THE ACTION OF TYPE-SPECIFIC HEMOPHILUS INFLUENZAE ANTISERUM

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In a previous study (1) of type-specific strains of *Hemophilus influenzae*, it was observed that the organisms of all the strains isolated from cases of "influenzal meningitis" were encapsulated, formed colonies which have been described as smooth, and produced a soluble specific substance which precipitated the homologous antiserum. At the time the above report was made, organisms from nine patients not closely associated had been studied, and all of the strains were similar and belonged to the group which has been designated as Type b. This suggested that most, or at least the majority of, meningitis strains were of the same serological type.

In 1922, Dr. T. M. Rivers concentrated broth cultures of two meningitis strains and then removed from the concentrates the alcohol-precipitable fractions. 9 years later, these fractions were given to the author to be tested immunologically. Solutions of the fractions from both strains were made, and in each solution a heavy precipitate was formed when Type b antiserum only was added. This indicated, therefore, that the two strains studied by Dr. Rivers were also of the Type b group.

It was mentioned previously (1) that Wollstein (2), Povitsky and Denny (3), Rivers and Kohn (4), and others, found a considerable degree of immunological relationship among the strains isolated from cases of meningitis. There may be some question, however, whether the relationship which they noted was that based on the possession of the common soluble specific substance which we have described. Many of the strains had been isolated a number of months before they were immunologically tested, and as organisms of the S form usually tend to change to those of the R form with great rapidity, it is probable that some of their cultures contained only R forms. Furthermore, the agglutination reactions studied by these observers were carried out at high temperatures (45°C. or higher), under which conditions type-specific reactions are at least partially masked.

Ward and Wright (5), and Ward and Fothergill (6), have also reported that they have observed a close immunological relationship between meningitis strains.

Our observation that the majority of meningitis strains are type-specific and of the same type has been further substantiated in the continuation of this study. Up to the present, 41 strains of Pfeiffer bacilli obtained from patients suffering from lesions of the central nervous system have been examined, and all but four have been of Type b.[1] Three of these exceptions will be discussed later, and in the fourth instance the bacterium isolated was not of the species *Hemophilus influenzae*, but of the species *Hemophilus parainfluenzae*. This organism was isolated by Dr. J. D. Trask from the spinal fluid of a child who had a brain abscess.

The discovery that the organisms present in influenzal meningitis are usually type-specific, and that most of them are immunologically identical, suggested that a highly immune horse serum might have therapeutic value. Earlier attempts by other investigators to treat influenzal meningitis with immune serum were not very successful.

Wollstein (7), and also Rivers (8), did not find *H. influenzae* antiserum very effective in treating cases of influenzal meningitis in children, although Wollstein (9) did find that immune serum was effective in treating monkeys suffering from meningitis experimentally produced.

Reports of patients treated with the Wollstein serum have been made by Torrey (10), Packard (11), and Dunn (12). Torrey and Packard each treated one case, and both, children 11 years old, recovered. Dunn treated eleven cases and all died. He considered that in none of these was the serum treatment started early enough to expect the best results.

In 1921, Neal (13) reported that five patients had been treated with anti-influenzal serum by the Meningitis Division of the New York Department of Health. One patient who was treated with a number of intraspinal injections of vaccine and a few injections of antiserum recovered. In 1933, Neal (14) stated that among 90 cases of influenzal meningitis under their care, there had been three recoveries. These recoveries she did not definitely ascribe to the use of influenza antiserum, but she mentions that she was impressed with the marked, though temporary, improvement in clinical symptoms which followed the use of the serum.

Notwithstanding the earlier failures and the fact that the anatomical conditions in influenzal meningitis are such as to render the local application of any specific treatment difficult, if not impossible, it was

[1] Certain of these strains were received from Dr. Martha Wollstein, Babies Hospital, New York, Dr. Ann G. Kuttner, Pediatric Clinic of Johns Hopkins Hospital, and the physicians listed in footnote 2, to all of whom the author is greatly indebted.

decided that in the light of the newer knowledge concerning these organisms, a further study of this problem should be made.

Production of Immune Serum in a Horse

In February, 1931, the immunization of a horse with Type b organisms was started.

The antigen used for the earlier as well as the later injections has been prepared by growing Type b meningitis strains for 18 hours on Levinthal agar (1) made with horse blood, and washing the bacteria from each plate with 6 cc. of 0.4 per cent formalin in 0.85 per cent NaCl solution. A fresh lot of vaccine has been prepared for each series of inoculations. Up to the present, twenty-seven different strains have been used. For the first vaccine, five strains were employed; then, as new strains were obtained, the old strains were discarded and the new ones substituted. Great precautions have been taken to employ only pure S cultures in the preparation of the vaccine.

The horse has been immunized by giving a series of five daily intravenous injections of the vaccine followed by a rest period of 9 days before beginning a new series. As marked reactions occurred following the injections, very small inocula of vaccine had to be employed. For the first series, two inoculations of 0.05 cc. of vaccine were made, then the amount was slightly increased for each of the remaining three injections of this series. For the first inoculation of each of the following series of injections, the amount given was a trifle greater than that of the first inoculum of the preceding series, and the dosage was then increased daily. This method of increase was continued until a large bleeding was made, after which the horse was allowed to rest for several weeks. In renewing the process of immunization following the rest, very small inocula were again at first employed, but the size of the dose was increased more rapidly than before.

After each injection, the horse has had a febrile reaction which has reached its maximum in 4 to 7 hours. This reaction has usually been greatest on the 1st and 2nd days of each series of injections. Besides the febrile reaction, the horse has at times had very labored breathing, increased heart rate, weakness of the legs, and marked injection of the blood vessels of the sclerae.

In order to follow the progress of the immunization, a small amount of blood was withdrawn from the horse before the first inoculation of each series, and the serum was tested for its content in type-specific antibodies by means of precipitation and agglutination reactions. For $3\frac{1}{2}$ months, a gradual increase in the precipitating and agglutinating power of the serum occurred. Since then, there has been no apparent change. Precipitation occurs when the Type b purified

capsular polysaccharide in dilutions up to one part in one million is added to the serum. Type b bacilli are agglutinated with disc formation in dilutions of the serum up to 1–80, and with granular clumping in higher dilutions up to 1–320.

The serological reactions are carried out at 37°C. for 2 hours, and the tubes are then kept in the ice box overnight before the final readings are made. If the tests are made at temperatures higher than 37°C., disc formation becomes less striking and agglutination becomes largely of the granular type.

The precipitation reaction appears to be the more specific test, and after 2 years of immunization the serum contains no precipitating antibodies for soluble specific substances derived from influenza bacilli other than Type b. On the other hand, after the horse had been under immunization for about a year, it was found that the serum had acquired the ability to agglutinate influenza bacilli of other types. This non-type-specific agglutination is probably due to the presence in the serum of an antibody against some fraction of the cell other than the soluble specific substance. Preliminary experiments indicate that this fraction is carbohydrate in composition, that it is present to some degree in all influenza bacilli, and that it is probably analogous to the C substance of Streptococcus (15) and Pneumococcus (16).

It has thus been possible to produce in a horse an immune serum which is highly specific for Type b influenza bacilli, as shown by precipitation tests. It now seemed important to determine, if possible, whether the serum would exert specific effects in the infected animal as well as in the test-tube. In case the serum were found to have therapeutic value, it also seemed important to learn whether quantitative differences in the relative value of the several lots of serum could be demonstrated.

Consequently, a series of experiments has been made to determine the action of the serum in infected animals.

The Effect of Immune Serum on Mice Infected with Hemophilus influenzae

Since the susceptibility to infection with *H. influenzae* varies markedly in individual mice, and since large inocula are necessary to produce lethal results, it seemed probable that a method of testing based on protective power for mice would not be suitable for determin-

ing the immunological value of this serum. Attempts were made, therefore, to evaluate the action of the serum in preventing or inhibiting invasion of the blood following intraperitoneal infections. The bacteria and serum were injected simultaneously, and at various intervals cultures were made of blood from the end of the tail, and the number of bacteria in the blood determined. In a few of the experiments, cultures were also made at the same time from the peritoneal cavity. The results of one of these experiments are given in Table I.

TABLE I

Protection of Mice against Hemophilus influenzae

Culture inoculum	Antiserum Lot 2	Blood cultures—hrs. after inoculation								
		Peripheral								Heart
		1	2	3	4	5	6	7	24	48
cc.	*cc.*									
0.5		++	+++	++++	++++	D				
0.5		++	+++	++	++++	++	+	+	+++	−
1.0		+++	++++	++++	++++	D				
1.0		++++	++++	++++	++++	D				
0.5	0.2	−	−	−	(2)	−	(1)	−	−	−
0.5	0.2	−	−	−	−	−	−	−	−	−
1.0	0.2	−	(2)	(5)	+	++	++	++	(1)	D 31 hrs. (2)
1.0	0.2	(2)	(1)	(6)	(5)	(6)	(2)	−	+	−

−, +, ++, +++, ++++ = none, few, moderate number, many, very many colonies that grew from 1 loopful of blood on Levinthal agar.

Numerals within parentheses indicate the actual number of colonies which grew from 1 loopful of blood.

D = death of animal.

The surviving mice were killed after 48 hours.

It is seen that in the serum-treated mice the invasion of the circulating blood by the bacteria was either prevented or limited, and that in those mice in which invasion occurred the severity of the septicemia was slight, and that three out of four mice survived. The fourth mouse lived for 31 hours, at no time did it have a severe septicemia, and from the heart's blood culture made at autopsy only two colonies developed. In other experiments, however, a few of the treated mice

have died as rapidly as the untreated ones. Yet in all experiments the serum-treated mice which succumbed have never had more than a mild septicemia, and in some instances the blood cultures have been sterile. Moreover, in the treated mice which have died the number of bacteria in the peritoneal cavity has been markedly reduced, and the bacteria have been swollen and globoid, and undergoing phagocytosis.

On the other hand, in the experiment recorded in Table I, within 1 hour the untreated mice had moderate to severe septicemia, and at the end of 5 hours three of the four mice were dead. The remaining mouse had a marked septicemia at the end of 24 hours, but the heart's blood culture was sterile when the mouse was killed at the end of 48 hours. In all of the experiments, blood cultures from the untreated mice which succumbed were positive, and cultures made from the peritoneal fluid of these animals showed heavy growths.

All of the animals, treated as well as untreated, have exhibited certain toxic symptoms such as diarrhea and conjunctivitis, yet these symptoms have disappeared more rapidly in the treated animals than in the untreated animals which recovered.

Control experiments have shown that the action of the horse serum is type-specific, since no effect of the serum could be demonstrated in mice infected with Type a or R strains. Moreover, it has been shown that the administration of normal horse serum has no effect on mice infected with Type b strains.

While it was possible to show that the immune horse serum has a specific effect on mice infected with Type b influenza bacilli, it was difficult to estimate this effect quantitatively. Consequently, it was decided to attempt to determine the effect of the serum on larger animals, and since the rabbit, in proportion to its weight, is apparently the least resistant of the ordinary laboratory animals to infection with *H. influenzae*, this animal was chosen for the experiments.

The Effect of Immune Serum on Rabbits Inoculated Intravenously with Hemophilus influenzae

Rabbits were inoculated intravenously with given amounts of culture, and after 30 minutes to an hour blood cultures were made on plates. Definite amounts of immune serum were then injected intravenously. Shortly after the serum treatment, and then at hourly

intervals, blood cultures were made. Febrile and other symptomatic reactions were also observed at hourly intervals.

The culture, Strain 306S, employed in these experiments, was originally obtained from the spinal fluid of a patient suffering from meningitis. It was passed through a number of rabbits, and its virulence for rabbits at the time these experiments were made was such that 0.5 cc. to 0.25 cc., and at times even 0.1 cc. of a broth culture, killed.

The rabbits employed were approximately 1500 gm. in weight, and those used in a single experiment were of the same litter, or they were of the same breed and nearly of the same age.

It was observed that 30 minutes to an hour after infection the rabbits had a high grade septicemia, but that 5 minutes after the administration of immune serum the number of bacteria in the blood was reduced, and that after an hour the blood contained few organisms, or was sterile. Most of the serum-treated rabbits survived, but even in the ones that died the reduction in the number of bacteria in the blood was noteworthy. In the control rabbits, which received no immune serum, the degree of septicemia increased, and rarely did a rabbit recover. The protocol of one of these experiments is given in Table II.

In this experiment, each of six rabbits was inoculated with 0.5 cc. of a broth culture. 30 minutes later cultures were made on plates with a loopful of blood from the ear vein, and then three of the rabbits were treated intravenously with immune serum, two with normal serum, and one was left untreated. A seventh uninfected rabbit received serum only. After another interval of 30 minutes, and then at hourly intervals, the blood cultures were repeated.

As shown in the table, 30 minutes after infection all the rabbits had a massive septicemia. But in the rabbits treated with immune serum this bacteremia was so quickly reduced that 30 minutes after treatment only a few or no colonies developed on the plates, and, except in one instance, all subsequent cultures were sterile. In this exceptional case, the rabbit receiving the smallest amount of serum, six colonies developed in the culture obtained 24 hours after treatment. Despite the fact that in all the serum-treated animals the blood was sterilized, one of the animals was found dead at the end of 24 hours.

In contrast to the rapid disappearance of the bacteria in the serum-

TABLE II

Protection of Rabbits against Hemophilus influenzae

Rabbit No.	Culture	Serum	Blood cultures—hrs. after inoculation of culture									
			½	1	2	3	4	5	6	7	24	48
	cc.	*cc.*	Inoculation of serum									
1	0.5	Immune* 0.5	+++	(5)	−	−	−	−	−	−	(6)	−
2	0.5	1.0	+++	+	−	−	−	−	−	−	D−	
3	0.5	2.0	+++	−	−	−	−	−	−	−	−	−
4	0.5	Normal 1.0	+++	+++	+++	++++	++++	D++++				
5	0.5	2.0	+++	+++	+++	++++	++++	++++	++++	++++	++++	+
6	0.5	−	+++	+++	+++	++++	++++	++++	++++	++++	D++++	
7	−	Immune 2.0										

−, +, ++, +++, ++++ = none, few, moderate number, many, very many colonies which grew from 1 loopful of blood.

Numerals within parentheses indicate the actual number of colonies which grew from 1 loopful of blood.

D = death.

* Immune serum from Lot 5.

treated rabbits, the degree of bacteremia increased in the rabbits which were not treated, or which were treated with normal serum.

One of these rabbits died at the end of 5 hours, and another within 24 hours. Both had many organisms in their blood at the time of death. The third rabbit had a massive septicemia at the end of 24 hours, but at the end of 48 hours only a few organisms grew from the blood. In this animal the blood cultures continued positive for several days. It then developed another infection and was killed. The rabbit which served as the serum control showed no abnormal reactions.

A number of experiments similar in plan to the one just described were made, and the results were similar. Furthermore, similar results were observed when the serum was administered before, or simultaneously with, the culture. In one experiment, however, in which the dosage of culture employed was larger, all the rabbits died. Even under these circumstances, however, there occurred a marked reduction in the number of bacteria in the blood of the animals treated with immune serum, as compared with the number of bacteria in the blood of the control animals. It has been found, as the immunization of the horse has progressed, that the amounts of serum necessary to bring about sterilization of the blood have become smaller.

The immune horse serum, therefore, possesses the power of sterilizing the blood of rabbits infected with homologous organisms, unless the initial inoculum of culture is so great that the animal is completely overcome by the toxicity of the injected bacteria. On the other hand, the serum apparently does not prevent the toxic symptoms which follow the inoculation of animals with *H. influenzae*, since these symptoms occur in the treated as well as in the untreated animals.

These symptoms, which have been described by others, are rapid and labored breathing, loss of muscle tonus, increased peristalsis, refusal to eat, and increased secretion from the conjunctivae. Another feature, apparently not previously mentioned, is injection, and at times hemorrhages, of the scleral vessels. This reaction begins about 1 hour after inoculation, and usually persists for about 24 hours. It apparently corresponds to the injection of the blood vessels and hemorrhages in the internal organs which are seen at autopsy in rabbits after inoculations of *H. influenzae*. A further toxic manifestation which occurs shortly after infection is a change of temperature, which may be either lowered or elevated. In the rabbits which survive, there is a rapid elevation of the temperature, which usually returns to normal in less than 48 hours, and the latter occurs even though the bacteremia may still be present.

Although the administration of serum does not prevent the occurrence of these toxic symptoms, it apparently does shorten their duration. This is probably associated with the inhibition of growth of the bacteria by the serum.

The Action of Immune Serum in Preventing the Lesions Induced by Intracutaneous Injections of Hemophilus influenzae

The first description of skin lesions induced by the injection of Pfeiffer bacilli was made in 1894 by Kruse (17). He inoculated large doses of bacteria subcutaneously into rabbits. The resulting localized lesions were edematous and hyperemic, yet cultures made from them after 24 hours were sterile. After a few days the inflammation subsided, but a hard mass, potato-like in consistency, persisted for a number of days.

In a previous paper (1), the author has described the lesions resulting from the intracutaneous injection of cultures of S and R influenza bacilli in rabbits, and has drawn attention to the employment of this technique for determining the relative virulence of different strains.

Although previous observations had been made of the skin lesions induced by *H. influenzae*, it seemed important, before studying the action of the immune serum on the development of these lesions, to make a more detailed study of the effects produced by the injection of different amounts of various strains, both living and dead.

It was found that when a massive inoculation, consisting of the living S organisms concentrated from 1 cc. of a broth culture, is given intradermally, the localized lesion may reach 20 to 40 mm. or more in diameter. The lesion is markedly edematous, at first bright red, then purple-red in color. Later, the center becomes necrotic and a scab forms which covers a thick fibrinopurulent exudate—rarely is there any discharge. The reaction usually begins to decrease after 48 hours, but some inflammatory reaction remains for 5 to 7 days. As the inflammation subsides, a large hard palpable area of induration becomes evident. This may persist for as long as 20 days.

When massive inocula of R organisms are injected, lesions of almost equal severity develop. Furthermore, similar reactions develop if large amounts of dead organisms, either S or R, are given. However, the intensity of the lesions induced by the different forms bears a very definite relationship to the amount of culture injected. When small amounts are given, it is observed that the living S forms have a much greater capacity to induce lesions than do the dead S, or living or dead R forms. Of the three latter forms, there seems to be no significant difference in the reactions which they induce.

To illustrate the capacity of these forms of bacteria to induce lesions, Table III is given, in which is indicated the relative severity of the reactions usually observed.

Since dead forms of these bacteria are capable of inducing lesions, it is obvious that at least some of the effects of intracutaneous inoculations are due to preformed substances present in the bacterial cells. Further, since the lesions induced by living R forms are no more severe than those induced by the dead R forms, it seems probable that in the case of the R forms the lesions are entirely due to preformed substances, the living R forms being quickly killed after the inoculation.

TABLE III

Comparison of Skin Lesions Induced by S and R Forms of Hemophilus influenzae

Amount of culture in 0.2 cc. volume	Qualitative differences in the lesions induced by		
	Living S	Living R	Dead S or R
cc.			
1.0	++++	+++	+++
0.1	+++	+	+
0.01	++±	±	±
0.001	++	−	−
0.0001	+	−	−
0.00001	±	−	−

−, ±, +, ++, +++, ++++ = no reaction, very mild, mild, moderate, severe, and very severe reaction.

In the case of the S forms, however, it is noted that a much smaller dosage of living than of dead bacteria will induce an evident lesion, and in this instance it seems that living S forms are able to multiply and thus produce sufficient irritating substance to give rise to reactions.

Preliminary experiments showed that if a certain amount of immune serum were added to massive doses of the culture, some effect on the extent of the lesion was observable, but that if smaller doses of culture were employed the inhibiting effect of the immune serum was much greater, and that with still smaller doses the production of a lesion might be completely prevented. It was found that the actual amount of serum injected made little difference in the severity of the lesions; if the dose of culture was not too great, small amounts of serum within

certain limits were as effective as large amounts. Furthermore, it was observed that when the dose of culture was so large as to produce lesions in spite of the admixture with serum, the lesions were very similar in size to those induced by like numbers of heat-killed bacteria given alone. Hence, it appeared that the serum could only inhibit the action of a definite number of bacteria, and that the action of the serum consisted in preventing the growth of the bacteria rather than in neutralizing the toxic or irritating substances.

It was therefore evident that in employing skin inoculations to make quantitative tests of the inhibitory action of different lots of serum, it would be necessary to determine with considerable care the optimal dosage of culture to be employed in the tests. A series of observations, therefore, was made, employing various doses of culture alone, and also the same amounts of culture mixed with varying amounts of immune serum.

The culture employed in the tests was Strain 225S, isolated from a patient suffering from meningitis. The strain was kept under optimal conditions to prevent the development of R variants. The technique employed in cultivating and studying the characteristics of the culture was the same as that described previously (1). For the tests, 24 hour broth cultures were employed, and physiological salt solution was used as a diluent rather than broth, as the whole broth alone sometimes causes slight reactions. To prevent injury to the bacterial cells taking place in the salt solution, the injections were made as quickly as possible after diluting the culture. In all cases, 0.1 cc. of the culture or culture dilution was mixed with 0.1 cc. of salt solution, or with 0.1 cc. of whole or diluted serum, the amount of fluid injected in all cases being 0.2 cc.

On the day preceding the tests, the hair was removed from the flanks of the rabbits with electric clippers. Gray rabbits with thick white skins have been found most suitable for the tests.

It was found that when the largest inoculum of culture (0.1 cc.) was injected alone, there was induced a hyperemic edematous lesion 20 to 30 mm. in diameter, with a small central area of necrosis. This inflammatory reaction lasted for 4 to 6 days, and one large palpable area of induration, or several smaller areas, persisted for about 10 days longer. Smaller amounts of the culture induced lesions of less severity and with less induration, but the lesions did not differ markedly in surface area unless the inocula were less than 0.0001 cc. of culture. Amounts less than 0.0001 cc. sometimes induced lesions as large as 15

mm. in diameter, but these rapidly diminished and rarely exceeded 10 mm. at the end of 72 hours, and no palpable areas of induration persisted.

When the serum was injected together with the culture, it was found that 0.001 cc. of culture was the largest dose that could be completely neutralized by the addition of the immune serum, no matter how large an amount of serum was employed. In other words, if doses larger than 0.001 cc. of culture are used, there is apparently sufficient preformed substance present to induce lesions, even though the growth of the bacteria may be completely inhibited. It therefore became obvious that 0.001 cc. was the largest dose of culture which could be used for carrying out a series of tests with different sera. And since this amount was at least ten times greater than an amount (0.0001 cc.) which could produce a persistent lesion, it was decided that 0.001 cc. of culture would be the most satisfactory dose to employ. It was arbitrarily decided that a reaction was to be considered negative if at no time it exceeded 10 mm. in diameter and had completely disappeared within 72 hours.

When the horse was bled at the different intervals, tests were made with various dilutions of the serum, employing 0.001 cc. as a constant standard dose of culture. Control tests were also made with mixtures of culture and normal serum, and with the culture alone. The results of three of these tests are given in Table IV.

The lesions are described by means of linear measurements, but this method of expressing the difference in the lesions is inadequate, as it does not indicate differences in degree of hyperemia and edema.

It will be seen that whereas the administration of normal serum had no effect in reducing the size of the lesions resulting from the intradermal injection of 0.001 cc. of the standard culture, the addition of immune serum had a marked effect on the lesions. Moreover, distinct differences in the effects produced by sera from various bleedings were evident. Employing the standards mentioned above, it is seen that while 1/200 cc. of serum of Lot 1 was necessary to render the skin reaction negative, only 1/600 cc. of serum of Lot 2 was required, and 1/800 cc., or possibly less, of Lot 3.

It is obvious, therefore, that by means of this technique the protective action of immune serum may be demonstrated, and, moreover,

that quantitative differences in the relative protective action of different lots of serum may be detected. Lot 1 serum was obtained 4 months after the immunization of the horse was undertaken, Lot 2 serum 3 months later, and Lot 3, 3 months after Lot 2. The results indicate, therefore, that with the continued immunization there occurred a progressive increase in the power of the serum to prevent

TABLE IV

Influence of Immune Serum in Preventing Lesions in the Skin of Rabbits

Rabbit No.	Readings	Serum	Area of lesion induced by: Serum plus 0.001 cc. of culture: 1/10 cc.	1/100 cc.	1/200 cc.	1/400 cc.	1/600 cc.	1/800 cc.	1/1000 cc.	Culture alone: 0.001 cc.	0.0001 cc.	0.00001 cc.
	hrs.		*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*
1	24	Immune		10x10	4x7	14x11	13x12			25x22	18x16	
	72	Lot 1		—	—	10x10	12x12			22x21	10x10	
2	24	Lot 2		8x8	9x9	7x7	8x8	10x12		18x16	10x11	
	72			—	—	—	—	8x10		19x17	10x10	
3	24	Lot 3	—	3x3	—	—	2x2	7x7	10x11	*	15x15	12x12
	72		—	—	—	—	—	—	—		11x12	7x8
	24	Normal	20x15	15x20	15x13							
	72		20x15	15x15	13x11							

Total volume of each inoculum = 0.2 cc.

The sera alone caused no reactions.

Protective titre of immune serum: Lot 1 = 1–200; Lot 2 = 1–600; Lot 3 = 1–800+.

* Material was lost.

the occurrence of skin lesions following the injection of a definite number of Type b influenza bacilli.

The immune serum was also tested with other Type b strains, and the results were the same as when the standard Strain 225S was employed. That the action of the serum was type-specific was demonstrated by observing the lesions induced by the injection of mixtures of serum and living and heat-killed organisms of the other types and R

forms. Under these conditions, no preventive action of the serum could be detected. However, the serum used in these cross-protection tests was from the second bleeding, which was made before the appearance of the antibody which seems to be similar to the C antibody, and it is not known what influence this antibody might have on heterologous type skin infections.

The Action of Immune Serum in Patients Suffering from Meningitis

The experimental studies have indicated that the serum of a horse immunized against Type b *H. influenzae* exerts a specific action on these organisms, not only in the test-tube, but also in infected animals. It was decided to study the therapeutic action of this serum in a small series of patients suffering from meningitis due to *H. influenzae,* Type b. As cases of this disease occur mostly in young children and have not been available in this hospital, the cooperation of certain physicians likely to meet with cases of this disease has been enlisted. The writer desires here to express thanks to the physicians who have kindly supplied the histories of the patients.[2]

Eighteen patients have now been treated by means of intrathecal injections of immune horse serum supplied by us. Owing to difficulties in making prompt diagnosis of the bacteria concerned, and lack of opportunities for careful study of the cultures, certain of the cases cannot justifiably be included in discussing the action of the serum. In three cases, the influenza bacilli isolated proved to be not of the Type b form. In one of these cases, the organisms were found to be of Type a, in another of Type f, and in the third case the organisms which grew from the spinal fluid obtained on four occasions early, as well as late in the disease, produced only R colonies. Inasmuch as the serum is considered to be type-specific, no action was expected in patients

[2] The author is gratefully indebted to Dr. Rustin McIntosh, Babies Hospital, New York; Dr. J. D. Trask, Jr., New Haven Hospital, New Haven; Dr. Elizabeth R. Brackett, Babies Hospital, Newark; Dr. C. L. Wilson, Jersey City Hospital, Jersey City; Dr. M. H. Genkins, Montgomery Hospital, Norristown; Dr. I. Cohen, Beth-El Hospital, Brooklyn; Dr. Fred Schwartzberg, Hospital for Joint Diseases, New York; Dr. John J. Byrne, St. Mary's Hospital, Philadelphia; Dr. E. L. Noone, Delaware County Hospital, Drexel Hill; Dr. Joseph C. Regan, St. Catherine's Hospital, Brooklyn; Dr. B. Kramer, Jewish Hospital, Brooklyn.

suffering from infections due to heterologous organisms. Nevertheless, in one of these cases, following treatment with the immune serum, the patient apparently recovered from an infection with *H. influenzae* of a heterologous type.

This case, W. B., treated at the Jersey City Hospital, was not typically one of primary meningitis in a child, but was a case of secondary meningitis following traumatic fracture of the skull and laceration of the face occurring in a man 51 years of age. 6 days after the injury he developed signs of meningitis, and 4 days later cultures from the cloudy spinal fluid revealed the presence of *H. influenzae* Type a. The physician was anxious to employ treatment with immune serum, and intraspinal injections of 20 cc. each were made once or twice daily over a period of 19 days. 7 days after the treatment was begun, cultures from the spinal fluid became sterile and remained so. During the period of treatment marked improvement in the patient's physical and mental condition occurred, but about 3 weeks after the treatment was stopped his condition again became worse and he died 1 week later. At autopsy, there was no evidence of meningitis, but a brain abscess was found, cultures from which showed the presence of streptococci and staphylococci, but no influenza bacilli.

Whether or not the serum had any effect in this case is uncertain. Influenza bacillus meningitis rarely occurs in adults, and it is less frequently fatal than in children. Its occurrence in adults has been reviewed by Wollstein (18), Rivers (19), Bloom (20), and others.

The other two cases of this group, the one in which Type f organisms were found, and the one in which the organisms were all of the R form, ended fatally, and there was little evidence that the administration of the serum had any effect on the course of the disease.

There were two further cases in which a thorough study was not made of the infecting organisms. The determination of type was made solely on the agglutination reaction in the immune horse serum, and, in the light of further experience, the conclusion that the organisms were of Type b must be considered doubtful.

There remain thirteen cases in which the infecting organisms were shown conclusively to be of Type b. Unfortunately these patients were treated under widely varying conditions, and by different methods. The ages of these patients ranged between 2 months and 7 years, nine of them being 3 years, or younger. Among these thirteen cases, there was only one in which recovery occurred.

Case I.—This patient, M. P., 2 years of age, was admitted to the Jewish Hospital of Brooklyn on Apr. 24, 1933. There had been a discharge from the ear for 4 weeks. For 3 days the child had had signs of muscular incoordination, as he was reported to have fallen several times. The night before admission he vomited and had a chill, which was followed by a rise in temperature. The following morning he seemed well, but in the evening his temperature again rose and he appeared very limp, drowsy, and listless. The next day he was admitted to the hospital. A diagnosis of meningitis was made, and he was treated with meningococcus antiserum. On the 2nd day, the organisms which grew in the culture from the spinal fluid, and also in the culture from the blood, were identified as influenza bacilli, and treatment with specific immune serum was started. On this day he was given two intravenous and two intralumbar injections of immune serum, all of 20 cc. each. On each of the 4 succeeding days he was given two intralumbar treatments of 20 cc. of immune serum to which 2 cc. of fresh human serum had been added, on each of the next 2 days one treatment of 10 cc. of immune serum plus 2 cc. of fresh serum, and on the following day one intralumbar injection of 10 cc. of immune serum alone. On the 2nd day of specific treatment he was also given an intravenous injection of 10 cc. of immune serum. After the first 24 hours of treatment, cultures from the spinal fluid were sterile. This was after two positive cultures had been obtained from the spinal fluid, and before any fresh serum had been injected. The blood culture was positive on admission, and no further cultures were made from the blood until 1 week later, at which time no growth occurred. During the period of specific treatment, the number of leucocytes in the spinal fluid gradually decreased from 8900 to 460 per c.mm., and 10 days later the cell count was 5. Clinically, the child progressively improved, the meningitic symptoms gradually disappearing. The temperature slowly dropped, and after May 2 remained normal. The patient, apparently well, was discharged May 13.

Shortly after admission to the hospital, nose and throat cultures were made from which Type b influenza bacilli were grown. 10 days after discharge, cultures were again made from the nose and throat, and from the nose culture Type b organisms were again recovered. The latest cultures were made on June 29 from excised tonsils and adenoids, and on the plates a few colonies of Type b organisms and many colonies of R forms developed.

In two of the thirteen cases, the spinal fluid became sterile following the administration of immune serum, and remained so for periods of 7 to 14 days, but in both instances the organisms reappeared and death ensued. Brief reports of these cases follow.

Case II.—R. S., 2½ years old, was admitted to the Babies Hospital, New York City, Mar. 8, 1932. A diagnosis of influenzal meningitis was made, and the child was treated intraspinally with immune serum to which fresh human serum was

added. For 4 days the number of influenza bacilli in the spinal fluid diminished, on the 5th day no growth was obtained, and for the next 6 days the cultures were sterile. During this time the serum treatment was continued and the child improved clinically. However, the child then developed otitis media, streptococci were recovered from the discharge, and influenza bacilli again appeared in cultures from the spinal fluid. After this, cultures from the spinal fluid were again sterile for several days, but the influenza bacilli reappeared once more and were present in all subsequent cultures until death, which occurred on Apr. 9.

Case III.—J. B., 3½ years old, was admitted to Beth-El Hospital, Brooklyn, Dec. 5, 1932. The child had been ill for 4 days, and for 3 days meningitic symptoms had been present. On admission, a lumbar puncture was made, a cloudy fluid was withdrawn, and the child was treated with meningococcus antiserum. But, on examination of a smear of the spinal fluid, a diagnosis of influenzal meningitis was made, and later that day the child was treated with specific immune serum. For the next 7 days the child was given two daily intralumbar injections of serum, of approximately 20 cc. each. Positive cultures of *H. influenzae* were obtained from the spinal fluid daily for 4 days after the beginning of treatment, then the cultures became negative (Dec. 10) and remained so until Dec. 24. On Dec. 12 the spinal fluid was clear, the cell count 100, and the sugar content normal, but as the temperature continued elevated a block was suspected. A cisternal puncture was made and fluid of the same appearance as that obtained from the lumbar tap was withdrawn. This fluid was replaced by serum. Cultures made from this fluid were sterile. From Dec. 12 to 22 the child was given one daily intralumbar injection of immune serum, 20 cc. on each occasion. During this time the child showed improvement and ate and slept well. The strabismus which had been present disappeared, but double otitis media developed and both drums were punctured on Dec. 18. On Dec. 24 the child's condition became much worse. Bilateral mastoiditis developed, and it was found that the spinal fluid had again become positive for influenza bacilli. 4 days later both mastoids were opened. The serum treatment was continued after the operation, but the child progressively grew worse and died Jan. 1, 1933. No fresh serum was used in the treatment of this case.

In another case, there was a reduction in the number of bacteria in the spinal fluid following intraspinal treatment, and on one occasion no organisms were recovered either from slant or broth cultures.

Case IV.—R. F., 5 years of age. The onset of this child's illness was very sudden, and she was admitted to the New Haven Hospital Nov. 25, 1931, on the 1st day of illness. A diagnosis of meningitis was made, and she was given an immediate intraspinal treatment with meningococcus antiserum. An examination of a smear of the spinal fluid, however, showed the presence of Gram-negative bacilli, and treatment with *H. influenzae* antiserum was begun. The next day the

child was given two intraspinal treatments, the 5 succeeding days one intraspinal or intracisternal treatment daily, and the 8th day two intraspinal treatments. No definite clinical improvement was noted, however, and the serum treatment was discontinued. The child died on the 26th day. On admission, the blood culture was positive, but on the 3rd day after treatment, and also on three subsequent occasions, it was negative, the last negative culture being obtained 10 days after the cessation of serum treatment. However, 4 days later a positive blood culture was again obtained, and death occurred the 4th day following. The spinal fluid cultures following the administration of the serum showed a reduction in the number of bacteria present, and on one occasion, after three intraspinal treatments, the cultures were sterile. But on all subsequent occasions the spinal fluid cultures were positive.

In each of three other cases (Cases V, VI, and VII) in which treatment was commenced on the 7th, 5th, and 2nd days of illness, respectively, there occurred a temporary decrease in the number of bacteria in the spinal fluid following the treatment with immune serum. Later, the organisms again became numerous in the spinal fluid and remained so until death. In Cases V and VII, the cultures from the blood, which were positive before treatment was commenced, later became sterile.

In Case V, two positive blood cultures were obtained on 2 successive days. 2 hours after the patient had received 20 cc. of immune serum intraspinally, and 20 cc. intravenously, the cultures from the blood were sterile. Blood cultures were also sterile 2 days later, but on the following day, 24 hours after serum treatment had been discontinued, the blood cultures were again positive. The patient died 2 days later.

In Case VII, 2 days after a total amount of 60 cc. of influenzal antiserum plus 15 cc. of fresh human serum had been given intraspinally, and 120 cc. of influenzal antiserum intravenously, the blood culture was negative. Blood cultures on the following day were also negative. The child lived 2 days longer, during which time the specific treatment was continued, but no reports of further blood cultures were made.

Among the remaining six cases, there occurred no significant changes in the condition of the patients or in the character of the spinal fluid following the administration of immune serum. In two of these only intracisternal treatments were given on account of inability to withdraw fluid by the lumbar route, and in one, two intraspinal injections were made, after which the serum treatment was discontinued because of inability to withdraw spinal fluid by this route.

It is realized that the results obtained in the treatment of these thirteen cases do not indicate that this form of specific therapy, carried out under the given conditions, was of great practical value. It must be borne in mind, however, that influenzal meningitis is a very serious condition, and that without specific treatment almost all of those afflicted die. Moreover among the cases here reported two were complicated by pneumonia and empyema. Six were treated very late in the course of the meningitis, and in certain instances treatment was carried out only over short periods of time. It is possible that in a group of cases treated earlier in the course of the disease with greater intensity, and over prolonged periods, the results might be better.

The most important evidence presented by this study, indicating that the administration of the serum may exert an influence on the course of the disease, is given by the results of cultures from the blood and spinal fluid before and after the administration of serum. In seven of the cases, influenza bacilli grew in cultures from the blood before specific serum treatment was administered. In two of these cases no further reports were obtained on the course of the blood infection. In one case, the patient was suffering also from pneumonia, and the septicemia was uninfluenced by the administration of serum. In the remaining four cases with positive blood cultures, the blood became sterile after treatment with serum; in one instance (Case I) the patient recovered, in another (Case VII) there was no report of a recurrence of blood invasion, while in two cases (Cases IV and V), in which treatment was discontinued, the blood cultures again became positive before death. The total number of cases is small, but it seems not unlikely that, in the four cases mentioned, the administration of immune serum had the effect of at least temporarily sterilizing the blood.

The results of the intrathecal injection of immune serum on the bacteria in the spinal fluid varied, but in certain instances, at least, the serum seemed to have a definite effect. In one case (Case I) the bacteria disappeared from the spinal fluid following treatment, and recovery occurred. In two cases (Cases II and III) the spinal fluid became sterile and remained sterile for 7 and 14 days, respectively. In one case (Case IV) there occurred a reduction in the number of bacteria, and on one occasion the culture was sterile. In three other cases (Cases V, VI, and VII) there occurred a reduction in the number of bacteria, as shown by smears and cultures. In the remaining six

cases, no changes in the number of bacteria in the spinal fluid were noted.

Recently, Ward and Fothergill (6), and Ward and Wright (5), have reported concerning the treatment of eight cases of influenzal meningitis with an immune serum produced by immunization of a horse with meningitic strains of *H. influenzae.* In one case recovery occurred, and in five, after treatment, the cultures of the spinal fluid were sterile for periods of from 1 to 14 days. In all these five cases the bacteria later appeared in the cultures and death ensued. In the treatment of these cases fresh human serum was added to the immune serum. The previous experimental studies of these writers had led them to the conclusion that the action of *H. influenzae* antiserum is bactericidal, brought about by the action of antibody and complement upon the antigen. As they had been unable to demonstrate the presence of complement in the spinal fluid of patients suffering from influenzal menigitis, they considered that complement should be added to the immune serum before making intrathecal injections.

In the treatment of the patients reported in the present paper, in certain cases small amounts of fresh serum were mixed with the immune serum, and in other cases the immune serum was given alone. Our data do not permit definite conclusions to be drawn concerning the importance of the addition of fresh serum. In the one case (Case I) which recovered, however, the spinal fluid cultures became sterile after the injection of the immune serum without the addition of fresh serum, and in another instance (Case III) the spinal fluid cultures became sterile and remained so for a period of 14 days following the administration of immune serum alone. In Case IV, in which a reduction in the number of organisms occurred, and in which the fluid was sterile on one occasion, no fresh serum was added to the immune serum. In three other cases in which there resulted temporary sterilization of the spinal fluid or a reduction in the number of bacteria present, fresh serum was added to the immune serum.

SUMMARY

In this communication, further evidence has been given which supports the view that the majority of the strains of *Hemophilus influen-*

zae giving rise to meningitis are of the same serological type. Forty strains have now been examined, and thirty-seven have been of Type b.

A horse has been artificially immunized with Type b strains isolated from the spinal fluid of patients. By precipitation tests with the capsular carbohydrate, the serum has been shown to be highly type-specific. For the first $3\frac{1}{2}$ months of immunization, the *type-specific* antibody content of the serum increased steadily. Later, in spite of continued immunization, there occurred no apparent increase.

By means of animal inoculations, it has been shown that the antiserum has an anti-infectious action. If mice, inoculated intraperitoneally with Type b organisms, were also given serum, the bacteria did not invade the blood, or did so to only a limited degree. But the recovery of the treated mice was found to be inconstant. In rabbits infected intravenously and later treated by the same route, the number of bacteria in the blood stream was quickly reduced and sterilization followed. In the experiments it was necessary that the dosage of the culture be not too large, as influenza bacilli contain a substance which, artificially introduced into mice and rabbits, gives rise to marked toxic reactions. This substance is apparently not neutralized by the antiserum. However, it was found that among the surviving animals, those treated with immune serum returned to the normal state more quickly than did the animals not so treated.

The anti-infectious action of the serum has further been demonstrated by a study of its effect on the lesions which follow inoculations of type-specific bacteria into the skin of rabbits. Again it was found that for any effect of the serum to be manifested it is necessary that the dosage of bacteria be limited, since if large numbers of bacteria are introduced into the skin the development of lesions cannot be completely inhibited, no matter how large doses of serum are employed. As the number of living S organisms which cannot be neutralized is roughly equivalent to the number of R or heat-killed bacteria which may produce a lesion, it seems that there is some preformed irritating substance in the bacterial cells which may give rise to lesions, even if the bacteria are killed or inhibited in their growth. In order to demonstrate the protective action of immune serum, therefore, it has been found necessary to employ a dosage of culture so small that if the bacteria are immediately killed, or their growth inhibited, no lesion

results. Employing immune serum under these conditions, it has been found that the ability of the serum to prevent the occurrence of skin lesions has progressively increased with continuing immunization of the horse.

A series of eighteen patients suffering from influenzal meningitis has been treated with Type b antiserum. Following the use of serum, recovery occurred in one patient of the series, and in two, although the patients ultimately died, the spinal fluid cultures became sterile and remained so for periods of 7 to 14 days. In four other cases, the spinal fluid cultures showed, temporarily, either no growth of bacteria, or a reduction of their number. Among five patients in whom septicemia was present before treatment, in four the blood cultures, after treatment with serum, became sterile.

The number of patients treated has been small, and the treatments were carried out under widely varying conditions. It is difficult, therefore, to draw conclusions regarding the actual value of this form of therapy, or the best methods of procedure. The clinical results, however, indicate, as do the experimental, that the serum has a definite anti-infectious action. The experience is too limited to permit final conclusions regarding the importance of the addition of fresh (complement-containing) serum to the immune serum. Further experience, under more accurately controlled conditions, may show that the serum has greater practical value in treatment than is shown by the mortality results in this series of cases.

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