A COMPARISON OF CUTANEOUS SENSITIZATION AND ANTIBODY FORMATION IN RABBITS IMMUNIZED BY INTRAVENOUS OR INTRADERMAL INJECTIONS OF INDIFFERENT OR HEMOLYTIC STREPTOCOCCI AND PNEUMOCOCCI*

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Swift and Derick (1) have shown that rabbits receiving repeated intravenous injections of culture or nucleoprotein of non-hemolytic streptococci reacted to subsequent intracutaneous inoculations of homologous streptococci with lesions that were smaller and firmer than those of normal animals similarly inoculated. Moreover they did not develop the general manifestations of hypersensitiveness that are shown by animals previously inoculated into the tissues with similar cultures. They have called this the "immune" type of reaction and assume that the animals have become resistant to sensitization. No mention is made of attempts to produce skin sensitivity in such intravenously injected animals by repeated intracutaneous injections.

Schultz and Swift (2) studied the serum agglutinins following the intracutaneous and intravenous injection of equal amounts of small doses of hemolytic streptococcus vaccine. They used what they considered to be the smallest dose for effective skin sensitization. The agglutinin titres were determined after 45 and 70 days. They were seldom above 1:80, in only one animal reached 1:640, and in many instances the readings were plus-minus. Similar studies were made by the injection of small amounts of cultures of *Streptococcus viridans*. The agglutinin titres of the sera on the 18th day were relatively low and about the same in both the intracutaneously and intravenously injected groups. After 44 days the agglutinins were generally higher in the latter.

McEwen and Swift (3) in a study of differences in the cutaneous sensitivity of immune and hypersensitive rabbits to intracutaneous injections of an indifferent streptococcus or its fractions found that, when rabbits were injected intravenously with large doses of both killed and living cocci, they had high agglutinin and precipitin titres, whereas comparatively few developed circulating antibodies when injected intracutaneously. In these experiments each intravenously injected rabbit received a total of from 139 to 215 cc. of culture, whereas the intradermally injected rabbits received only from 0.017 to 0.121 cc.

Julianelle (4) has also reported experiments in which the antibodies were studied following both intradermal and intravenous injections of suspensions of heat-killed

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pneumococci. After intradermal injection he was able to detect anti-R, or species specific, agglutinins in the blood. No anti-S, or type specific, agglutinins were present, and he concluded that the soluble specific substance had probably been destroyed in the skin. Both species and type specific antibodies were present, however, when the vaccine was injected intravenously.

Freund and Bonanto (5) recently reported that antitoxin formation in the rabbit is more rapid and abundant after intravenous than after subcutaneous injection of alum-precipitated toxoid. The relationship of the route of injection to its effectiveness is the reverse when plain toxoid is employed.

It has seemed desirable to extend these studies: (*a*) to determine whether skin sensitivity could be produced in rabbits previously immunized by intravenous injection; and (*b*) to study antibody formation in both intravenously and intradermally immunized rabbits when approximately the same quantities of antigen were injected.

Method

Normal Havana and albino rabbits, weighing from 1800 to 2400 gm., were used.

Cultures.—An indifferent streptococcus (3), a *Streptococcus hemolyticus* (strain AB 13) (6), and a pneumococcus (Type I) were used.

Preparation of Vaccine.—18 hour broth cultures of both the indifferent streptococcus and the pneumococcus were washed in saline and resuspended to 1/10 of the original volume. They were killed in a water bath at a temperature of 56°C. for 30 minutes. Similar suspensions of hemolytic streptococci were killed with 0.2 per cent formalin.

Injections.—To produce skin sensitization, the hair was first removed with an electric clipper, and repeated intracutaneous injections were made with the killed suspensions of microorganisms, contained in a volume of 0.1 cc. The intravenous injections were given in a volume of 1 cc.

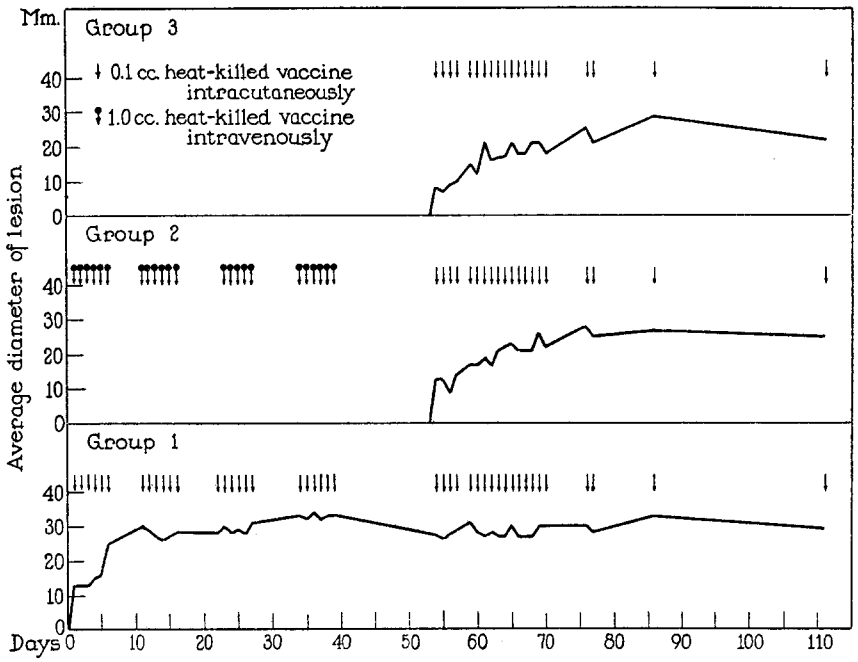
Preparation of Filtrates.—48 hour broth cultures were passed through a Seitz filter. 0.1 cc. of the sterile filtrate was injected intradermally to test the degree of skin sensitivity. The skin reactions were read 48 hours after injection.

Preparation of Nucleoprotein.—The nucleoprotein was obtained from the hemolytic streptococcus and from the pneumococcus cultures in a similar manner. The sediment from 1 liter of broth culture was ground by hand in a mortar (in later experiments with a ball mill (7)), and the nucleoprotein extracted according to the method of Avery (8). The pneumococcus nucleoprotein contained 0.22 mg. of nitrogen per cc., and the streptococcus nucleoprotein 0.24 mg. of nitrogen per cc.

Sensitization Experiments with Indifferent Streptococci

The following experiment was first done on 3 groups of 3 rabbits each, and later was repeated with similar results with 6 rabbits in each group. To simplify the description of the experiment all the animals will be included in 3 groups.

Nine rabbits in group 1 received 4 courses of 6 intradermal injections of 0.1 cc. of a heat-killed suspension of indifferent streptococci over a period of 40 days (Text-fig. 1). Skin sensitivity to the heat-killed organisms appeared first on about the 6th day and increased thereafter. The rabbits in group 2 were injected intravenously with 1 cc., or 10 times the amount, of a similar suspension on the same days. On the 52nd day after the first injection these animals, together with 9 controls (group 3), were given daily intracutaneous injections of 0.1 cc. of vaccine for 16 days. All the animals received additional single intradermal injections of vaccine on the 74th, 75th, 84th, and 109th day. The average diameter of the lesions resulting from the injections of each group is shown in Text-fig. 1. The injections are indicated by arrows and the size of the skin lesions is represented by a solid black line.



TEXT-FIG. 1. The average diameter of the skin lesions produced by intradermal injections of 0.1 cc. of heat-killed indifferent streptococci in 3 groups of 9 rabbits each. Group 2 received 24 intravenous injections of 1.0 cc. of vaccine before the intradermal injections. The injections are indicated by arrows. The size of the cutaneous lesions is represented by a solid black line.

The initial reactions of the intravenously injected group to intracutaneous injection of heat-killed microorganisms were small, firm, and elevated, with none of the characteristics of hypersensitiveness; in other words,

they corresponded to the immune reaction described by Swift. These rabbits became increasingly sensitive to intradermal injections of vaccine and after a number of injections the cutaneous reactions were similar to those regularly seen in animals made sensitive by repeated intradermal injections. They reacted about equally to either vaccine or broth filtrate.

TABLE I

The Agglutination Reactions of 12 Rabbits Immunized with Heat-Killed Indifferent Streptococci

No. of rabbits	Route of injection	Total amount received by each animal	Duration	Final serum dilutions					
				1:40	1:80	1:160	1:320	1:640	1:1280
		cc.	*days*						
6	Intradermal	2.4	50	1	2	1	1	1	
6	Intravenous	24.0	50				4	1	1
6	Intradermal	4.5	136			1	2	3	
6	Intravenous and intradermal	26.1	136			1	3	2	

TABLE II

The Precipitin Reactions of 12 Rabbits Immunized with Heat-Killed Indifferent Streptococci

Rabbit No.	Route of injection	50 days after injection			136 days after injection		
		Tube I	Tube II	Tube III	Tube I	Tube II	Tube III
1	Intravenous	+	±	−	++	−	−
2	"	++	+	−	++	−	−
3	"	+	±	−	++	+	−
4	"	+±	+	−	+++	+	−
5	"	++	+	−	+	−	−
6	"	++	+	−	+++	+	−
7	Intradermal	+±	+	−	++	+	−
8	"	++	−	−	++	±	−
9	"	−	−	−	+++	++	−
10	"	−	−	−	++	+	−
11	"	+	−	−	+++	++	+
12	"	±	−	−	++	−	−

The skin reactions of the normal control animals to intracutaneous injections of vaccine were similar in size to those of animals that had previously received the intravenous injections.

Antibodies.—The agglutinin titres on 6 intradermally and 6 intravenously injected rabbits were determined 50 and 136 days after injection (Table I). At 50 days, the average titre of the former group was less than that of the latter. After 136 days the titres in the two groups were essentially the same.

Precipitin tests were done on the same sera (Table II) against a hydrochloric acid extract of indifferent streptococci, according to the method of Lancefield (9). The amount of precipitin in both groups did not vary greatly after 136 days, whereas at 50 days it was considerably less in the intradermally injected group.

To determine the effect on antibody formation when equal amounts of antigen were injected intradermally or intravenously, 2 groups of 5 animals each received the equivalent of 1.0 cc. of vaccine at the same times as the above group. The agglutinin titre was determined after 30 and 50 days. After 30 days, the average titre of the intravenously injected group was only slightly higher than that of the intradermally injected group, whereas after 50 days it was essentially the same.

Skin Reactions and Antibodies in Rabbits That Received Intracutaneous or Intravenous Injections of Streptococcus hemolyticus Vaccine

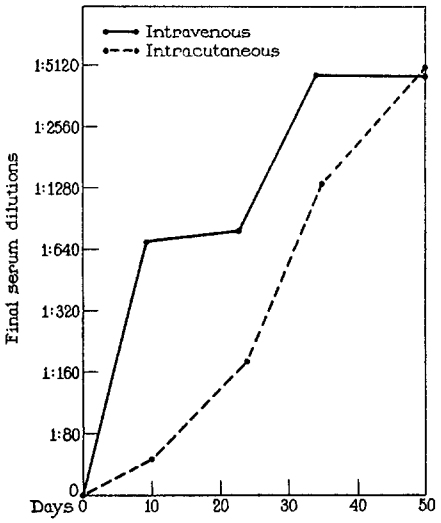
Twenty-eight rabbits received a total of 24 intradermal injections, and 22 received a similar number of intravenous injections of 1.0 cc. of formalin-killed hemolytic streptococci (strain AB 13). The injections were given daily for 6 days and the animals allowed to rest for a week. Four such courses were given. The intradermally injected animals became highly skin-sensitive to streptococcus nucleoprotein and moderately sensitive to broth filtrate. Some of the intravenously injected rabbits developed slight skin sensitivity to the nucleoprotein but were not sensitive to the filtrate. The agglutin titres of the majority of the animals were determined at intervals of about 10, 24, 35, and 50 days, and the average titres are shown in Text-fig. 2. Agglutinins appeared earlier in the intravenously injected group, and reached a fairly high level sooner than in animals that received the intradermal injections. However, there was a gradual increase in the agglutinin titres in the latter group so that after 50 days they were at about the same level in both groups.

Precipitin tests against streptococcus nucleoprotein were done on the same sera. The results are given in Text-fig. 3, and were very similar to those obtained with the agglutinins except that precipitins did not appear as early as agglutinins in the intradermally injected group.

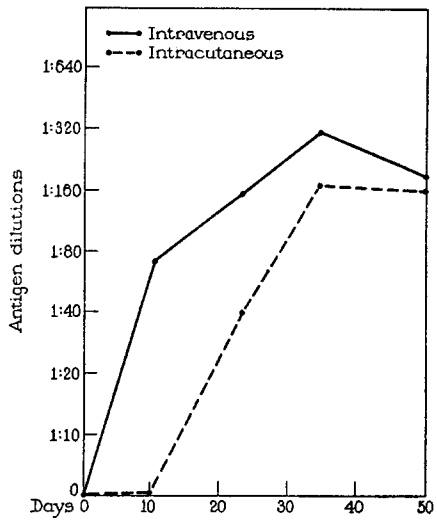
Skin Reactions and Antibodies in Rabbits Injected Intradermally or Intravenously with Type I Pneumococcus Vaccine

Another experiment was performed to study the skin reaction as well as the agglutinin and precipitin titre in rabbits immunized intracutaneously or intravenously with Type I(S) pneumococcus vaccine. Eight animals

received 24 intradermal injections and 8 others 24 intravenous injections of 1.0 cc. of heat-killed vaccine over a period of 7 weeks. Injections were given daily for 6 days and the animals then permitted to rest for a similar period. The intradermally injected animals all became highly skin-sensitive to nucleoprotein and moderately sensitive to broth filtrate. The intravenously injected animals did not react to intradermal injection of



TEXT-FIG. 2



TEXT-FIG. 3

TEXT-FIG. 2. The average agglutination titres of 28 intradermally injected and 22 intravenously injected rabbits during immunization with formalin-killed hemolytic streptococci. The sera of the majority of the rabbits were tested at 10, 24, 35, and 50 days.

TEXT-FIG. 3. The precipitin reactions of 28 intradermally injected and 22 intravenously injected rabbits during immunization with formalin-killed hemolytic streptococci. The sera were tested in most instances at 10, 24, 35, and 50 days.

either nucleoprotein or filtrate after 48 hours, although they did show a moderate immediate reaction to nucleoprotein.

Both groups of animals were bled 33 and 63 days after injection. The sera of all the animals were tested against both an R and an S strain of pneumococcus Type I for the presence of anti-R (species specific) and anti-S (type specific) agglutinins. The results are recorded in Tables III and IV. It is evident that the anti-R, or species specific, antibodies were about the same in both the intradermally and the intravenously injected groups after 33 and 63 days (Table III). No type specific antibodies were demonstrable in the intracutaneously injected rabbits after 33 days, but they were

present in titres up to 1:160 after 63 days (Table IV). In the intravenously injected group they were present in titres up to 1:40 after 33 days and up to 1:640 after 63 days.

TABLE III

Anti-R Agglutination Reactions on 16 Rabbits Immunized with Heat-Killed Pneumococci

No. of rabbits	Route of injection	Total amount received by each animal	Duration	Final serum dilutions			
				Neg.	1:20	1:40	1:80
		cc.	*days*				
8	Intradermal	12.0	33	3	2	1	2
8	Intravenous	12.0	33	3	3	2	
8	Intradermal	24.0	63	4	1	2	1
8	Intravenous	24.0	63	3	2	3	

TABLE IV

Anti-S Agglutination Reactions on 16 Rabbits Immunized with Heat-Killed Pneumococci

No. of rabbits	Route of injection	Total amount received by each animal	Duration	Final serum dilutions						
				Neg.	1:20	1:40	1:80	1:160	1:320	1:640
		cc.	*days*							
8	Intradermal	12.0	33	8						
8	Intravenous	12.0	33	2	1	5				
8	Intradermal	24.0	63		3	3	1	1		
8	Intravenous	24.0	63				3	3	1	1

TABLE V

Precipitin Reactions against Pneumococcus Nucleoprotein on 16 Rabbits Immunized with Heat-Killed Pneumococci

No. of rabbits	Route of injection	Total amount received by each animal	Duration	Antigen dilutions				
				Neg.	1:10	1:50	1:100	1:200
		cc.	*days*					
8	Intradermal	12.0	33		5	2	1	
8	Intravenous	12.0	33		2	6		
8	Intradermal	24.0	63			2	6	
8	Intravenous	24.0	63			2	3	3

Precipitin tests were also done against the nucleoprotein, using various dilutions of antigen. Both groups developed precipitins and they were only slightly less in the intradermally injected animals (Table V).

DISCUSSION

We have been able to confirm the experiments of Swift in respect to the immune type of reaction produced by repeated intravenous injections. However, it is evident from our experiments that following repeated intravenous injections, animals can be made highly skin-sensitive with intracutaneous injections. This indicates either that the immune reaction occurs because the skin of the experimental animal has never been sensitized by intradermal injections, or that repeated intravenous injections have kept the animal in a permanent state of desensitization.

The studies of Swift and his associates indicate that intravenous injections of cultures or vaccines of various strains of streptococci usually produce a skin immunity unassociated with hypersensitiveness. Our experiments, on the other hand, show that intravenously injected animals with high circulating antibodies are not more refractory to skin sensitization than normal animals. In other words, animals that show an immune cutaneous reaction are not immune to subsequent sensitization. Further experiments of this nature may enable us to obtain a clearer understanding of the significance of sensitization in relation to immunization.

Observations on circulating antibodies show that they develop following either intravenous or intradermal inoculation, although they appear earlier and maintain for a time a somewhat higher level when the injections are made intravenously. It is probable that with repeated intracutaneous injections of vaccine, there is a gradual increase in the amount held at the site of injection and a decrease in the dissemination of injected antigen to the lymph nodes and blood stream (10). If this assumption is correct, most of the antibodies would be limited to the skin and presumably few would be demonstrable in the circulation until the skin lesions were numerous or large, their extent, of course, being dependent upon the amount of antigen injected.

On the other hand, when microorganisms are injected intravenously, they probably localize in the liver, spleen, bone marrow, and lymph nodes, and because of this widespread distribution, can incite greater antibody formation than when localized in a restricted site such as the skin.

The experiments with pneumococci show that after 33 days no type specific antibodies are present in the serum and that only species specific antibodies can be demonstrated in the blood after intracutaneous injection, whereas both types of antibodies are demonstrable after intravenous injection. When the intradermal injections were continued over a longer period, however, type specific agglutinins were demonstrable in fairly high titres, although they were at a slightly lower level than in animals injected

intravenously. The presence of type specific antibodies after 63 days can be explained by the fact that we gave more frequent injections and a larger amount of pneumococcus vaccine than Julianelle.

In none of the experiments described have we been able to demonstrate any correlation between circulating antibodies and skin sensitiveness. Swift and his coworkers came to a similar conclusion from their experiments. It is true, as Julianelle has shown, that when hypersensitiveness develops there is a gradual and parallel increase in antibodies; however, many of the most highly skin-sensitive animals had a low antibody titre, whereas animals with either little or no skin sensitivity frequently showed a high agglutinin or precipitin titre.

SUMMARY AND CONCLUSIONS

1. Rabbits that had received repeated intravenous injections of heat-killed indifferent streptococci with no resulting sensitization were subsequently made skin-sensitive to them by repeated intracutaneous injection of heat-killed vaccine.

2. Serum agglutinins and precipitins developed earlier in rabbits that had received repeated intravenous injections of killed streptococci and pneumococci than in those injected intracutaneously. However, when such injections were continued over a longer period, the antibodies in both groups of animals reached a similar level.

3. Species specific agglutinins reached about the same level after either intracutaneous or intravenous injections of heat-killed Type I(S) pneumococcus vaccine. Type specific agglutinins were present only in the intravenously injected animals after 33 days but were present in all the animals after 63 days although the titres were somewhat lower in those injected intradermally. Both groups developed precipitins which were only slightly less in the intradermally injected rabbits.

4. These experiments indicate that rabbits intravenously injected with heat-killed streptococci can be made highly skin-sensitive in the same manner as animals injected intracutaneously and that they are not immune or refractory to skin sensitization. When antigen was injected either intracutaneously or intravenously into rabbits for a fairly long time, the amount of circulating antibody in both groups was approximately equal.

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