when administered daily to rats beginning at the time of the tumor inoculation.

1. Nungester, W. J., Gordon, Jean D., and Collins, Katherine E., *J. Infect. Dis.*, 1950, v87, 71.

2. Landsteiner, K., Raubitschek, H., *Centralblatt F. Bakt.*, 1907, v45, 660.

3. Boyd, W. C., Reguera, R. M., *J. Immunol.*, 1949, v62, 333.

4. Boyd, W. C., *J. Immunol.*, 1950, v65, 281.

5. Krebs, H. A., *Biochemica et Biophysica Acta*, 1950, v4, 249.

6. Li, G. J., and Osgood, E. E., *Blood*, 1949, v4, 670.

Received July 28, 1953. P.S.E.B.M., 1953, v83.

Development of Antibodies in Children Convalescent from Whooping Cough.* (20517)

JEANETTE L. WINTER. (Introduced by L. Emmett Holt, Jr.)

From the Department of Pediatrics, New York University College of Medicine and the Willard Parker Hospital, New York City.

The nature of the immunity which develops as a result of infection with *H. pertussis* has never been clearly defined. Many investigators believe that resistance to this disease is determined by the level of circulating antibacterial agglutinins. Evidence that this type of antibody frequently fails to appear as a result of the disease is, however, present in the literature. Other antibody responses which have been studied are complement fixation, opsonocytophagic reaction, and mouse protection. Recently Keogh and North(1) studied antihemagglutination and reported that in experimental murine pertussis the protective power of serum, given to mice intraperitoneally, was a function of the antihemagglutinin content of the serum—a conclusion not supported by the work to be reported here.

The present study is concerned with a comparison of 3 types of serum antibody in patients convalescent from pertussis, namely: 1) antibacterial agglutinin, 2) antihemagglutinin, and 3) mouse protective antibody (the capacity of serum to protect mice against intranasal infection with *H. pertussis*). It was the purpose of this study to ascertain which of these antibodies appeared most consistently with clinical recovery; hence which was the most significant measure of acquired immunity.

Methods and materials. Sera were obtained on patients ill with whooping cough from whom *H. pertussis* was isolated on nasopharyngeal culture.† The patients varied in age from 3 months to 9 years; their illnesses were characterized as moderately severe or severe. Blood was drawn immediately on admission and at 2- to 3-week intervals during convalescence. The sera were stored in the dry ice chest; all the specimens collected on each individual patient were tested together.

A. Antibacterial agglutinins. H. pertussis strain No. 18323 (obtained from Dr. Pearl Kendrick, Michigan Department of Health) and strain No. 484 (obtained from Dr. Anne Kimball, Minnesota Department of Health) were employed to prepare the standard antigen. The 24-hour growth from Bordet-Gengou Media was suspended in saline to a concentration of 20 billion organisms/ml, 0.3% neutral formaldehyde was added, and the bacterial suspension stored in the refrigerator. The antigen was used in a final concentration of 5 billion organisms/ml and was added to serial 2-fold dilutions in saline of the unknown serum. The tubes were shaken, and after 1-hour incubation at 37°C, the test was

* This investigation was supported in part by a research grant from the National Institutes of Health, Public Health Service.

† These patients were studied at Willard Parker Hospital. We should like to express our thanks to the members of the house staff and nursing staff of the hospital for their cooperation in obtaining specimens for the study. In particular we are indebted to Dr. Zacharias Matthews, Dr. Doris Zenger and to Miss Wilma H. Penzes, R. N.

stored in the refrigerator overnight. Readings were made after 24 hours. Known positive and negative sera and antigen in saline were included as controls.

B. Antihemagglutinins. The method described by Keogh and North(1) was employed in the determination of antihemagglutinins, excepting that horse red blood cells were substituted for the human cells used in the original method; horse cells were found to be a more sensitive indicator of the presence of hemagglutinin. It was necessary however first to absorb out any horse cell agglutinins normally present in serum before testing for the presence of antihemagglutinin. The nonspecific lipoid hemagglutinin inhibitor present in many of the sera was extracted with acetone by the method of Chanock and Sabin(2). Supernatants of the 4-5-day growth in Cohen and Wheeler fluid media of *H. pertussis* strain No. 61 (obtained from Dr. E. V. Keogh, Department of Health, Commonwealth of Australia) served as the source of hemagglutinin in these tests. All dilutions were prepared in 0.02 molar phosphate buffer (pH 7), and readings were made by observing the pattern of the settled cells after the test had remained at room temperature for 1 hour.

C. The intranasal mouse protection test. The method of North, Keogh, Anderson, and Williams(3) was utilized in demonstrating mouse protective antibody. Swiss mice, Carworth Farms c.f.w. strain, 3-4 weeks old were injected intraperitoneally with 0.2 ml of graded dilutions of serum 4 hours prior to infection with the challenge culture. Serum dilutions were prepared in saline and ten mice were used for each dilution of serum; 0.2 ml of undiluted serum was the maximum amount tested.

Preparation of challenge: *H. pertussis* strain No. 18323 passed once through mouse brain and once through mouse lung was employed as the challenge strain. The LD/50 of the strain as determined by the intracerebral challenge(4) was 250 organisms before lung passage and 125 organisms after lung passage. Stock cultures of the reisolated strain were stored in the dry ice chest and a new vial was started for each test. The revived culture was grown on modified Bordet-Gengou media. The 24-hour growth from the 5th or 6th subculture on this media was used to prepare the challenge suspension. The growth was harvested into 1% casamino acids (pH 7-7.2) and standardized with the Klett photo-electric colorimeter to equal 10 billion organisms/ml. Further dilutions as needed were made in the 1% Casamino Acids.

Infective doses of 5 million, 50 million and 500 million organisms in 0.05 ml volume were used to determine the virulence of the culture. A dose of 50 million organisms in 0.05 ml volume (representing 2.5 to 5 LD/50 of the culture) was used as the standard infecting dose for testing the passive protective power of the unknown serum. This challenge dose regularly killed 80% to 100% of the control mice.

Intranasal inoculation was performed by the method of Burnet and Timmins(5). The mice were observed daily for 21 days; at the end of this period the PD/50 of the serum (the amount of serum needed to protect 50% of the mice against the 50 M challenge) and the LD/50 of the culture were calculated by the method of Reed and Muench(6). The acute and convalescent serum of each patient were assayed simultaneously. A standard

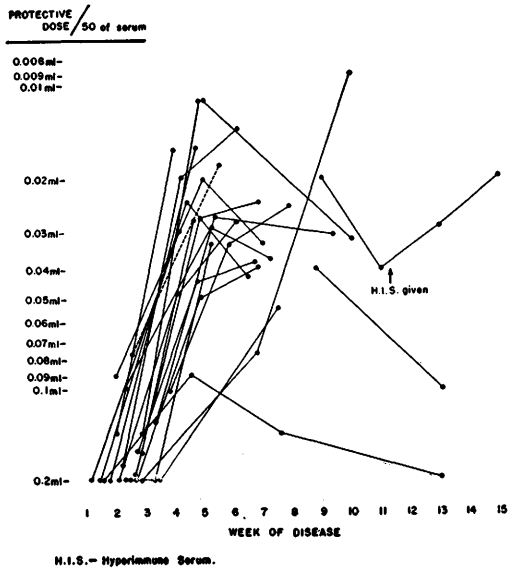

FIG. 1. Development of mouse protective antibody in patients convalescing from pertussis. One patient [broken line (- - - - - -)] had received 2 doses of vaccine prior to developing whooping cough.

TABLE I. Comparison of Mouse Protection with Other Serological Methods. Sera of children with whooping cough.

Patient	Age	Wk of disease	Antibacterial titer*	Antihemagglutinin titer*	Mouse protection,† PD/50 of serum
					ml
1. J.D.	4 mo	2	< 20	< 20	>.2
		4	20±	< 20	>.2
		6	40±	< 20	.033
2. V.Z.	4 "	3	< 20	< 20	>.2
		4	< 20	< 20	>.2
		8	< 20	< 20	.054
3. A.R.	5 "	2	< 20	< 20	>.2
		5	320	< 20	.016
4. M.R. I	8 "	3	< 20	< 20	>.2
		7	< 20	qns	.076
		10	< 20	80	.009
5. T.G.¶	9 "	3	640	< 20	.076
		6	320	80	.018
6. L.J.	12 "	3	< 20	< 20	.19
		5	< 20	< 20	.027
7. S.A.	14 "	3	< 20	< 20	.1
		6	320±	< 20	.029
		8	80	< 20	.037
8. P.A.	15 "	2	< 20	< 20	>.2
		5	< 20	< 20	.03
9. D.D.	20 "	3	< 20	< 20	.13
		5	< 20	< 20	.027
		9	< 20	< 20	.031
10. M.L.	2 yr	2	< 20	< 20	>.2
		5	< 20	< 20	.024
		7	< 20	< 20	.043
11. B.B.	2 "	2	40	< 20	>.2
		5	320	< 20	.09
		8	160	< 20	.14
		12-14	< 20	< 20	>.2
12. M.Q.	2 "	3	< 20	< 20	.158
		5	20±	< 20	.027
		7	40±	< 20	.024
13. R.R.	2 "	3	< 20	< 20	>.2
		5	< 20	< 20	.051
		7	< 20	< 20	.04
14. C.W.	2 "	2	< 20	< 20	>.2
		4	80	< 20	.049
		6	320	< 20	.028
15. L.R.	3 "	2	< 20	< 20	.14
		4	< 20	< 20	.016
16. M.D.	3 "	2	< 20	< 20	.18
		4	< 20	320±	.02
		6	< 20	320±	.014
17. M.R. II	6 "	3	< 20	80	.14
		5	< 20	160	.011
18. S.N.	6 "	3	< 20	< 20	.16
		5	< 20	< 20	.044
		7	< 20	< 20	.038
19. H.G.	8 "	3	< 20	80	.09
		6	80	320	.02
		8	40±	320	.033
20. V.H.	9 "	4	< 20	80	.1
		6	< 20	320	.033
		8	< 20	320	.025
Patients studied only during convalescence					
a. R.A.	2 yr	5	40	< 20	.011
		10	< 20	< 20	.032
b. G.P.	4 mo	9	40±	< 20	.04
		13	< 20	< 20	.1
c. S.S.	5 "	9	< 20	< 20	.02
		11	< 20	< 20	.04
		H.I.S.‡			
		13	80±	< 20	.029
		15	< 20	< 20	.02
Controls					
1. Normal infant serum			< 20	< 20	>.2
2. Human gamma globulin			< 20	< 20	>.2
3. Normal rabbit serum			< 20	< 20	>.2
4. Rabbit anti *H. influenza* serum			80±§	< 20	>.2
5. Rabbit *H. pertussis* immune serum			10,240	< 20	.009
6. Human hyperimmune serum			320	40	.057 .045‖

* Reciprocal of titer.

† PD/50 = Amount of serum which protected 50% of the mice against intranasal challenge with 50 M. organisms of *H. pertussis* #18323.

‡ H.I.S. = Human hyperimmune serum administered.

§ Cross agglutination due to presence of agglutinins for *B. bronchisepticus*.

‖ Maximum and minimum PD/50 values obtained for this serum in 10 tests.

¶ Received two doses of pertussis vaccine prior to developing whooping cough.

serum of known protective potency was included in all the tests.

Results. A. The development of antibacterial agglutinins and antihemagglutinins during convalescence. In a group of 50 patients studied 31 or 62% developed antibacterial agglutinins, and only 7 or 14% developed measurable antihemagglutinins. Some of the pa-

tients who failed to show the development of antibacterial agglutinins when tested with the standard antigen were retested with antigen prepared from their own strain; again the results were negative. The demonstration of the antihemagglutinins was complicated by the presence in many sera of the non-specific lipoid inhibitor reported by Fisher and Keogh (7). With acetone extraction of the serum (method of Chanock and Sabin(2)) and controlled conditions of testing (Ludewig and Chanutin(8)) the interference of this inhibitor was avoided. It was observed that whereas many of the sera obtained during the acute phase contained non-specific inhibitor in high titer, it was usually absent from sera obtained during the convalescent stage of the disease.

B. Development of mouse protective antibody during convalescence. In twenty of the aforementioned patients mouse protective antibody titers were determined concomitantly with the antibacterial agglutinins and the antihemagglutinins. Fig. 1 and Table I show the results obtained in these patients and in 3 additional patients tested only during convalescence. Sera tested in the acute phase of the disease contained only minimal amounts of antibody. During convalescence mouse protective antibody titers increased in every case, the increases as a rule being from 4- to 10-fold. Some children reached their maximum concentration of protective antibody during the fifth or sixth week and subsequently showed some decline in titer, while others continued to show increases during the seventh to eighth week and later. In general the development of the antibody titers was parallel to the course of recovery. Of the three cases tested only during convalescence, two showed decreasing titers. The third child showed a decrease in titer associated with an increase in severity of her disease; human hyperimmune sera was given and there was a temporary improvement followed by another relapse; the patient finally recovered at a time when the mouse protective antibody titer had risen to its initial high level. The poorest response observed was in a 2-year-old child with a severe attack complicated by a protracted bronchopneumonia; in this child the maximum increase was only 2-fold and occurred in the fifth week of illness.

TABLE II. Comparison of Development of Mouse Protective Antibody with Other Serological Methods. Children with whooping cough.

Antibody measured	Total No. of patients studied	Patients showing rise in antibody titer	
		No. of patients	% of patients
Antibacterial agglutinins	50	31	62
Antihaemagglutinins	50	7	14
Mouse protective antibody	20	20	100

Table I compared the antibacterial agglutinins, antihemagglutinins, and the mouse protective antibody titers observed in each patient. Increases in mouse protective antibody occurred in patients who failed to develop measurable antibacterial agglutinins (12 patients), antihemagglutinins (14 patients), or both of these antibodies (8 patients). In cases where both the antibacterial agglutinin and mouse protective antibody developed the rise and fall of these 2 titers occurred simultaneously; no correlation could be made however between the magnitude of the respective titers. Antihemagglutinin was the most irregular in development.

The total number and the proportion of patients who developed each of the 3 antibodies studied is shown in Table II.

Discussion. The uniform development of mouse protective antibody during convalescence as demonstrated by the intranasal method of challenge suggests that this antibody may play an important role in immunity to the disease. The ability of patients to develop increases in mouse protection in the absence of antibacterial agglutinins and antihemagglutinins further supports this hypothesis. The lack of quantitative correlation between the mouse protective antibody and the antibacterial agglutinin titers found in the present study has been reported also by North *et al.* (3) in adult contacts and immunized children, by Mishulow *et al.*(9), and others. Similarly Hink and Johnson(10) working with mice given human hyperimmune plasma subsequently challenged by the intracerebral route found that the gamma$_2$ globulin contained approximately 90% of the mouse protective

antibody and less than half of the Phase I agglutinins present in whole plasma.

It has not been possible to establish the relationship between antihemagglutinin and mouse protective antibody postulated by Keogh and North(1). The fact that only a small number of the patients developed measurable antihemagglutinins was unexpected and contrasted sharply to the ease with which this antibody could be produced in high titer as a result of vaccination of both human beings and laboratory animals with hemagglutinin containing fractions of *H. pertussis*. Although hemagglutinin is antigenic, additional evidence that the mouse protective power of serum is not determined by the antihemagglutinins has now been reported by Masry(11).

From limited data it would appear that a marked decline in circulating mouse protective antibody may occur within two or three months of the disease although some individuals have shown measurable titers when tested as long as 4 years after their illness. While circulating mouse protective antibody frequently disappears in the course of time, the immunity to whooping cough is known to persist. It is possible by means of a recall (booster) reaction to demonstrate a sharp increase in protective antibody in individuals with a known history of disease and in most adults(12). A corresponding increase in antibacterial agglutinins and antihemagglutinins may or may not occur. Whether this conditioned antibody response can explain the continued state of immunity is still undetermined. Further work along these lines is being carried out.

The mouse protection test is being applied to a study of children immunized against whooping cough as an important tool in the evaluation of different methods of vaccination against this disease.

Summary. 1. In a study of the development of antibacterial agglutinin, antihemagglutinin, and mouse protective antibody in children convalescing from whooping cough, mouse protective antibody was the only one to occur regularly as a result of the disease; it appears to be the most significant measure of immunity studied. 2. Mouse protective antibody titers increased during convalescence in all instances, the increases generally being from 4- to 10-fold. 3. The intranasal mouse protection test has been found to give reproducible results and is being applied to the study of children immunized against whooping cough.

We gratefully acknowledge the very valuable help and the encouragement given throughout this study by Dr. Robert Ward and Dr. L. Emmett Holt, Jr. Thanks are also due Mr. Clarence Johnson for his technical assistance.

1. Keogh, E. V., and North, E. A., *Australian J. Exp. Biol. and Med. Sc.,* 1948, v26, 315.

2. Chanock, R. M., and Sabin, A. B., *J. Immunol.,* 1953, v70, 302.

3. North, E. A., Keogh, E. V., Anderson, E., and Williams, S., *Australian J. Exp. Biol. and Med. Sc.,* 1939, v17, 275.

4. Kendrick, P. L., Eldring, G., Dixon, M. K., and Mesner, J., *Am. J. Pub. Health,* 1947, v37, 803.

5. Burnet, F. M., and Timmins, C., *Brit. J. Exp. Path.,* 1937, v18, 83.

6. Reed, L. J., and Muench, H., *Am. J. Hyg.,* 1938, v27, 493.

7. Fisher, S., and Keogh, W. V., *Nature,* 1950, v165, 248.

8. Ludwig, S., and Chanutin, A., *J. Biol. Chem.,* 1949, v179, 271.

9. Mishulow, L., Siegel, M., Leifer, L., and Berkey, S. R., *Am. J. Dis. Child.,* 1942, v63, 875.

10. Hink, S., Jr., and Johnson, F., *J. Immunol.,* 1950, v64, 39.

11. Masry, F. L. G., *J. Gen. Microbiol.,* 1952, v7, 201.

12. Winter, J. L., Unpublished data.

Received July 28, 1953. P.S.E.B.M., 1953, v83.