

## CUTANEOUS REACTIONS IN RABBITS TO THE TYPE-SPECIFIC CAPSULAR POLYSACCHARIDES OF PNEUMOCOCCUS

By THOMAS FRANCIS, JR., M.D., AND WILLIAM S. TILLET, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

(Received for publication, July 7, 1931)

It has previously been shown (1) that, following the intradermal injection of the type-specific polysaccharides of *Pneumococcus*, there develops in the skin of patients convalescent from pneumonia an immediate wheal and erythema reaction to the polysaccharide homologous in type to that of the pneumococcus causing the infection. The reaction is type-specific and its occurrence is always associated with the presence in the patient's serum of specific antibodies for the homologous type of *Pneumococcus*.

Since the purified carbohydrates are capable of eliciting this striking response in the skin of human individuals who, upon recovery from lobar pneumonia become spontaneously immune, it seemed desirable to study the cutaneous reactivity of experimental animals which had been artificially immunized to type-specific pneumococci. The purpose of this paper, therefore, is to report the occurrence of a local cutaneous reaction to the type-specific polysaccharides in rabbits actively or passively immunized to the pneumococcus.

### *Materials and Methods*

#### *1. Immunization of Rabbits.—*

(a) *Active Immunization.*—Groups of rabbits were immunized with pneumococci of Types I, II or III. Other animals were immunized with a non-type-specific R strain of pneumococcus. Immunization was carried out by the method of Cole and Moore (2). 10 days after the final injection, blood was obtained from an ear vein and the serum tested for the presence of type-specific antibodies.

(b) *Passive Immunization.*—Antipneumococcus rabbit serum was obtained from animals immunized as above. Antipneumococcus horse serum for each of Types I, II and III was obtained from the New York State Board of Health. Rabbits were passively immunized by the intravenous injection of 5 to 15 cc. of

one or other of these sera. The following day blood was obtained from an ear vein for the determination of circulating type-specific antibodies.

2. *Determination of Type-Specific Antibodies.*—

(a) Type-specific antibodies were detected by the precipitin test; 0.2 cc. of serum diluted to 0.5 cc. with physiological saline was mixed with 0.5 cc. of varying dilutions of the type-specific polysaccharides. Readings were made after 2 hours in the water bath at 37°C. and overnight in the ice box.

(b) Type-specific agglutinins were determined by the usual method using dilutions of serum and suspensions of heat-killed organisms.

3. *Skin Testing Materials.*—Skin tests were made with the capsular polysaccharides of pneumococcus derived in purified form from *Pneumococcus* Types I, II and III. These substances are chemically and serologically distinct. They are protein-free. The Type II and Type II polysaccharides are also nitrogen-free; the Type I material contains 5 per cent non-protein nitrogen which is part of the sugar molecule (3). The polysaccharides were dissolved in physiological saline in a concentration of 1:200 and 0.1 cc. of this solution, containing 0.5 mg. of polysaccharide, was used for each injection. The animals were closely observed for the occurrence of an immediate or delayed reaction. Measurements were recorded 24 hours following the injection and daily thereafter.

In most instances the specific capsular polysaccharides of Types I, II and III *Pneumococcus* were injected simultaneously into neighboring sites in the rabbit's skin.

*Skin Reactions in Actively Immunized Rabbits*

Three series of rabbits were actively immunized with pneumococci of Types I, II and III, respectively. After immunization, injections of the type-specific polysaccharides into the skin were made.

Protocols of rabbits representative of the three different immune groups illustrate the conditions of experiment and characteristic results.

Rabbit 4-74 was actively immunized to Type I *Pneumococcus* by the intravenous route. 10 days after the final immunizing injection, the animal's serum contained specific precipitins for the Type I polysaccharide in a titer of 2,560,000. The following day injections of 0.5 mg. of each of Types I, II and III polysaccharides were made into the shaved skin of the side. 2½ hours later a few faint purpuric spots were observed at the site of injection of the Type I material. The lesion increased in size until at 24 hours a purpuric edematous area 2.5 cm. in diameter was noted. In 48 hours the diameter had reached 3.0 cm., the edema was less but the purpura had darkened and infiltration had increased. A gradual clearing took place over the next 4 days. At the sites of injection of the Type II and Type III materials no evidence of reaction was observed.

Rabbit 4-41 was actively immunized against Type II Pneumococcus. 10 days after immunization was completed the serum contained precipitins for the Type II polysaccharide in a titer of 1,280,000. The following day, injections of 0.5 mg. of each of the Type I, II and III polysaccharides were made into the skin of the side. 24 hours later, at the site of injection of the Type II polysaccharide, a firm edematous lesion, bright red in color, and measuring 4.5 cm. x 2.3 cm. in cross diameters was noted. No purpura was present. The lesion had practically cleared in 4 days. No reaction was elicited by the carbohydrates of Types I and III.

Rabbit 14-85 was immunized with a strain of Type III Pneumococcus which had, by repeated animal passage, acquired virulence for rabbits. On the 10th day after the last immunizing dose the serum contained precipitins for the Type III polysaccharide. With a 1:80,000 dilution of the polysaccharide a + + + + reac-

TABLE I

*Skin Reactions to Type-Specific Polysaccharides in Rabbits Actively Immunized with Pneumococci of Homologous Types*

Type of Pneumococcus used for immunization	Type-specific precipitins present		No type-specific precipitins present	
	No. of rabbits	No. giving skin reaction	No. of rabbits	No. giving skin reaction
I	11	10	4	0
II	9	8	3	0
III	5	3	8	0
R	—	—	5	0

tion was obtained. Higher dilutions were not tested. The following day 0.5 mg. of each of the polysaccharides of Types I, II and III was injected into the shaved skin of the side. After 24 hours at the site of injection of the Type III polysaccharide, there was noted a slightly elevated, firm, purpuric area 2.6 x 3.4 cm. in diameter, surrounded by a white zone of edema. The lesion gradually subsided until the 6th day when only a faint pigmentation remained. The reaction was type-specific; no reaction was produced by the Types I and II carbohydrates.

The foregoing protocols demonstrate clearly the form of reaction obtained and the conditions under which it was produced. Table I shows the frequency with which skin reactions were elicited by the intradermal injection of the homologous polysaccharide in rabbits actively immunized with pneumococci of Types I, II or III, or with an R strain of pneumococcus.

It is seen in Table I that of twenty-five animals yielding serum in which type-specific precipitins were present, all but four gave a typical local inflammatory reaction to the injection of the carbohydrate of the homologous type. In these four instances the titer of antibodies was lower than in the reactive animals. On the other hand, those animals in which circulating type-specific precipitins could not be demonstrated failed in every instance to give a positive response. No reaction was obtained in any case except to the homologous type-specific polysaccharide.

In contrast to the immediate wheal and erythema reaction produced by the carbohydrates in patients convalescent from lobar pneumonia, the reaction produced in the artificially immunized rabbits is usually first detected after 6 to 8 hours. The reaction makes its appearance as a faint blush and gradually increases in size, producing a pink edematous area frequently surrounded by a zone of pale edema. The edema tends to increase and erythema is replaced by a purplish discoloration. The lesion reaches its height in 24 to 48 hours and at that time it may be 4 to 5 cm. in diameter and 0.5 to 1.0 cm. in elevation. A gradual regression takes place in 4 to 7 days until little or no trace of the reaction remains. With the doses employed necrosis of the skin has not been observed. The cutaneous reaction was produced in rabbits only by the polysaccharide homologous in type to that of the pneumococcus employed in the process of immunization. The severity of reaction appears to be related to the titer of type specific precipitins for the homologous polysaccharide in the serum of the animal. In certain instances, despite the presence of type-specific precipitins, no cutaneous response was evoked by the carbohydrate, but in no case was a reaction produced in the absence of circulating type-specific precipitins.

#### *Skin Reactions in Passively Immunized Rabbits*

Attempts were made to transfer reactivity, passively, to normal rabbits by the intravenous injection of serum from immune rabbits known to give positive skin tests and the serum of which contained a high titer of type-specific precipitins. 5 to 40 cc. of serum were injected. In none of the recipient animals, however, was a positive skin reaction induced, nor were precipitins demonstrable in their sera.

The failure in these instances may be due to the fact that insufficient immune serum was injected.

Attempts were next made to induce skin reactions to the type-specific polysaccharides in animals passively immunized with serum of horses immune to *Pneumococcus* Types I, II or III. Fourteen rabbits were injected: five with Type I serum, three with Type II serum and six with Type III serum. 10 to 15 cc. of immune serum were injected intravenously, although in one rabbit only 5 cc. of serum were used. The following day the serum of all the passively immunized rabbits was found to contain a high titer of precipitins for the homologous polysaccharide and in each instance a well marked cutaneous response to the intradermal injection of the polysaccharide occurred. The character of the reaction was identical in every respect with that obtained in actively immunized animals. No reactions to the polysaccharides of heterologous types were observed.

Local passive transfer of sensitivity was carried out in rabbits by infiltrating each of two areas of skin with 1.0 cc. of immune horse serum. After 24 hours there were seen at the sites of serum infiltration small flat areas of erythema. Into one of these areas 0.5 mg. of the homologous polysaccharide was injected and into the other, as control, an equal amount of a heterologous polysaccharide. 24 hours later, at the site of inoculation of the homologous polysaccharide, a deep red, elevated edematous area 2 to 4 cm. in diameter was observed and this persisted for 48 to 72 hours. At the control site there remained only a pale erythema due to infiltration of the serum.

Guinea pigs were passively immunized by intraperitoneal injections of antipneumococcus horse or rabbit serum. The animals which received immune horse serum showed no signs of local or general reaction to the subsequent intradermal injection of the homologous type-specific polysaccharide. Similarly, no cutaneous reaction to the polysaccharide was observed in those guinea pigs passively immunized with rabbit serum. In several of the latter group, however, mild signs of general anaphylaxis (restlessness, coughing, scratching of nose, etc.) were noted shortly after the introduction of the specific substance into the skin. Tillett and Avery (4) were able to induce fatal anaphylactic shock with minute amounts of the type-specific polysaccharide in guinea pigs passively sensitized with homologous

antipneumococcus rabbit serum. If horse serum was employed for sensitization no response occurred. In the present instances the observation that anaphylactic symptoms were produced in guinea pigs receiving antipneumococcus rabbit serum but not in those prepared with immune horse serum, appears to be another demonstration of the facts reported by these workers.

#### DISCUSSION

The results reported in the present paper demonstrate that in rabbits actively or passively immunized to a type-specific pneumococcus the intradermal injection of specific polysaccharide of homologous type elicits a definite form of cutaneous reaction. In contrast to the immediate wheal and erythema reaction observed in humans convalescent from pneumonia, the reaction produced in the immune rabbit is delayed and resembles in its general appearance the form of reaction first described by Arthus (5). The reaction is characterized by slow development, edema, erythema and purpuric discoloration. The capacity of the individual rabbit to give a positive skin reaction is closely associated with the presence of demonstrable type-specific precipitins in its serum. In no instance was a positive reaction observed in the absence of demonstrable type-specific precipitins. In the guinea pig no local skin reaction was elicited but signs of general anaphylaxis were observed.

It is plain that one can correlate the reactions induced in immunized individuals of three species (man, rabbit and guinea pig) by the intradermal injection of a protein-free carbohydrate of bacterial origin. The result is the same, apparently, whether the immunity has been actively or passively acquired. In all three species the form of response to carbohydrate is typical of the reactions commonly considered to be of an anaphylactic nature when produced by protein materials.

The reactions are elicited only in the presence of specific antibodies for the capsular polysaccharide of *Pneumococcus* and then only by the specific polysaccharide homologous in type to that of the pneumococcus inducing the antibody response. Julianelle has shown (6) that animals which have received repeated intravenous injections of unencapsulated R forms of *Pneumococcus*, or of the nucleoprotein

TABLE II  
*Relation of Hypersensitiveness and Immunity to Pneumococcus in Rabbits*

Material used for immunization	Route of immunization	Antibodies produced		Immunity against virulent pneumococci		Skin reaction to pneumococcus protein	Skin reaction to capsular polysaccharide	Summary
		Species-specific	Type-specific	Active resistance	Ability of serum to protect mice			
Pneumococcus nucleoprotein	i. v. or i. c.	+	-	-	-	+	-	Hypersensitiveness to pneumococcus protein but no immunity to pneumococcus infection
R form of pneumococcus	i. v. or i. c.	+	-	+	-	+	-	Broad increased active resistance to all types of Pneumococcus with protein hypersensitiveness
S Pneumococcus	i. c.	+	Present in low titer in only 12%	+	Protection against high dilutions in only 21%	+	-	Same as above
S Pneumococcus	i. v.	+	+	+	+	+	+	Broad active anti-R resistance. Anti-S type-specific immunity. Hypersensitiveness to protein. Reaction to polysaccharide

of the pneumococcus, or have received intracutaneous injections of unencapsulated or even of encapsulated organisms develop only species-specific, antisomatic antibodies and a hypersensitiveness for the proteins of the pneumococcus. Type-specific antibodies for the capsular polysaccharide could not be detected in the serum of animals so treated, nor were any skin reactions produced by the injection of type-specific polysaccharides. Similarly, in the present experiments no reactions to the carbohydrates were obtained in animals immunized to the R organisms nor in fact, in any animal the serum of which contained no type-specific precipitins. Furthermore, in man, the reaction obtained with nucleoprotein does not take the form of a wheal and erythema, but is a delayed reaction reaching its height in 18 to 24 hours. It is evident, therefore, that the species-specific antisomatic, or antiprotein, antibodies play no rôle in the production of the skin reactions to the capsular polysaccharides. Moreover, the data presented stress again the sharp type specificity of all reactions involving the pneumococcus capsular polysaccharides.

The purpose of this report is not to enter into a discussion of the relation between hypersensitiveness and immunity but a few noteworthy facts may be mentioned. These are presented in tabular form in Table II. Hypersensitivity to the protein of the pneumococcus can be induced in rabbits by repeated injections of nucleoprotein. Under these conditions, antibodies for the species-specific R Pneumococcus develop but neither active immunity nor any capacity of the serum to confer passive immunity upon another species can be demonstrated (7, 8). In this case, hypersensitivity to the bacterial protein is not associated with increased resistance to pneumococcus infection.

If an R form of Pneumococcus is employed for immunization, only species-specific (anti-R) antibodies appear in the serum of the animal. Protein hypersensitivity can be demonstrated in this animal but no reaction is elicited by any of the capsular polysaccharides (6). In addition, the animal can be shown to have acquired an increased resistance to virulent pneumococci of Types I, II and III (8). Hence, this resistance is not type-specific. The height of the immunity is not great and is passively transferable only with great difficulty within the same species. The serum of such an animal does not confer passive protection upon mice. Here, then, is an example of protein



hypersensitiveness associated with a broad, non-type-specific increased active resistance.

When an animal has been effectively immunized with a virulent, encapsulated type-specific pneumococcus, type-specific anticapsular antibodies appear in the circulating blood. The animal has developed not only a highly efficient type-specific active immunity but its serum affords passive protection to mice against many lethal doses of pneumococci of the same type. This type-specific immunity is the form observed in convalescents from lobar pneumonia. In association with immunity due to type-specific antibodies, the intradermal injection of the type-specific capsular polysaccharide incites a response characteristic for the species. The reaction to the intradermal injection of the capsular polysaccharides of *Pneumococcus* may be termed, therefore, a hypersensitive or anaphylactic response occurring only in the presence of type-specific immunity.

#### SUMMARY

The injection of the type-specific capsular polysaccharides of *Pneumococcus* Types I, II and III into the skin of rabbits, actively or passively immunized to one of these types of *Pneumococcus*, elicits a type-specific cutaneous reaction. The form of reaction resembles that described by Arthus. The reaction is produced only when type-specific precipitins for the homologous polysaccharide are demonstrable in the blood of the rabbit. In 84 per cent of actively immunized rabbits, the serum of which contained type-specific precipitins, a reaction was elicited. A positive result was obtained in 100 per cent of rabbits passively immunized with antipneumococcus horse serum whereas, attempts passively to transfer reactivity from immune rabbit to normal rabbit were unsuccessful. The recipients, in the latter group, possessed no demonstrable circulating type-specific precipitins. The reaction produced by specific capsular carbohydrates is always associated with a well grounded type-specific immunity.

A brief summary of the relation of hypersensitiveness and immunity to pneumococcus is given.

#### BIBLIOGRAPHY

1. Tillett, W. S., and Francis, T., Jr., *J. Exp. Med.*, 1929, **50**, 687.
2. Cole, R., and Moore, H. F., *J. Exp. Med.*, 1917, **24**, 527.

3. Heidelberger, M., Goebel, W. F., and Avery, O. T., *J. Exp. Med.*, 1925, **42**, 727.
4. Tillett, W. S., and Avery, O. T., *J. Exp. Med.*, 1929, **49**, 251.
5. Arthus, M., *Compt. rend. Soc. biol.*, 1903, **55**, 817.
6. Julianelle, L. A., *J. Exp. Med.*, 1930, **51**, 625.
7. Julianelle, L. A., *J. Exp. Med.*, 1930, **51**, 449.
8. Tillett, W. S., *J. Exp. Med.*, 1927, **46**, 343.