned above, we found that as the suspension aged the bacterial cells were more readily phagocytized. Kendrick, Gibbs and Sprick did not have this difficulty. It may be explained by denaturation of the bacterial protein.

5. *Variations in the time interval* between drawing the blood and adding the bacterial suspension apparently were not important factors in our experience. When the antigen was added one hour after blood was obtained, the degree of phagocytosis noted was similar to that when the antigen was added immediately. Veitch (30), however, rightly insists on a standard time interval.

After considering these inherent and technical variables it is obvious that the experimental error is great. Because of this the writers feel that little significance can be attached to a single determination. It does not seem wise to grade results as Kendrick, Gibbs and Sprick (29) did into negative, weak, moderate and strong reactions on the basis of enumeration of bacteria ingested, except in relation to a change in degree. The actual amount of phagocytosis observed is far less important than the increase observed with a given technique.

It should be mentioned that the opsono-cytophagic test has proved to be of value in the diagnosis of brucellosis. Huddleson et al. (35) have reported a high specific opsono-cytophagic index occurring in convalescents and also in individuals in contact with infected cattle and infected meat (dairy and slaughter house workers). Meyer et al. (41) found high indexes in 21 of 22 active cases.

Recently Ward and Lyons (42, 43) have emphasized the importance of phagocytosis in resistance to streptococcal infections. They make the statement that the leukocyte in the presence of the type specific opsonin is the basis of streptococcic antibacterial immunity. Lyons (44) has utilized the opsono-cytophagic test as a means of determining the presence of specific opsonin in the sera of donors to be used for transfusion. Donors are then selected for the treatment of septic streptococcic infections on the basis of the opsonin content of their sera.

Our determinations on the opsono-cytophagic power of the blood after *H. pertussis* vaccination and after an attack of pertussis confirm the work of Kendrick, Gibbs and Sprick (29). We agree that the opsono-cytophagic test may be of value in estimating the potency of immunizing agents. We also agree that it seems inadequate as a test of immunity. To their report, however, we would append the following remarks:

1. Different strains of *H. pertussis* seem to vary in the ease with which they are sensitized and phagocytized by a given blood.

2. We have obtained evidence suggesting that the increased phagocytosis of suspensions of *H. pertussis* observed during *H. pertussis* vaccination may not be entirely due to the formation of specific opsonin. The observation that injections of

a mixed respiratory vaccine increased the phagocytosis of *H. pertussis* indicates that this organism is readily sensitized by nonspecific antibody (normal opsonin). This detail suggests that immunity to pertussis is not entirely specific. At the same time this detail may not be a theoretical disadvantage to the test.

The value of the opsono-cytophagic test depends entirely on the correlation between a marked degree of phagocytosis and immunity to adequate exposure. This can only be determined by future observations on a large number of children. On this point, the test in its present state seems inadequate, since an isolated determination on a blood sample means little. The technique must be further improved so that with different strains similar results are obtained on a single blood specimen. The use of a standard strain dried at low temperature *in vacuo* (as by the lyophile process) might facilitate standardization.

The test, on the other hand, appears definitely more delicate than the agglutination or complement fixation reactions for determining the presence of circulating antibody. It certainly shows that antibody capable of sensitizing *H. pertussis* remains in the blood stream longer after vaccination and longer after the disease than was formerly supposed. The question of whether immunity is dependent on the presence of circulating antibody is left unanswered.

SUMMARY

The recent adaptation of the opsono-cytophagic test to the study of pertussis has been scrutinized, and the following findings of Kendrick, Gibbs and Sprick (29) have been in general confirmed.

1. After injection of infants and children with a vaccine of *H. pertussis* in Phase I there was a definite increased phagocytosis of *H. pertussis in vitro*. This high opsono-cytophagic titer was maintained for at least six to nine months.

2. During and after an attack of pertussis there was a similar rise in the phagocytic activity of the blood.

3. A low degree of phagocytosis of *H. pertussis* was found in the blood of infants under 18 months of age if they had had neither the vaccine nor the disease. In children over 18 months of age more phagocytosis was occasionally observed even though the history was negative. It was, however, rarely as marked as after vaccination or disease.

It was also noted that:

4. After injection of *H. pertussis* undenatured bacterial antigen, no increased phagocytosis of *H. pertussis* occurred. Titers similar to those in the non-injected control children of the same age range were obtained.

5. After injection of a mixed respiratory vaccine increased phagocytosis of *H. pertussis* occurred. This stimulation of nonspecific opsonins indicates that the test is not specific for *H. pertussis* opsonins. This finding, however, does not necessarily invalidate the test, since an increase of non-specific opsonin may be commonly associated with an increase in resistance.

6. Variability of ease of sensitization and phagocytosis by the same blood was noted with different Phase I strains and with the same suspension of one strain as it aged. This together with other technical variables constitute drawbacks to the opsono-cytophagic test in its present form.

Our conclusions agree with those of Kendrick and coworkers that the test may be of value in estimating the potency of *H. pertussis* antigens when two or more determinations are made and the presence or absence of increasing phagocytosis is noted. The test at present is inadequate as a test of immunity, since immunological and technical variables obscure the value of a single determination or of multiple determinations with the same degree of phagocytosis. The ultimate value of the opsono-cytophagic test will depend on technical improvements and careful correlation with clinical immunity.

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