FACTORS INVOLVED IN THE PRODUCTION OF IMMUNITY WITH PNEUMOCOCCUS VACCINE.

I. Active and Passive Immunity during the First Seven Days after Injection of Antigen.

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Inasmuch as pneumonia is a self-limited disease ending by crisis or lysis generally between the 7th and 10th days, 3 to 5 days are frequently afforded in which an attempt may be made to produce active immunity before the natural termination of the disease takes place. At the suggestion of Dr. A. R. Dochez, a study was therefore undertaken of the onset and rate of development of pneumococcus immunity. As a result of recent work in oxygen therapy, it appeared to the author (1) that the life of the pneumonia patient was at times prolonged by the inhalation of 40 to 50 per cent oxygen, a circumstance that would further increase the possibilities of pneumococcus vaccine in an individual case. Any procedure that would initiate an earlier activity of the immunity mechanism seems therefore to have therapeutic possibilities. We wish to report preliminary animal experiments that have a bearing on this problem.

Historical.

In reviewing the subject, we shall present only such work as is concerned with the demonstration of specific immunization to the pneumococcus, and shall therefore omit the clinical reports of pneumococcus vaccine in the treatment of pneumonia as being apart from the present purpose. The development of immunity after introduction of pneumococcus organism has been shown by a number of workers. A great variety of methods of preparation of the organism has been used, employing the intact cell and extracts or solutions of the cell. The onset of demonstrable immunity in the studies of different investigators has been generally

between the 5th and 14th day after injection. Deviations from these results will be taken up in the individual instances. As we are concerned particularly with the attempt to secure a vaccine that would be applicable to the treatment of pneumonia, we shall stress chiefly the development of immunity during the first 7 days after injection.

The first demonstration that the serum of animals injected with pneumococcus possessed immune properties was made in 1891 by Foa and Carbone (2), Emmerich and Fowitzky (3), and G. and F. Klemperer (4). Neufeld (5) (1902) employed the bacteria separated from broth by centrifugalization, believing that the substances formed in the medium during growth were harmful. Wadsworth (6) (1912) found that the immunity produced by whole cultures was greater than that obtained by the injection of the bacteria alone. He also employed filtrates from the culture material after precipitation with ammonium sulfate. In both instances active immunity was produced in the rabbit 8 to 13 days after the injection. In 1913 Dochez and Gillespie (7) published their classification of pneumococci which made possible more specific study of individual strains. Lister (8), employing a similar classification in South Africa, made an intensive study of prophylactic inoculation against pneumococcus infection in the rabbit and in man. He found the factor of dosage was of considerable importance; doses limited to hundreds of millions of pneumococci were of little value, whereas 1 to 10 billion organisms administered intravenously both in the rabbit and in man were effective in immunizing the subject.

After three intravenous injections at weekly intervals, the rabbit was able to withstand a lethal dose of virulent pneumococcus culture. 4 to 5 days after the first injection, the serum of the animal in a few instances showed evidence of agglutinins when 3 volumes of serum were used to 1 of culture. One African native who received an intravenous inoculation of 10 billion the first dose, 20 billion the second dose, and 40 billion the third dose, showed agglutinins (with 3 volumes of serum) 6 days subsequent to the first injection. The vaccine was prepared from 18 hour blood agar cultures.

When the vaccine was administered subcutaneously, using from 2 to 12 billion organisms, no agglutinins were demonstrated even after three injections. When 20 to 30 billion doses were administered, agglutinins appeared 7 days after the second injection. In a later article, Lister (9) took his culture from human blood agar slants, inoculating it into trypsinated bullock heart broth for 8 hours, and then transferring this culture into similarly prepared broth for an incubation period of 6 to 7 hours. The cocci were finally suspended in saline, heated to 60° for 1 hour, and phenol added to a concentration of 0.5 per cent. Other preparations of vaccine such as (1) the filtrate after passing broth cultures through Berkefeld filters, (2) the cocci separated from broth cultures by electrolytic methods, (3) the pneumococci from 40 hours incubation in glucose broth cultures he found relatively inert as immunizing agents.

Still other types of vaccine have been employed: sensitized bacteria (Levy and

Aoki (10)), bacteria subjected to freezing (Cole (11)), bile extracts of pneumococcus (Neufeld (12), Vetrano (13), Cole (11)), glycerol extracts (G. and F. Klemperer (4)), and cultures precipitated by alcohol and dissolved in water (Wadsworth (6)). In all these instances, the immunity was slight, according to Cole (11), and never as good as from the injection of heat-killed bacteria. He found that better immunity was established in the rabbit by small daily doses than by larger doses at weeky intervals, agglutination (++ with equal parts of serum and culture) appearing on the 11th day after the first injection. The serum also protected a mouse against 0.001 cc. of virulent pneumococcus culture.

Wadsworth (14) recently recorded quantitatively the degree of immunity which he obtained with a standardized vaccine made of meat infusion broth with 0.5 per cent glucose. After three subcutaneous injections of Type II vaccine at weekly intervals, there was slight active immunity after the last injection. Thus, of three mice injected with 0.00001 cc. culture, one died, and of three injected with 0.0001 cc. culture, two died.

Larson and Nelson (15) reported that the addition of sodium ricinoleate to a virulent culture of pneumococcus so that the final dilution of the soap is 0.1 per cent caused the organisms to lose their pathogenicity. When 10 cc. of such a culture were injected intravenously into a rabbit, large amounts of agglutinins were present in the blood stream 24 hours after. Cecil (16) has produced active immunity in monkeys after three subcutaneous injections of Pneumococcus Types I and II and to a less extent in Type III. After a single large subcutaneous injection in man of 8 billion each of the three types, agglutination (+ in 1–1 dilution) was observed in some instances 7 days after. The vaccine was made by growing the organisms in 0.5 per cent glucose broth for 12 to 14 hours. Mackenzie (17) found that an intraperitoneal injection of 0.25 cc. of a heat-killed 18 hour culture produced active and passive immunity in guinea pigs 6 days after the injection although no agglutinins were demonstrable. Perlzweig (18) noted active immunity 10 days after a single subcutaneous injection of Pneumococcus Type I in mice. He also immunized mice actively with the protein fraction obtained by treating pneumococci with anhydrous sodium sulfate and by solution of pneumococci in bile salts and precipitation with alcohol. Ferry and Fisher (19) found that the centrifugate from broth cultures or saline suspensions of pneumococcus were more effective as an immunizing agent than the sedimented bacteria. White mice who were given two doses of 0.5 cc. of these extracts subcutaneously at weekly intervals were immune against 0.001 cc. of culture 1 week after the last injection. Wright (20) found that immunized rabbits cleared the blood of injected virulent pneumococci more effectively than normal rabbits. This increased resistance to septicemia was observed as early as the 3rd day following a single intravenous injection of heat-killed bacteria. Armstrong (21) has reported passive immunity in rabbits and active immunity in mice both beginning on the 4th day (occasionally on the 3rd day) and increasing to the 6th day after injection of killed pneumococcus vaccine.

In 1917 Dochez and Avery (22) pointed out that the pneumococcus elaborates a soluble specific substance which is found in the broth culture of the organism and frequently in the body fluids of the host as well. Recently, Avery and Heidelberger (23) have separated the constituents of the pneumococcus cell into two chemically and immunologically distinct substances, one protein and the other carbohydrate. The protein fraction is less specific than the intact bacterial cell, creating antibodies for the protein common to all groups of pneumococci when injected into animals. The carbohydrate, which has been shown to be of polysaccharide nature, is highly and specifically reactive only with the antibacterial serum of the same type of pneumococcus as that from which the substance is derived. However, when dissociated from combination with other cell elements, it is incapable of inducing antibody formation. They state that solutions and extracts of pneumococci behave as solutions of pneumococcus protein, *i.e.*, produce antibodies reactive only with the protein. Sera prepared from filtered solutions of disintegrated cells free from formed elements fail to exhibit any of the dominant type-specific properties which characterize sera obtained by immunization with whole bacteria. Schiemann and Casper (24) very recently reported that a specific soluble substance which they made employing a technique similar to Avery and Heidelberger was antigenic.

The literature recited above presents considerable divergence of opinion in regard to various problems in pneumococcus immunity. Our experiments have mainly emphasized two factors: (1) the onset and rate of development of pneumococcus immunity, (2) the character of immunity produced by the use of the intact organism as compared with that produced by a solution or extract from the bacterial cell.

Methods.

Numerous experiments have been conducted in the effort to prepare an antigen which would be particularly effective in producing early immunity. The detail of these findings will be considered at a later date. A fact originally mentioned by Neufeldt was suggested by our experience, namely, that the highly virulent organism provokes a more marked immunity than an organism of low virulence. We have attempted to prepare our antigen from a pneumococcus culture that was fatal to a mouse in 10–7 dilution, and preferably passed through a mouse immediately before using.

Human serum was employed in a concentration of 2.5 per cent in beef infusion broth in order to preserve virulence to the last stage and in order to augment

growth. The addition of glucose for stimulating growth did not appear to be satisfactory because of the readiness with which the organism was altered by growth in the culture medium. Young cultures were employed also with the idea of preventing autolysis. The incubation period was varied between 6 and 11 hours without appreciably influencing this factor.

Four different antigens were prepared in the following manner: A virulent Pneumococcus Type II (or Type I) culture was passed through a mouse in a preparation of the vaccine. 0.2 cc. of the heart's blood obtained from the animal immediately after death was inoculated into a test-tube containing 5 cc. of beef infusion broth with 2.5 per cent of human serum. After 8 hours incubation, this was used to inoculate flasks containing 250 cc. of similarly prepared broth, in the proportion of 0.1 cc. inoculum to 5 cc. broth. Incubation was carried on for 8 to 11 hours as was desired. The culture was then centrifuged, the supernatant broth poured off and saved. Care was taken not to loosen the sedimented bacteria at the bottom of the centrifuge tube which was now carefully rinsed with distilled water to wash off all broth adhering to it. Distilled water was finally added to dilute the bacteria to a concentration of 1 billion organisms to 1 cc. of water. (A nephelometer was used and checked by the Wright counting method.) The vaccine was sterilized by exposure to 60° for 1 hour. After it had cooled, tricresol was added to a concentration of 0.3 per cent. This is called in the text the 8 or the 11 hour serum vaccine.

The supernatant broth saved from the original culture was passed through a Berkefeld candle, bottled, and placed in the ice box. The preservative was added after culture had demonstrated no organisms had passed through the filter. This is called the broth filtrate.

A second lot of serum vaccine before sterilizing is shaken by hand for 5 minutes and centrifuged. The supernatant fluid is passed through a Berkefeld filter, bottled, and placed in the ice box. If sterile on culture, the preservative is added. This is called the filtrate of the shaken bacteria. The sediment remaining from the above is taken up in distilled water, heated for 1 hour at 60°, and allowed to cool before the addition of tricresol. This is called the washed bacteria vaccine.

Experiments.

Experiment 1.—A series of white mice were injected intraperitoneally with three different antigens derived from Pneumococcus Type II: (1) 11 hour serum vaccine, (2) Berkefeld filtrate of shaken bacteria, (3) washed bacteria vaccine. Each antigen was injected into five mice daily for 5 days. The dose of the serum vaccine and the washed bacteria vaccine was 0.2 cc., representing 200 million organisms. The dose of the filtrate was 0.4 cc., derived from 400 million organisms. At the end of the 5th day, all mice received an intraperitoneal injection of Type II culture in varying dilutions, as shown in Table I. The test culture was fatal to a mouse in 10–7 dilution.

TABLE I.

Active Immunity in Mice after Intraperitoneal Injection of (1) 11 Hour Serum Vaccine, (2) Berkefeld Filtrate of Shaken Bacteria, (3) Washed Bacteria Vaccine.

Antigen used and dose	Day after vaccination	Survival after Pneumococcus II injection of culture			Remarks
		.0001	.00001	.000001	
Vaccine 0.2 cc.	1st	24	28	30	All injections were administered intraperitoneally; antigen and test culture
		24			
	2nd	36	36	36	
			36	S	
	3rd	42	S	S	The surviving mice were subsequently injected with virulent Type I culture in corresponding doses. All died (within the same period that control mice died)
			42	S	
	4th	60	S	60	
			S	S	
	5th	S	S	68	
			S	S	
Filtrate of bacteria 0.4 cc.	1st	24	28	S	All antigens were prepared from an 11 hr. Pneumococcus Type II culture
			28	30	
	2nd		40	40	
			40		
	3rd	42	40	40	
			40	43	
	4th	S	S	S	
			S	S	
	5th		S	S	
		S	S	68	
Washed bacteria 0.2 cc.	1st	24	28	30	
			28	S	
	2nd	36	40	40	
			40	40	
	3rd	42	40	40	
			40	43	
	4th	S	62	62	
			S	S	
	5th	S	S	S	

Controls	.01	.001	.0001	.00001	.000001	.0000001
	15	15	25	35	68	85

As noted in Table I no protection was evidenced in any of the vaccinated animals on the 1st and 2nd days. Immunity began on the 3rd day in the case of the animals who received the serum vaccine and on the 4th day in those who received the washed bacteria vaccine and the filtrate of the shaken bacteria. The immunity was further increased on the 5th day. The mice who survived from this experiment were injected with a virulent Type I culture in corresponding doses to the Type II culture. All died.

Active immunity is therefore demonstrated on the 3rd day after injection of the serum vaccine, and on the 4th day in the case of the

TABLE II.

Active Immunity in Mice after Injection of 8 Hour Serum Vaccine.

Antigen used and dose	Day after vaccination	Survival after injection of Pneumococcus II culture				Remarks
		.001	.00001	.000001	.0000001	
Pneumococcus II 8 hr. serum vaccine 400 million organisms	1st	40	40	60	60	Vaccine and test culture administered intraperitoneally
	2nd	60	40	40	24	
	3rd		S	S	90	
	4th		S	S	S	
	5th		S	S	S	
	6th	40	S	S	21	
	7th	S	40	S	S	
Controls		40	21	40	96	

washed bacteria vaccine and the filtrate of the shaken bacteria. It is progressive in degree and specific for type. The failure of any of the vaccinated animals to survive an injection of Type I culture indicates that the protection against Type II culture was not due to increase in general resistance but rather to type-specific immunity. This was produced both by the use of the intact cell and by the water-soluble specific substance shaken off the bacteria.

Experiment 2.—(Table II.) An 8 hour serum vaccine was injected daily for 7 days into a series of white mice, four mice to each day. The dose was 0.4 cc., representing 400 million organisms, administered intraperitoneally. The test culture was of moderate virulence, 10–6 cc. being fatal to a mouse.

As shown in Table II, immunity is present from the 3rd to the 7th days inclusive.

Experiment 3.—(Table III.) An 8 hour serum vaccine was injected daily for 5 days into a series of white mice, five mice to each day. The dose was 0.2 cc.,

TABLE III.

Active Immunity in Mice after Intraperitoneal Injection of 8 Hour Serum Vaccine. Test Culture of Low Virulence.

Antigen used and dose	Day after vaccination	Survival after injection of Pneumococcus II culture					Remarks
		.01	.001	.0001	.00001	.000001	
Pneumococcus II 8 hr. serum vaccine 100 million organisms	2nd	S	S	S	S	S	Pneumococcus culture of relatively low virulence, 10–6 surviving and 10–5 fatal in 130 hrs.
	3rd	S	S	S	S	S	
	4th	S	S	S	S	S	
	5th	S	S	S	S	S	
Controls.....		36	22	36	130	S	

TABLE IV.

Active Immunity in Mice after Intraperitoneal Injection of 8 Hour Serum Vaccine. Test Culture of High Virulence.

Antigen used and dose	Day after vaccination	Survival after injection of Pneumococcus II culture				Remarks
		.001	.0001	.00001	.000001	
Pneumococcus II 8 hr. serum vaccine 200 million organisms	1st	14	44	44	44	Pneumococcus culture at maximum virulence, fatal in 10–7 cc.
	2nd	20	20	20	44	
	3rd	44	44	20	44	
	4th	20	53	S	S	
	5th	53	S	S	20	
Controls.....		14	14	20	20	

representing 200 million organisms. The test culture was of low virulence, 10–5 cc. being fatal in 130 hours. Immunity was demonstrated from the 2nd to the 5th day inclusive.

Experiment 4.—(Table IV.) A similar experiment to the above was performed, employing however, a highly virulent test culture, 10–7 cc. being fatal to a mouse. Immunity was obtained on the 4th and 5th days only.

TABLE V.

Passive Immunity in Rabbits after Intravenous Injection of (1) Serum Vaccine, (2) Berkefeld Filtrate of Shaken Bacteria, (3) Washed Bacteria Vaccine, (4) Berkefeld Filtrate of Broth Culture.

Antigen used and dose	Day after vaccination	Survival after injection of Pneumococcus II culture			Survival after injection of Pneumococcus I culture			Remarks
		.001	.0001	.000001	.001	.0001	.000001	
Rabbit 4 pneumococcus II 11 hr. serum vaccine 5 billion organisms	3rd	S	60	S	18	45	45	Mice employed in Pneumococcus I infections were used previously in Pneumococcus II experiments in which they survived injections of virulent Pneumococcus II culture All rabbit injections given intravenously
			S	S		45	45	
	5th	S	S	S	45	45		
			S	S				
	7th	S	S	S				
			S	S				
	9th	S	42	S				
			42	S				
	15th	S	S	S				
			S	S				
	20th	42	S	S				
			S	S				
Rabbit 7 pneumococcus II filtrate of shaken bacteria 8 cc.	3rd	42	42	42				Concentration of antigens—1 billion organisms to 1 cc. distilled water
			42	60				
	4th	42	42	42				
			60	48				
	5th	S	S	S	42	45	45	
			S	S		45	45	
	6th	S	S	S	45	45	45	
			S	S		45	45	
	8th	S	S	S				
			S	S				
	11th	S	S	S				
			S	42				
Rabbit 5 pneumococcus II washed bacteria vaccine 5 billion organisms	3rd	S	42	S	45	45	45	
			S	S		45	45	
	5th	S	S	S	45	45	45	
			S	S		45	45	
	7th	S	S	S				
			S	130				
	9th	S	S	S				
			S	S				

TABLE V—*Concluded.*

Antigen used and dose	Day after vaccination	Survival after injection of Pneumococcus II culture			Survival after injection of Pneumococcus I culture			Remarks
		.001	.0001	.000001	.001	.0001	.000001	
Rabbit 6 Pneumococcus II filtrate of broth culture 10 cc.	3rd	36	S	60				
			S	60				
	4th	36	42	36				
			60	S				
	5th	S	S	S				
			S	S				
	7th	S	S	S				
			S	S				
	15th	S	S	S				
			S	S				
	20th	S	S	S				
			110	S				
Rabbit serum	Before	42	42	42				
Controls Pneumococcus II			42	42				
Controls Pneumococcus II		24	24	24				
Controls Pneumococcus I		48	48	60				

The foregoing experiments indicate that the onset of active immunity alters with the virulence of the test culture, appearing on the 2nd day after vaccination when an organism of low virulence is used, on the 4th day with an organism of very high virulence, and on the 3rd day with an organism of moderately high virulence. Varying the virulence of the organism represents the progressive increase in active immunity from the 2nd to the 5th day better than varying the number of organisms injected, for a highly virulent culture may cause death of all the animals even in high dilutions and a culture of low virulence may result in complete survival. This is in accordance with the results of Neill and Gaspari (28) who found that within a certain range (10–6 to 10–3 cc. of culture) the number of invading bacteria was without influence in determining the occurrence of infection and fatality.

Experiment 5.—(Table V.) Four antigens were used in this experiment: (1) 11 hour serum vaccine, (2) filtrate of shaken bacteria, (3) washed bacteria vaccine, (4) filtrate of broth culture. The dose of the serum vaccine and the washed bacteria vaccine was 5 billion organisms, the filtrate of the broth and the shaken bacteria was in each case derived from 10 billion organisms. Each antigen was administered intravenously to a rabbit, and blood taken, before and from the 3rd to the 9th day or longer. The mouse protection test was carried out according to the technique of Dochez (25). Thus, 0.2 cc. of rabbit serum was mixed with

TABLE VI.

Passive Immunity in Rabbits after Injection of (1) Serum Vaccine and (2) Berkefeld Filtrate of Shaken Bacteria.

Antigen used and dose	Day after vaccination	Survival after injection of Pneumococcus II culture				Remarks
		.01	.001	.0001	.00001	
Rabbit 3 Pneumococcus II serum vaccine 2 billion organisms	1st	17	64	64	64	All antigens administered intravenously. Vaccine and filtrate made from 8 hr. serum broth culture
	2nd	30	30	30	64	
	3rd	30	S	S	S	
	4th	S	S	S	S	
	5th	50	30	S	S	
	6th	17	S	S	S	
	7th	S	S	S	S	
Rabbit 1 Pneumococcus II serum vaccine 2 billion organisms	3rd	64	64	S	S	
	4th	30	S	S	S	
	5th	46	S	S	S	
Rabbit 2 Pneumococcus II filtrate of shaken bacteria 2 cc.	4th	72	S	S	S	
	5th	S	S	S	S	
Controls.....		30	22	36	130	

varying dilutions of culture and injected intraperitoneally into mice. Survival in these as in the previous active immunity experiments is recorded when the mouse lives 6 days after injection. The blood drawn on the 3rd, 5th, and 6th days was also mixed with varying dilutions of Type I culture to determine whether the early immunity secured was a general non-specific increase in natural resistance or was entirely due to the development of Pneumococcus Type II specific protective substance. The test culture was of moderately high virulence, 10–6 cc. killing control animals as well as animals who received the serum of normal rabbits (*i.e.*, before their vaccination with pneumococcus antigen.)

As Table V indicates, the onset of passive immunity was on the 3rd day in the case of the serum vaccine and the washed bacteria vaccine. The broth filtrate and the filtrate of the shaken bacteria show a definite immunity on the 5th day. The immunity persists as long as observations in the experiments were conducted, from the 11th to the 20th day after injection. In a similar experiment noted in Table VI, the serum vaccine initiates passive protection on the 3rd day also, but the filtrate of the shaken bacteria produces an immune response on the 4th day. The rabbit sera of the animals whose blood protected against Type II culture failed to show any protection

TABLE VII.

Passive Immunity in Rabbit after Intravenous Injection of Serum Vaccine.

Antigen used and dose	Day after vaccination	Survival after injection of Pneumococcus II culture				Remarks
		.001	.000001	.0000001	.00000001	
Rabbit 1 Pneumococcus II 8 hr. serum vaccine 2 billion organisms	2nd	19	19	48	48	Virulence of pneumococcus culture was at its maximum, fatal in 10–7 cc.
	3rd	19	22	48	19	
	4th	19	48	48	49	
	5th	48	48	48	22	Mouse with immune horse serum was not protected against 10–6 culture.
	6th	19	48	72	72	
Immune serum		S	S	19	S	
Controls.....		22	48	48	72	

against Type I culture, demonstrating that the passive immunity produced was specific for type and not dependent upon increase of general resistance or on the common protein antibody.

Experiment 7.—(Table VII.) An 8 hour serum vaccine was injected intravenously to a rabbit, and blood drawn from the 2nd to the 6th day inclusive. The dose was 2 billion organisms. The test culture was at maximum virulence, 10–7 cc. being regularly fatal.

No animals survived in this experiment. The blood of rabbits varies to an extent in its power to elaborate protective substance, particularly in the case of a culture of maximum virulence.

Experiment 8.—(Table VIII.) A series of mice were inoculated intraperitoneally with an 11 hour (1) serum vaccine Type I pneumococcus and (2) filtrate of the shaken bacteria. The dose of the vaccine was 0.2 cc., representing 200 million organisms, and of the filtrate 0.4 cc., representing 400 million organisms. The test culture was of very high virulence, 10–7 cc. being fatal to a mouse.

Active immunity began on the 4th day in the animals vaccinated with the serum vaccine, and on the 5th day with the animals vaccinated with the filtrate of the shaken bacteria. This indicates again that the use of a test culture of very high virulence records the onset

TABLE VIII.

Active Immunity Following Intraperitoneal Injection of Type I Vaccine.

Antigen used and dose	Day after vaccination	Survival after injection of Pneumococcus I culture			Remarks
		.001	.0001	.000001	
Pneumococcus I 11 hr. serum vaccine 400 million organisms	1st	20	20	24	Vaccine and test culture administered intraperitoneally
			20	36	
	2nd	20	20	24	
		36	36	36	
	3rd	24	36	36	
			36	36	
	4th	36	S	S	
			S	48	
	5th	S	S	S	
			S	48	
Controls		20	24	36	
			20	24	

of immunity at a later date than one of lesser virulence, and thus registers the progressive increase in antibody response from the 3rd to the 5th day.

Experiment 9.—(Table IX.) In this experiment two antigens from Pneumococcus Type I were employed, (1) the Berkefeld filtrate of the shaken bacteria and (2) the filtrate of the broth culture. A comparison was made between the standard dose and a very large dose. A series of mice were inoculated intraperitoneally with 0.4 cc. of broth filtrate, a second series with 1.0 cc. of broth filtrate, a third series with 0.2 cc. of the filtrate from the shaken bacteria, and a fourth with 1.0 cc. of the filtrate of the shaken bacteria. Each antigen was injected daily into eight mice, and on the 7th day after the first injection, the test culture was administered to the

entire series, comprising mice injected 2 to 7 days after inoculation with antigen. The test culture was of high virulence, 10–7 being fatal to a mouse in 60 hours.

As will be seen in Table IX, all the mice injected on the 2nd day after vaccination died, 32 in number. Survival began on the 3rd day after injection in each series but usually in only one out of eight animals. In the case of the filtrate of the shaken bacteria, definite protection was present on the 4th day, increasing to its height on the

TABLE IX.

Effect of Variation in Dosage of Antigen on Active Immunity in Mice after Intraperitoneal Injection of (1) Berkefeld Filtrate of Shaken Bacteria, (2) Berkefeld Filtrate of Broth Culture.

Antigen used and dose	Day after vaccination	Survival after injection of Pneumococcus I culture						Remarks
		.01	.001	.0001	.00001	.000001	.0000001	
Broth filtrate 0.4 cc.	2nd	40	40	40	58			All injections administered intraperitoneally. Filtrates made from 11 hr. serum vaccine, Pneumococcus Type I.
		40	58	58	58			
	3rd	58	58	58	40			
		58	58	58	S			
	4th	18	40	58	58			
		58	58	58	S			
	5th	40	40	58	40			
		58	58	S	S			
	6th	40	40	58				
		40	80	S	S			
	7th	40	S	58	S			
		40	S	S	S			
Broth filtrate 1.0 cc.	2nd	40	40	40				
		40	58	58	58			
	3rd	40	40	40	40			
		40	40	S	58			
	4th	40	40	58	S			
		58	S	58	S			
	5th	40	40	40	S			
		58	40	58	80			
	6th	40	48	58	S			
		40	58					
	7th	40	58	S	S			
		40	S	S	S			

TABLE IX—*Concluded.*

Antigen used and dose	Day after vaccination	Survival after injection of Pneumococcus I culture						Remarks
		.01	.001	.0001	.00001	.000001	.0000001	
Bacterial filtrate 0.2 cc.	2nd	18	40	40	58			
		40	40	40	58			
	3rd	40	40	40	40			
		40	S	40				
	4th	40	40	40	S			
		40	40	S	S			
	5th	18	S	S	S			
		40	80	S	S			
	6th	40	58	80	S			
		S						
	7th	40	S	S	18			
		40	S		18			
Bacterial filtrate 1.0 cc.	2nd	40	40	40	58			
		40	40	58	58			
	3rd	40	40		S			
		40	40	40				
	4th	40	40					
		40	80	S	S			
	5th	40	18	S	S			
		40	S	S	S			
	6th	40	40	S	S			
		58	58					
	7th	40	58	40	S			
		40	58	58				
Controls.....		40	40	58	58	58	60	
		40	40		58			

5th day, and remaining approximately stationary to the 7th. In fact, in this experiment there was a slight falling off in survival from the 5th to the 7th day. The increased dose (1.0 cc. of filtrate) gave slightly inferior results than the standard dose (0.2 cc.).

The immunity resulting from the standard broth dose (0.4 cc.) was less marked than that from the filtrate of the shaken bacteria. Animals survived from the 3rd to the 6th day in small numbers and on the 7th day, five out of eight survived. The large dose of broth (1.0 cc.) gave a less marked immunity response but here too, five out of

TABLE X.

Effect of Variation in Dosage of Antigen on Active Immunity in Mice after Intraperitoneal Injection of Serum Vaccine.

Antigen used and dose	Day after vaccination	Survival after injection of Pneumococcus I culture			Remarks
		.001	.0001	.00001	
Serum vaccine dose 0.2 cc.	2nd	44	44	70	All injections administered intraperitoneally
		44	44	70	
		44	44	44	Vaccine grown for 8 hrs. in 2.5 per cent serum broth
	3rd	92	92	SS	
		SS	SS	S	Numbers in table refer to length of time in hrs. which mice lived after injection. S indicates survival for 6 days or more
	4th	SS	SS	SS	
		S	S	S	
	5th	SS	SS	SS	
		S	S	S	
Dose 0.02 cc.	2nd	44	44	44	
		44	44	44	
			44		
	3rd	44	44	44	
		44	44		
		S	S	SS	
	4th	SS	92	SS	
		S	SS	S	
	5th	92	SS	SS	
		SS	S	S	
Dose 0.002 cc.	2nd	44	44	44	
		44	44	44	
		44	44	44	
	3rd	SS	44	SS	
			92		
		S	S	S	
	4th	SS	SS	SS	
		S	S	S	
	5th	SS	SS	SS	
		S	44	S	
Dose 0.0002 cc.	2nd	44	44	44	
		44	44	44	
		44	44	44	
	3rd	44	SS	44	
		44		44	
		S	S	S	
	4th	SS	44	92	
		S	SS	SS	
	5th	44	70	92	
		92			
		S	SS	SS	

Normal controls.....	.001	.0001	.00001	.000001	.0000001	.00000001	.000000001
	44	44	44	44	44	44	44

eight survived on the 7th day. It is observed that in only one instance (1 of 192 mice), did an animal resist 0.01 cc. of test culture. On the 5th day, however, the filtrate of the shaken bacteria produced an immunity sufficient to withstand an injection of 0.001 cc. of a culture with a virulence of 10^{-7}

Experiment 10.—A series of mice were injected intraperitoneally with the following doses of Type I vaccine; (1) 0.2 cc., (2) 0.02 cc., (3) 0.002 cc., (4) 0.0002 cc.

TABLE XI.

Active Immunity after Intraperitoneal Injection of Pneumococcus Type I Culture to Which Sodium Ricinoleate Had Been Added.

Antigen used and dose	Day after vaccination	Survival after injection of Pneumococcus I culture			Remarks
		.001	.0001	.00001	
0.1 per cent sodium ricinoleate Pneumococcus Type I culture	1st	44	20	44	11 hr. Pneumococcus Type I broth culture exposed to a final concentration of 0.1 per cent sodium ricinoleate before injection
		44	44	44	
	2nd	44	44	44	
		44	44	44	
	3rd	44	20	44	
		44	44	84	
	4th	44	44	S	
		S	44	S	
Dose 0.4 cc.	6th	S	S	S	
		S	S	S	
	7th		44	44	
		S	S	S	
Dose 0.04 cc.	1st	20	20	20	
		20	20	20	
	4th	44	44	S	
		64	64	S	
Normal controls.....		.001	.0001	.00001	.000001 .0000001 .00000001
		44	44	44	44 44 44

1 cc. of the vaccine contained 5 billion organisms. Twelve mice were inoculated daily with each antigen, and on the 5th day a test culture of Pneumococcus Type I was injected intraperitoneally into the entire group. The virulence of the culture was such that 10^{-9} cc. was fatal to a control animal.

As seen in Table X all the 2nd day mice (48 in number) as well as all the control animals died. Survival began on the 3rd day and was

more complete on the 4th and 5th days. The immunity produced was fairly constant in the range of dosage between 0.2 cc. and 0.002 cc., but began to diminish when the dose of vaccine was lowered to 0.0002 cc. The degree of protection on the 3rd day was sufficient to protect against 100,000 minimal lethal doses. (When the maximal virulence of the organism 10^{-9} is used in the calculations, the protection is equivalent to 1 million minimal lethal doses.) The Type I organism produces a more marked early immunity than the Type II.

Experiment 11.—(Table XI.) The antigen used in this experiment was a virulent Type I culture exposed to a final concentration of 0.1 per cent sodium ricinoleate. Mice were inoculated with this culture intraperitoneally daily for 5 days, dose 0.2 cc. On the 5th day a virulent test culture was injected intraperitoneally.

Survival began on the 4th day, and was complete on the 6th. The use of sodium ricinoleate did not hasten the immunologic response. The total results were not as good as those which were produced by the use of the serum vaccine.

DISCUSSION.

The experiments reported in the present paper are concerned mainly with (1) the character of the pneumococcus antigen as a factor in the antibody response, (2) the onset and rate of development of immunity to the pneumococcus.

As reviewed in the early part of this paper, some observers have found the intact bacterial cell necessary for the development of type-specific immunity, whereas others have reported that solutions or extracts of the pneumococcus are adequate for this purpose. We have employed four antigens, two of them consisting of the intact cell, namely, the serum vaccine and the washed bacteria vaccine; and two of them containing water-soluble substances derived from the cell, namely, a Berkefeld filtrate of the shaken bacteria and a filtrate of the broth culture. The latter antigens are contained in a clear water solution free from formed elements.

Mice were actively immunized by a single intraperitoneal injection of each antigen. On the 5th day after the injection a high grade type-specific immunity was demonstrated in all. Survival of the mice to a virulent culture of the homologous organism was not deemed sufficient

to prove type-specific immunity, for it appeared possible that the increased resistance might be due to augmenting the natural defensive mechanisms or to the stimulation of the common protein antibody. Tillett (26) in this connection found that immunization of rabbits with Type I or Type II pneumococci, and with R forms derived from any of the fixed types, was equally effective in producing active immunity against Type III infection. All the mice, therefore, that survived injection of Type II culture after previous vaccination with Type II antigen were injected with virulent Type I culture. None survived. Wright (27) was also unable to demonstrate increased resistance to Type I pneumococci by previous injection with heterologous organisms.

After a single intravenous injection to rabbits of each of the four antigens described above, all sera on the 5th day showed type-specific protective substance. The sera which protected mice against Type II culture gave no protection against Type I culture. Thus, in both active and passive immunity experiments a Berkfeld filtrate containing a watery extract of the pneumococcus cell resulted in a type-specific antibody response.

The onset of definite type-specific immunity, both active and passive, appeared on the 3rd day after injection of the serum vaccine. It increased progressively to the 5th day and remained approximately unchanged to the 7th day or longer. The washed bacteria vaccine resulted in a similar slightly less marked response. The immunity resulting from the filtrate of the shaken bacteria and the broth filtrate began on the 4th day after injection, increased on the 5th, and remained stationery from the 5th to the 7th day. The degree of active immunity secured on the 5th day from Pneumococcus Type II vaccine was approximately that which protected a mouse from an intraperitoneal injection of 0.001 cc. of a culture with a virulence of 10–6 cc. In the case of Type I vaccine protection was obtained against 0.001 cc. culture with a virulence of 10–7 cc. The degree of antibody response cannot be measured accurately solely by recording survival after injection of varying doses of test culture, for as pointed out earlier in the paper and as observed by Neill and Gaspari, the incidence of infection between the range 10–3 and 10–6 is not wholly dependent upon dilution. We have considered as of almost equal

importance the total number of animals surviving of the entire group (10–3 to 10–6) irrespective of the dose administered. Lastly, varying the virulence of the organism was of especial help in determining the graded increase of early immunity. When active immunity was tested against a Pneumococcus Type II organism of moderately high virulence, (10–6), well marked specific immunity appeared on the 3rd day. With an organism of still higher virulence (10–7) an immune response was noted on the 4th day. In two instances, when a test culture of low virulence was employed, evidence of active immunity appeared on the 2nd day. (Test culture was fatal to a mouse, 10–5 cc. in 130 hours.) In the case of Type I pneumococcus vaccine, active immunity appears on the 3rd day even against a highly virulent 10–7 organism.

By evaluating these three criteria of resistance, we feel justified in stating that an antigen derived from the intact cell gives rise to a type-specific immunity both active and passive beginning definitely on the 3rd day, increasing to the 5th, and remaining approximately stationery to the 7th or longer. The antigen contained in the filtrate, free from formed elements, initiates a type-specific immunity on the 4th day, increases markedly to the 5th, and remains approximately stationery to the 7th day. The immunity produced by Pneumococcus Type I vaccine is greater than that produced by Type II. On the 3rd day, mice vaccinated with Type I vaccine resisted 100,000 minimal lethal doses, whereas mice immunized with Type II resisted 10,000 minimal lethal doses. On the 5th day, a larger percentage of mice survived these doses than on the 3rd day.

We are unable at this time to evaluate the factors which are responsible for the early induction of immunity to the pneumococcus. In the preparation of our vaccine we have employed highly virulent cultures, relatively short incubation periods, and the addition of human serum to the broth. The final vaccine was cleared of broth by carefully rinsing the centrifuge tube containing the sedimented bacteria instead of washing, since the washings contained considerable highly antigenic material. The average dose of vaccine administered to the rabbit was 2 to 5 billion organisms intravenously; to the mouse 200 to 400 million organisms intraperitoneally.

This study was undertaken with the object of determining whether

an active immunity to the pneumococcus could be established in a sufficiently short space of time as to make the injection of vaccine a therapeutic possibility in lobar pneumonia. As far as the time interval is concerned, our results support this hypothesis. Whether the patient with lobar pneumonia would react as experimental normal animals do raises a question not within the scope of this paper.

CONCLUSIONS.

1. The antigenic function of a pneumococcus vaccine made from the intact cell was compared with that derived fron a watery extract of the cell free from formed elements. In each instance, the immunity produced was dependent upon type-specific protective substance and not upon the elaboration of the common protein antibody.

2. The vaccine made from the intact cell resulted in both active and passive immunity which began on the 3rd day, increased markedly to the 5th, and remained approximately stationery to the 7th day. In the case of the Berkefeld filtrate of the shaken bacteria and the filtrate of the broth culture, the immunity began on the 4th day, increased to the 5th, and remained approximately stationery to the 7th day. The immunity produced by Pneumococcus Type I vaccine is greater than that produced by Type II. On the 3rd day, mice vaccinated with Type I vaccine resisted 100,000 minimal lethal doses, whereas mice immunized with Type II resisted 10,000 minimal lethal doses. On the 5th day, a larger percentage of mice survived these doses than on the 3rd day.

3. Certain factors related to the preparation and dosage of the vaccine are discussed.

4. As far as the time interval and the degree of immunity produced are concerned, these results suggest the possibility of employing pneumococcus vaccine in suitable doses in the treatment of lobar pneumonia. That an earlier activity of the immunity mechanism could actually be initiated in a patient with lobar pneumonia has still to be demonstrated.

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