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VI Typhoid Flagellar and Heterophile Antibodies in Serums of Children

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VI. TYPHOID FLAGELLAR. AND HETEROPHILE
ANTIBODIES IN SERUMS OF CHILDREN

BY

PETER PAUL ANTOSIA

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE
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OF

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ABSTRACT

The purpose of this investigation was to determine whether Vi, typhoid flagellar (H), and heterophile (sheep hemagglutinating) antibodies were present normally in children. Vi antibodies have been reported in the serums of normal adults. Typhoid H antibodies appear during immunization of adults against typhoid fever and then gradually disappear. Heterophile antibodies have been found in the serums of many normal adults.

One hundred and twenty-six children's serums were obtained from blood specimens submitted to the Rhode Island Hospital laboratory for a variety of diagnostic purposes. The children were between one week and 15 years of age. None was diagnosed as having typhoid fever or related diseases. Twenty-five serums were secured from adults who had been immunized with typhoid-paratyphoid vaccine within recent years, and 25 from adults undergoing such immunization.

Vi and typhoid H antibodies in the serums were titrated with test antigens consisting of human erythrocytes of group O sensitized with saline bacterial extracts containing the Vi and typhoid H antigenic substances, respectively. Heterophile antibody was titrated by agglutination of sheep erythrocytes.

Vi antibodies were found in 70.6 per cent of the 126 children examined, and typhoid H antibodies in 62.9 per cent of 35 children. Eighty per cent of the 25 adults previously immunized with typhoid-paratyphoid vaccine possessed Vi antibodies, but only 40 per cent contained typhoid H agglutinins. Vi antibodies were present in all 25 adults undergoing immunization; typhoid H antibodies were found in 44 per cent. Heterophile

antibodies were detected in 99.1 per cent of 110 children and 98 per cent of the 50 adults.

Vi and typhoid H antibodies appeared during late childhood, possibly as a result of an active immunization process by serologically related organisms. Although heterophile antibodies were widely distributed, both in children and adults, their titers were greater in very young children.

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I. INTRODUCTION

Antibodies of various kinds, such as agglutinins, antitoxins, and bactericidal antibodies, are frequently found in the serum of normal men and animals. These antibodies occur naturally in the blood serum of man and animals with no known history of infection or artificial immunization by bacterial organisms with which the antibodies react.

It is well established that certain kinds of natural antibodies are produced in accordance with definite genetic factors and in the absence of any environmental stimulus. The best examples are provided by the blood group isohemagglutinins of man and animals. Other natural antibodies, like sheep hemolysins and hemagglutinins are known to occur in humans, but their origin is obscure.

The source of the natural antibacterial antibodies is unknown. Their presence is thought by some to reflect subclinical infection or infection with related organisms; for example, typhoid flagellar (H), somatic (O) and Vi antibodies have been found in adults but have not been reported in children. It was thought that some indication of their time of appearance might be provided by a study of their occurrence in children. Consequently this investigation was undertaken.

II. REVIEW OF LITERATURE

Smith and Reagh (1903) discovered two kinds of antigen by agglutination studies of the Salmonella choleraesuis bacillus; one was contained in the flagella, and the other in the cell body. Beyer and Reagh (1904) extended these observations and demonstrated the flagellar antigens to be thermolabile and the somatic antigens thermostable. Weil and Felix (1917) designated the flagellar antigens as H antigens and the somatic antigens as O antigens. All motile organisms so far investigated have possessed both these antigens.

Members of the *Salmonella* group are classified into species according to their antigenic structures. Since most *Salmonella* are motile, they contain both the O and the H antigens. Salmonella typhosa has long been known to contain at least two heat-stable O antigens and one heat-labile H antigen.

Felix and Pitt (1934) presented evidence of another antigenic component previously undescribed. These workers concluded from mouse protection tests that the virulence of S. typhosa was intimately associated with this new antigenic fraction and it was accordingly named "Vi." The Vi antigen was found to occur in many typhoid strains and also in other *Salmonella* species and even in extragenetic sources like certain coliform and paracolon species. Kauffmann and Möller (1940) reported a new species of *Salmonella* (S. ballerup), later transferred to the genus *Paracolonobacterium* (Bruner, Edwards, and Hopson, 1949) which contained Vi antigen serologically identical with the Vi antigen of S. typhosa and S. paratyphi. C. Stuart et al. (1943) found a Vi-like antigen apparently identical with that of S. typhosa and S. ballerup in a number of apparent-

ly normal strains of Escherichia coli and paracolon bacilli. Landy and Lamb (1952) isolated Vi antigen from E. coli (strain 5396/38) and found this organism to be the richest available source of the antigen. The Vi antigen was originally reported to be destroyed by a temperature of 60°C. However, Peluffo (1941) observed that this antigen is not destroyed by heat if the organisms are suspended in absolute alcohol, acetone, or glycerin.

Antibodies in blood serums of individuals against Vi, typhoid H and O antigens may be detected by employing the agglutination test. Vi antibodies may be titrated by use of a saline suspension of living Vi-containing bacteria with incubation at 37°C. for two hours followed by room temperature or refrigeration overnight. They can also be titrated by use of a test suspension consisting of human or various animal erythrocytes to which soluble Vi antigen has been adsorbed. H antibodies are determined by means of formalized (0.3 per cent formalin) test antigens and incubation at 50°C. for two to four hours. O antibodies are demonstrated with test antigens which have been heated at 60° to 120°C. for one-half hour to two and one-half hours. The tests are incubated at 50° to 55°C. for 18 to 24 hours.

Forsman (1911) discovered that the blood serum of man and other mammals usually contains small amounts of naturally occurring antibody that produces hemolysis of red blood cells from different animal species. These so-called heterophile antibodies are known to occur normally but in low concentration in human adult blood serum. Two types of sheep cell heterophile antibodies are abnormally found in man. The Forsman antibody or sheep cell hemolysin in high titer results from immunization of man with animal serum, such as horse serum. The heterophile antibody

(sheep hemagglutinin) of infectious mononucleosis is serologically different from the Forssman antibody. Davidsohn (1938) stated that a titer of 1:224 (semiquantitative presumptive test using test tube method) would confirm the presence of infectious mononucleosis in a patient with no history of horse serum injections but with a clinical picture of infectious mononucleosis. Ham (1951) concluded that "normal subjects may have heterophile antibody titers as high as 1:28, occasionally 1:56." The Forssman antibody levels are not increased in infectious mononucleosis.

Felix, Krikorian, and Reitler (1935) found Vi agglutinins in low titer in most serums studied from typhoid patients and convalescents. Felix (1938) first observed Vi antibodies in carriers of the typhoid bacilli. Bhatnagar (1938) noted that Vi agglutinins were present in chronic carriers of typhoid fever and suggested that such individuals might be detected by Vi agglutination. Eliot (1940), Eliot and Cameron (1941), and Coleman (1942) corroborated these previous findings and suggested the application of the Vi agglutination tests as a screening procedure for the detection of chronic carriers of typhoid fever.

Klein (1943) observed that the serum of three per cent of 100 normal individuals possessed Vi antibodies and 25 per cent contained H antibodies.

Mackenzie and Taylor (1945) noted that 33 of 492 subjects with a history of typhoid-paratyphoid immunization produced H and O antibodies in significant titer but only 2.9 per cent produced Vi agglutinins.

Landy and Lamb (1952) utilized a purified Vi antigen from E. coli (strain 5396/38) for the immunization of 300 individuals. They found 96 per cent of all individuals developed antibodies reactive with Vi sensi-

tized human O erythrocytes. They later (1953) found that of 252 serums from normal individuals, 1.6 per cent were positive Vi reactors. Vi agglutinins were also detected in the serums of 19 of 20 typhoid carriers.

Stuart and Serakis (1954) employed Vi hemagglutination and observed that approximately 75 per cent of 1200 serums of adults, typhoid-paratyphoid vaccine immunized or not, contained Vi agglutinins.

Accurate knowledge of the levels of H agglutinins among the normal population in any geographical area is not available. Many investigations and studies have been conducted relative to flagellar H agglutinins, however, on various animals and some humans, and all H agglutinin levels have been relatively low. Some data are available, however, on normal H agglutinin levels from isolated groups.

Giglioli (1933) observed that 54.6 per cent of 350 uninoculated natives in British Guiana possessed H agglutinins in titers greater than 1:20.

Alves (1936) surveyed a group of 530 uninoculated natives in Southern Rhodesia. The lowest titer recorded in this series was 1:50 but only 12.6 per cent of this group produced H agglutinins.

Lewin (1934), working in South Africa, noted that 15 per cent of 442 probably uninoculated natives contained H agglutinins reactive with S. typhosa.

H antibody titers of 1:1000 or greater in typhoid agglutination tests usually indicate with diagnostic certainty a typhoid fever infection. A full knowledge of the history of a patient, especially with reference to previous vaccination against typhoid fever must be had. A repetition of the agglutination tests should be made in order to secure

III. THE INVESTIGATION

A. Object:

The object of this investigation was to detect Vi, typhoid H, and heterophile agglutinins in the serums of children in the age group from one week to 15 years. Such antibodies are known to be present in serums of older individuals, but little study has been made of their presence in children. For purposes of comparison a similar study was also made on a group of adults previously immunized with typhoid, paratyphoid A and paratyphoid B and a second group of adults now actively undergoing such immunization.

B. Apparatus and Material:

In addition to the customary serological equipment and reagents, the following materials were employed: human erythrocytes of group O, sheep erythrocytes, Vi antigen, S. typhosa H antigen, rabbit anti-Vi immune serum, and serums from 126 children and 50 adults.

Group O human erythrocytes were secured from the Rhode Island Hospital blood bank and were employed within one to seven days after bleeding. They were prepared for use by washing three times with saline by centrifugation at 2000 RPM for 10 minutes and were finally packed in the centrifuge at 2000 RPM for 15 minutes.

Sheep erythrocytes were secured from the Charles V. Chapin Hospital and were used within one to seven days after bleeding. They were washed and packed in the same manner as the human red cells.

Vi antigen was secured through the courtesy of Dr. C. A. Stuart of Brown University. It represented a saline extract of a boiled suspension of P. ballerup, strain 481, in the "opaque" colonial phase. The method

by which this reagent was prepared is described in detail in Appendix A.

S. typhosa H antigen was prepared from a saline extract of S. typhosa, strain H901 (which lacks Vi antigen), provided by Dr. Stuart. The extract was boiled one hour and preserved with 0.3 per cent formalin.

Rabbit anti-Vi immune serum was obtained through the courtesy of Dr. Stuart. The method of preparation is described in Appendix B.

Children's serums were obtained from blood specimens submitted to the Rhode Island Hospital laboratory for a variety of diagnostic purposes. None of the children was diagnosed as having typhoid fever or related diseases. Serums were carefully removed from the clots after centrifugation and were stored in the refrigerator until they could be examined (within 2 weeks).

Twenty-five adult serums were obtained from the Veterans Hospital, Providence, and were secured from individuals who had been immunized against typhoid fever within recent years. Twenty-five additional serums were provided by the Quonset Point Base Dispensary, and were from individuals currently undergoing typhoid-paratyphoid immunization.

The test antigen for Vi agglutinins consisted of human group O erythrocytes sensitized with Vi antigen. Washed red cells were diluted with an equal volume of saline. One ml. of this suspension was placed in a 50 ml. beaker containing four ml. of 0.9 per cent saline. This solution was well mixed and to this was added slowly, drop by drop, with constant careful swirling, five ml. of a 1:5 dilution of the Vi extract. The container was then placed in a 37°C. water bath for one to two hours after which the cells were washed three times to remove excess antigen, and finally packed. A one per cent suspension of these sensitized cells was prepared.

The typhoid H test antigen was prepared similarly by sensitizing human O erythrocytes with extract of S. typhosa H901.

Sheep red blood cells for the determination of heterophile antibodies were diluted with saline to a concentration of one per cent.

C. Method of Procedure:

All serums to be examined were inactivated at 56°C. for 30 minutes to destroy complement prior to performance of the hemagglutination tests. "Master" dilutions (e.g. 1:5, 1:10, 1:20, etc.) of each inactivated serum were prepared and dispensed in 0.5 ml. amounts into the required numbers of serological tubes. Each tube then received 0.1 ml. of the appropriate test antigen. Control tubes containing 0.5 ml. of saline and 0.1 ml. of test antigen were always included. Control titrations to confirm the reactivity of the Vi test antigen were also performed using the anti-Vi rabbit immune serum. In all cases the antigen was agglutinated by antiserum dilutions between 1:5120 and 1:20,480. All tubes were shaken several minutes and were then incubated four hours at 37°C. and overnight at room temperature. Readings were made by flipping the tubes in groups of three and noting the degree of clumping of cells. The last tube in each series showing agglutination was the end point and the reciprocal of the serum dilution contained in that tube was considered the titer of the serum.

Serums with Vi titers of 40 or more were checked by Dr. C. A. Stuart, with all results in complete agreement.

D. Results:

The results of the investigation are presented in Tables 1, 2 and 3. It is apparent that Vi and typhoid H antibodies were present in the great majority of serums examined whether from children with no history

Table 1. The relation between Vi antibody titer and age of 126 unimmunized children, and the relation between Vi antibody titer and typhoid-paratyphoid immunization history of 50 adults.

Titer	Children				Adults (19 yrs. or older)	
	Under 1 year	1-5 years	6-10 years	11-15 years	Previously immunized	Under immunization
	Number of Serums					
0	1	9	20	10	5	0
5	0	5	9	7	4	1
10	3	4	7	4	5	8
20	0	4	8	5	7	11
40	1	3	3	8	4	4
80	0	2	3	5	0	1
160	0	0	0	2	0	0
320	0	0	0	1	0	0
640	0	0	0	1	0	0
1280	0	0	1	0	0	0
Log average titer	8.32	6.15	5.38	12.60	7.79	17.87

Table 2. The relation between typhoid H antibody titer and age of 35 unimmunized children, and the relation between typhoid H antibody titer and typhoid-paratyphoid immunization history of 50 adults.

Titer	Children				Adults (19 yrs. or elder)	
	Under 1 year	1-5 years	6-10 years	11-15 years	Previously immunized	Under immunization
	Number of Serums					
0	0	3	6	3	15	14
5	0	2	4	3	0	4
10	0	1	3	2	5	2
20	0	0	1	5	2	3
40	0	0	0	0	2	2
80	0	0	1	1	0	0
160	0	0	0	0	1	0
Log average titer	0.00	2.52	3.99	7.79	4.00	2.99

Table 3. The relation between heterophile antibody titer and age of 110 unimmunized children, and the relation between heterophile antibody titer and typhoid-paratyphoid immunization history of 50 adults.

Titer	Children				Adults (19 yrs. or older)	
	Under 1 year	1-5 years	6-10 years	11-15 years	Previously immunized	Under immunization
	Number of Serums					
0	0	0	1	0	1	0
5	1	2	1	1	3	3
10	0	1	3	6	7	4
20	1	4	5	8	7	11
40	0	3	12	7	4	6
80	1	4	11	9	3	1
160	0	3	9	2	0	0
320	1	2	2	2	0	0
640	0	4	1	1	0	0
1280	0	1	1	0	0	0
Log average titer	40.0	81.6	61.1	40.0	16.2	17.7

of infection or immunization with typhoid or related bacteria, or from adults previously immunized or currently under immunization with typhoid-paratyphoid vaccine. Moreover, the average titers of Vi and typhoid H antibodies did not vary significantly with age among the children and were about the same in the previously immunized adults. Vi antibodies were present in somewhat higher titer in adults undergoing immunization than in all other groups, although their typhoid H titers did not differ markedly from those of the other groups.

Vi antibodies were found in 86 (68.2 per cent) of the 126 children's serums examined, in 20 (80 per cent) of the 25 serums from previously immunized adults and in all of the serums from adults undergoing immunization. Typhoid H antibodies were present in 23 (66 per cent) of the 35 children's serums tested, and in only 10 (40 per cent) of the 25 previously immunized adults and 11 (44 per cent) of the group of 25 under immunization.

Heterophile antibodies were present in all except two individuals, one child and one adult. It is noteworthy that the titers were greatest in children from one to five years of age and gradually declined in higher age groups. It should also be pointed out that most of the highest heterophile titers were found in children suffering from febrile conditions (pneumonia, rheumatic fever, etc.).

Table 4 summarizes the results of the investigation and shows the percentage distribution of Vi, typhoid H, and heterophile agglutinin titers in all of the children and all of the adults. It is of interest that among the children the incidence of Vi and typhoid H titers was similar. Among adults, however, the incidence of Vi antibody titers resembled that of heterophile antibody titers.

Table 4. Vi, typhoid H and heterophile agglutinin titers in serums of children (unimmunized) and adults (previously immunized or under immunization).

Group	Agglutinin	No. of serums	Agglutinin titers										Log average
			0	5	10	20	40	80	160	320	640	1280	
Children	Vi	126	Per cent of serums										7.9
	typhoid H	35*	29.4	19.0	14.3	13.5	11.9	7.9	1.6	0.8	0.8	0.8	4.6
	heterophile	110*	37.1	22.8	17.1	17.1	0.0	5.8	0.0	0.0	0.0	0.0	44.7
Adults	Vi	50	0.9	4.5	9.0	16.4	20.0	22.7	12.7	6.4	5.5	1.8	12.3
	typhoid H	50	10.0	10.0	26.0	36.0	16.0	2.0	0.0	0.0	0.0	0.0	3.1
	heterophile	50	60.0	6.0	14.0	10.0	8.0	0.0	2.0	0.0	0.0	0.0	17.5

*Only 35 serums were examined for typhoid H antibody and 110 serums for heterophile antibody because these phases of the investigation were suggested after the Vi agglutinin titrations were in progress.

IV. DISCUSSION OF RESULTS

The diagnostic significance of antibodies in the serums of patients cannot be evaluated without studies of natural agglutinin levels in the general population. The results of the present investigation would seem to indicate that Vi, typhoid H and heterophile antibodies are normally present in a high percentage of children. Of 126 serums of children studied, 70.6 per cent showed Vi agglutinins. Several very high titers (160 to 1280) were obtained. As far as could be ascertained none of the children with Vi agglutinins had had any previous history of typhoid fever nor a history of typhoid immunization. The Vi antigen is widely distributed, occurring in S. typhosa, other Salmonellas, and also in certain strains of E. coli and P. ballerup. The Vi antibodies in the positive cases, therefore, may have been produced through antigenic stimulus, at one time or another, with any one of the Vi-containing organisms. This suggestion is supported by the observation that the average titer in serums from the 11 to 15 year group was considerably greater than in specimens from younger children. It is also of interest that Vi titers in adults under active immunization were higher than in those immunized at some time in the past. The percentage of specimens containing Vi antibodies in this investigation is quite consistent with the report of Stuart and Serakis (1953), who found 75 per cent Vi reactors among 1200 adults, either immunized or unimmunized.

Too much reliance should not be placed upon the typhoid H antibody data because of the small number of children's serums examined. However, 66 per cent of the specimens contained such antibodies. All were from children over one year of age, which indicates that they were probably

not acquired passively from the maternal circulation, since antibodies acquired in this way are known to disappear within a few months after birth. Two children possessed titers of 80, usually considered on the borderline of diagnostic significance. As with Vi agglutinins, it is noteworthy that the average titer of the 11 to 15 year children was greater than that of younger children, although admittedly both the titers and number of samples are small. The poor response of the adults under immunization with typhoid-paratyphoid vaccine is puzzling. The presence of typhoid H antibody in such a relatively large percentage of children without known history of infection or immunization with typhoid organisms may be attributed to infection (possibly unrecognized and/or unreported) with other Salmonellas, some of which possess the same flagellar antigen.

Heterophile antibodies were found in all except two blood specimens, one from a child and one from an adult, which points to their almost universal distribution. Titers of 80 or greater were found in 54 (49 per cent) of the 110 children, but in only four (eight per cent) of the adults examined. This is of interest in connection with Ham's (1951) statement that normal subjects only occasionally possess heterophile antibody titers as high as 56. It would appear that his statement is correct for adults, but may not be so for children. This seems particularly true in younger children, since the average titer in the one to five year group was 81.6. Thereafter the average titer gradually diminished toward an adult level of about 17. Fifteen of the children's serums possessed titers of 320 or greater. In the presence of clinical symptoms such values would be considered diagnostic of infectious mononucleosis (Davidsohn, 1938). The origin of sheep hemagglutinins in this disease (sometimes called "glandular

fever") is unknown. It may be suggested that the high heterophile antibody content of children's serums reflects the great incidence of febrile conditions during childhood, some of which may be related to infectious mononucleosis but are never recognized as such. In the present group of children there were none with a known previous or present history of infectious mononucleosis.

Future work on this problem should include the examination of a considerably larger number of blood samples, particularly from children in the first year of life. It would be desirable to devise a micro-technique for titrating antibodies of various kinds so that specimens from newborn babies could be studied. This might give interesting results with regard to the time of appearance and possible inheritance of antibodies, as in the case of sheep hemagglutinins, for example. The present investigation showed that such antibodies were present in all four of the first year serums obtainable, but this number is too small for generalization.

It would also be of interest to examine a large number of children for typhoid O as well as H antibodies and Vi antibodies, and then to subject them to typhoid immunization with subsequent reexamination of their serums. O and Vi antibodies are considered of greater protective value than H antibodies.

In other surveys of this type it is suggested that individuals possessing Vi titers of 160 or greater be tested for a possible typhoid carrier state. Bhatnagar (1938) reported titers of 50 or 100 in several typhoid carriers examined during the first year after recovery.

V. CONCLUSIONS

1. Vi and typhoid H agglutinins are present to a titratable degree in the serums of many children without known history of typhoid infection or immunization.
2. Vi antibodies apparently persist longer than typhoid H antibodies in adults previously immunized against typhoid fever.
3. Immunization with typhoid-paratyphoid vaccine causes an increase in Vi agglutinins.
4. Heterophile antibodies (sheep hemagglutinins) are widely distributed, both in children and adults. Their titers are apparently greater in very young children.
5. Vi and typhoid H antibodies seem to appear during late childhood as a result of an active immunization process, possibly by serologically related organisms.

VI. SUMMARY

1. The serums of children between one week and 15 years of age were examined, using a hemagglutination technique employing P. ballerup (a non-typhoid strain rich in Vi antigen), S. typhosa H901 (a strain devoid of Vi antigen), and sheep cells, for the detection of Vi, typhoid H, and heterophile agglutinins respectively.

2. Vi antibodies were found in 70.6 per cent of the 126 children examined, and typhoid H antibodies in 62.9 per cent of 35 children.

3. Eighty per cent of the 25 adults previously immunized with typhoid-paratyphoid vaccine possessed Vi antibodies, but only 40 per cent contained typhoid H agglutinins. Vi antibodies were present in all 25 adults undergoing immunization; typhoid H antibodies were found in 44 per cent.

4. Heterophile antibodies were detected in 99.1 per cent of 110 children and 98 per cent of the 50 adults. The titers were greater in children, particularly in the one to five year age group.

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APPENDIX A

PREPARATION OF VI ANTIGEN

Three nutrient agar petri dishes were heavily inoculated with P. ballerup, strain 481, and incubated at 22-30°C. for 18 hours. Three ml. of saline were added to each plate and the growth was carefully emulsified in the saline with a glass hoe. The three suspensions were pooled and boiled for one hour, centrifuged at 8500 revolutions per minute for 15 minutes and the supernatant, containing Vi antigen (Stuart, 1953) was removed and retained. To the supernatant was added 0.009 ml. of a solution containing 50 per cent by weight of phenol in ether per milliliter of Vi extract. The extract was stored at 4°C. until ready for use.

APPENDIX B

PREPARATION OF VI IMMUNE SERUM

Rabbits were injected intraperitoneally with 0.1 ml. of a 1:100 dilution of boiled Vi extract. The initial injection was followed by a second of 0.1 ml. of a 1:10 dilution, and a third injection of 0.1 ml. of undiluted Vi extract. Injections were given at intervals of five to six days. The animals were bled from time to time after the last inoculation and tested for Vi agglutinins. When high titer serum (approximately 1:20,000) was obtained, the animals were bled out and immune serum collected.