REACTIONS OF RABBITS TO NON-HEMOLYTIC STREPTOCOCCI.

II. Skin Reactions in Intravenously Immunized Animals.

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In previous papers it has been shown (1, 2) that rabbits inoculated in practically any manner, except intravenously, with sufficiently large doses of certain non-hemolytic streptococci developed a condition of tissue hypersensitiveness. This was made evident by the occurrence of secondary reactions at the site of the primary inoculation, by the presence of corneal sensitivity after a certain period, by much larger reactions following intracutaneous re-inoculation than occur in normal animals inoculated with similar doses of culture; and finally by death of many of the animals following intravenous inoculation with amounts of culture well tolerated by normal rabbits. It was also shown that if the primary inoculation of the animals had been by the intravenous route, using amounts of culture and time intervals comparable with those employed in the hypersensitized (*i.e.*, intracutaneously inoculated) rabbits, these intravenously inoculated animals responded with none of these reactions of hypersensitiveness. From such observations one might have concluded that they reacted to re-inoculation in the same manner as normal animals; but on closer study it was found that their reactivity differed from that of either normal or hypersensitive animals. The object of this communication is to present the macroscopic evidence that these intravenously inoculated rabbits react differently than do normals to intracutaneous inoculation.

EXPERIMENTAL.

The methods employed were comparatively simple: animals were inoculated intravenously with varying amounts of centrifugate of 18 to 24 hour blood broth cultures of non-hemolytic streptococci, and

after varying periods were inoculated into the previously depilated skin with decreasing amounts of similar cultures of homologous microorganisms. The test doses were usually 10^{-1} cc., 10^{-2} cc., 10^{-3} cc., and 10^{-4} cc., the first in 0.1 cc. volume, the other three in 0.05 cc. volumes, as it was found that with these amounts distinct differences in reaction of the various animals were made quite clear. At the time of the skin testing a group of normal control animals was inoculated in a similar manner; hence the strength of the culture used in any given experiment was determined.

Experiment 1.—Two rabbits, Q515 and Q516, were inoculated intravenously on Feb. 5 and Feb. 15, 1926, with the centrifugate of 5 cc. of culture of Streptococcus V92/0/11. On Feb. 19 they, together with 3 normal controls, Q531, Q532, Q533,

TABLE I.

Average Size of Lesions following Intracutaneous Inoculation of Rabbits Previously Inoculated Intravenously, Compared with Controls.

	No.	Size of inoculum		
		10^{-1} cc.	10^{-2} cc.	10^{-3} cc.
		*mm.**	*mm.*	*mm.*
Intravenously inoculated	2	33	15	Negative
Normals	3	39	20	9

* Indicates average sum of 2 longest diameters of the lesions.

were inoculated intracutaneously with 10^{-1} cc., 10^{-2} cc., and 10^{-3} cc. of the homologous strain. The lesions were measured and described daily. 2 days later a striking difference in the reactions of the 2 groups of animals was evident: At the site of the 10^{-1} cc. inoculation of Rabbits Q515 and Q516 there were red, hard, shotty lesions; at the site of the 10^{-2} cc. lesions there were flat, barely palpable, pink, maculopapules, and where 10^{-3} cc. had been injected there was no macroscopic evidence of injury. The controls at this time showed larger and softer lesions resulting from inoculation of 10^{-1} cc. and 10^{-2} cc. doses and with one exception also distinct soft papules at the site of the 10^{-3} cc. inoculation.

Because all of the rabbits received intravenous inoculations 7 days later the subsequent development of the lesions was probably altered, hence the main differentiation between the 2 groups appears in the physical characteristics of the reactions and in the average size of comparable lesions in the 2 sets of animals, as shown in Table I.

The results of a different time interval between the immunizing and testing doses are shown in Experiment 2.

Experiment 2.—Each of 4 animals, Q380, Q381, Q385, and Q386, received intravenously the centrifugate of 10 cc. of blood broth culture, V92/0/10. 20 days later the reactivity of their skins, together with that of 4 controls, was tested with doses of 10^{-1} cc., 10^{-2} cc., and 10^{-3} cc. of homologous culture. The results are shown in Table II.

TABLE II.

Comparison of Skin Reactivity of Rabbits Intravenously Immunized 20 Days Previously with That of Normal Animals.

	Rabbit No.	Sum of diameter of lesions			Secondary reaction	Retested after 10 days; sum of diameter of lesions		
		Size of inoculum				Size of inoculum		
		10^{-1} cc.	10^{-2} cc.	10^{-3} cc.		10^{-1} cc.	10^{-2} cc.	10^{-3} cc.
		mm.	mm.	mm.		mm.	mm.	mm.
Immunized intravenously	Q380	55	22	16	0	44	23	16
	Q381	30	30	16	0	37	26	22
	Q385	46	26	24	0	52	24	16
	Q386	32	23	16	0	33	29	17
Average.....		41	25	18		41	25	18
Normal	Q415	36	22	16	+	76	30	24
	Q416	37	20	0	+	46	27	18
	Q417	49	25	27	0	47	25	16
	Q418	50	19	0	0	46	27	21
Average.....		43	21	11		54	27	20

It is at once obvious that the differences in the size of primary reactions between the intravenously inoculated group and normals was not so marked as in Experiment 1. Neither was the nodular character of the lesions of the immunized group so marked as in the first experiment. Nevertheless, 2 of the 4 normal rabbits showed secondary reactions at the sites of the 10^{-1} cc. and 10^{-2} cc. inoculations, while none of the immunized animals showed secondary reactions.

It thus appears that differences in the interval between immunizing inoculation and skin testing had a distinct influence on the character of the reaction following intracutaneous inoculation. When this period was short, as in Experiment 1, the differences between the

immune and normal animals were greater than in Experiment 2, where an interval of 20 days had elapsed. It is probable that the relatively slight immunity that followed the single intravenous injection of 10 cc. of streptococci was passing off by this time. Still it was sufficient to prevent the development of secondary reactions, and an accompanying general hyperergy; for the immunized group retested 10 days later showed practically the same sized lesions as when first tested, while the controls similarly retested showed distinctly larger lesions.

The effect of more prolonged continuous intravenous inoculation is brought out better in Experiment 3.

Experiment 3.—A group of 7 animals, R651, R652, R653, R654, R655, R657, and R658, were selected for immunization with culture of Streptococcus V110A. Five of them were inoculated intravenously as follows: 1st day 1 cc., 3rd day 1 cc., 5th day 2 cc., 7th day 4 cc., 12th day 5 cc. Two received only 1 cc. each on the 11th and 14th days respectively. On the 16th day all were tested with intracutaneous inoculations of 10^{-1} cc. and 10^{-2} cc. of blood broth culture of homologous streptococci; 7 normal rabbits were tested with similar doses. On the 18th day the first 5 rabbits of the first group each received the centrifugate of 4 cc. of culture intravenously and the other 2 cc. On the 27th day all received 4 cc.; thus the immunization was continued during the period in which secondary reactions might have been expected to appear.

The results of these intracutaneous inoculations are shown graphically in Charts 1 and 2. In Chart 1 are given the curves formed from daily measurements of the sum of the 2 longest diameters of the lesions, while in Chart 2 the volumes of the lesions resulting from the 10^{-2} cc. inoculations are indicated.[1] A distinct difference is immediately evident. Only 3 out of the 7 rabbits inoculated intravenously showed secondary reactions, but 2 of these had received only 2 small inoculations before the skin testing; the third showed only a slight and late secondary reaction. Six out of the 7 normal animals, on the other hand, developed secondary reactions, which in most instances usually appeared about the 8th to the 10th day; none of the immune animals, on the other hand, showed secondary reactions before the 14th day. The primary reactions of the 2 groups of animals also showed similar differences in consistency and size to those previously noted. These differences in size are brought out more strikingly by comparing their

[1] The method of calculating these volumes is given in a previous paper (2).

volumes as shown in Chart 2, where not only the greater initial size of the primary lesions, but also that of the secondary reactions in the

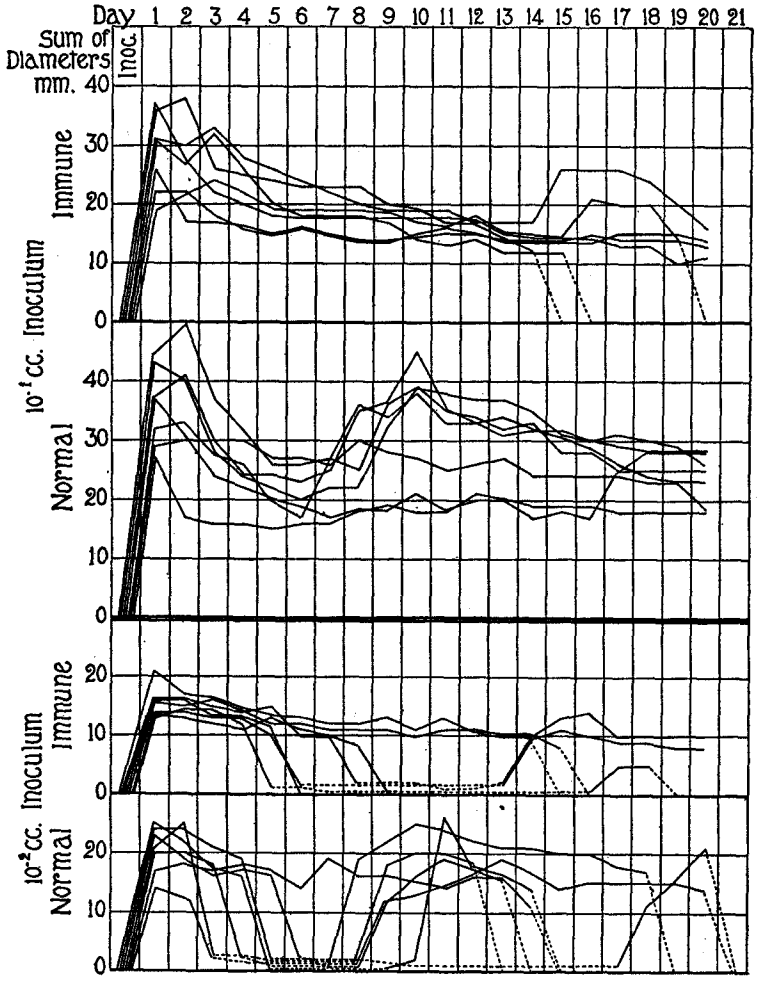

CHART 1. Comparison of sizes of lesions of immune and normal rabbits following intracutaneous inoculation with Streptococcus V110A.

normal animals is made evident. The immunized animals showed only relatively small increases in their lesions at the time of their secondary reactions, while the normal animals showed very marked in-

creases. This experiment indicates how necessary it is not only to measure the diameters, but also to pay careful attention to the thickness of these lesions. The nodular character of the primary reactions in the immune group compared with softer lesions in the normal animals was observed here as in previous experiments.

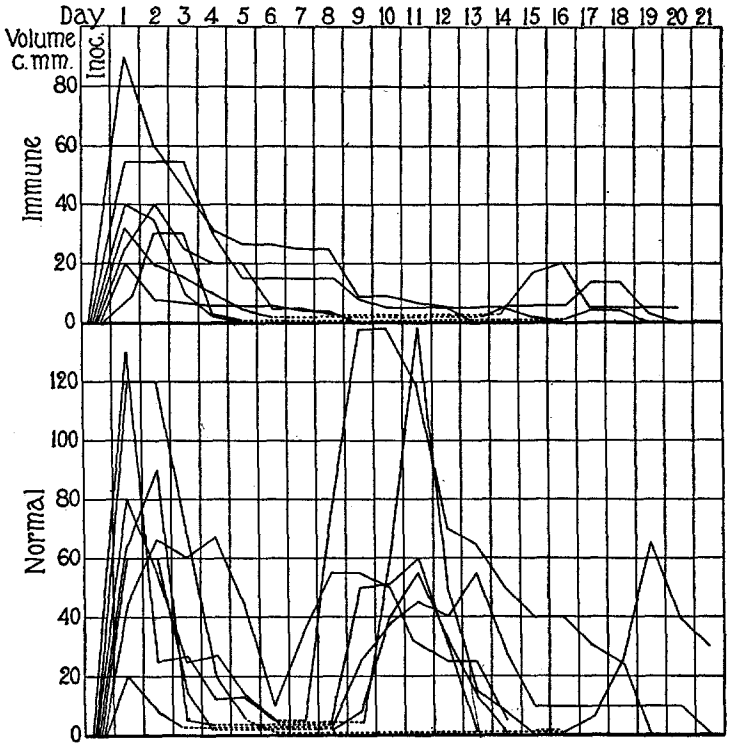

CHART 2. Comparison of volumes of lesions of immune and normal rabbits following intracutaneous inoculation with 10^{-2} cc. of blood broth culture of Streptococcus V110A.

In view of the fact that such differences in the tissue response could be demonstrated when animals were previously inoculated intravenously with living organisms, it was decided to investigate whether or not a similar alteration in responsive capacity could be detected if a bacterial fraction were used as the immunizing agent. It had been previously demonstrated by Lancefield (3) that the nucleoprotein ex-

tract of green streptococci was the only fraction with a definite antigenic capacity. She, therefore, prepared a fairly large quantity of nucleoprotein of Streptococcus V92 with which a group of rabbits was immunized intravenously, as indicated in Experiment 4.

Experiment 4.—Eight rabbits were injected intravenously with a normal saline solution of green streptococcus nucleoprotein as follows: 1st, 2nd and 3rd days, 10 mg. each day; 9th, 10th, 11th and 12th days, 20 mg. each day; 17th, 18th, 19th and 20th days, 30 mg. each day. On the 26th day each rabbit received intracutaneous inoculations with the centrifugate of blood broth culture of Streptococcus V92 in the following amounts, 5 cc., 5 cc., 10^{-1} cc. and 10^{-2} cc.; 4 normal controls were similarly inoculated. The curves showing the reactions of 4 of the immunized animals compared with the 4 controls are shown in Charts 3 and 4. Only the curves of the 10^{-1} cc. and 10^{-2} cc. lesions are given because the intensity of the response to 5 cc. of culture was so marked in all rabbits that differences in the 2 groups were not made so evident as with the smaller inocula.

While differences in the diameters of the primary lesions in the 2 groups of animals were not very marked, it became apparent, as the evolution of the lesions was followed, that there was a distinct difference in the response of the animals. All of the normal controls showed well marked secondary reactions in all of their lesions, while only 3 of the 8 immunized animals developed secondary reactions in the smaller lesions; and in 2 instances these were slight and delayed. The striking difference in the character of these secondary reactions is brought out in Chart 4, where the volume of the 10^{-2} cc. lesions of 4 of the immunized rabbits is compared with similar lesions in the 4 controls.

While the average volume of the primary lesions was 69 c.mm. in the immunes compared with 94 c.mm. in the controls, that of the secondary reactions was 25 c.mm. in those developing these reactions compared with 230 c.mm. in the controls. It is evident, therefore, that immunization with nucleoprotein over a period of approximately 4 weeks had altered the rabbits' type of response towards intracutaneous inoculation in the direction of a diminished intensity of reaction. It must be realized that the amount of nucleoprotein used represented large bacterial growths, and that probably a similar period of immunization with a corresponding amount of vaccine would have been more efficacious. The main point to be gathered from this ex-

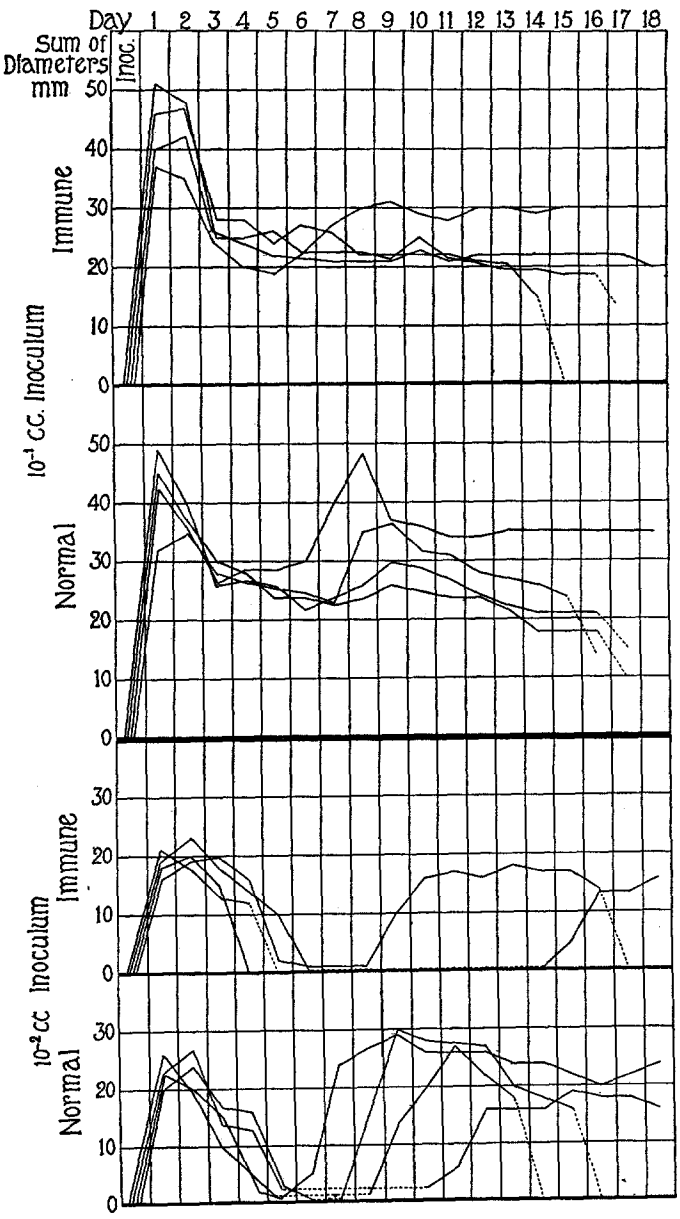

CHART 3. Comparison of sizes of lesions of rabbits immunized with nucleoprotein of green streptococci and normal rabbits following intracutaneous inoculation with Streptococcus V92.

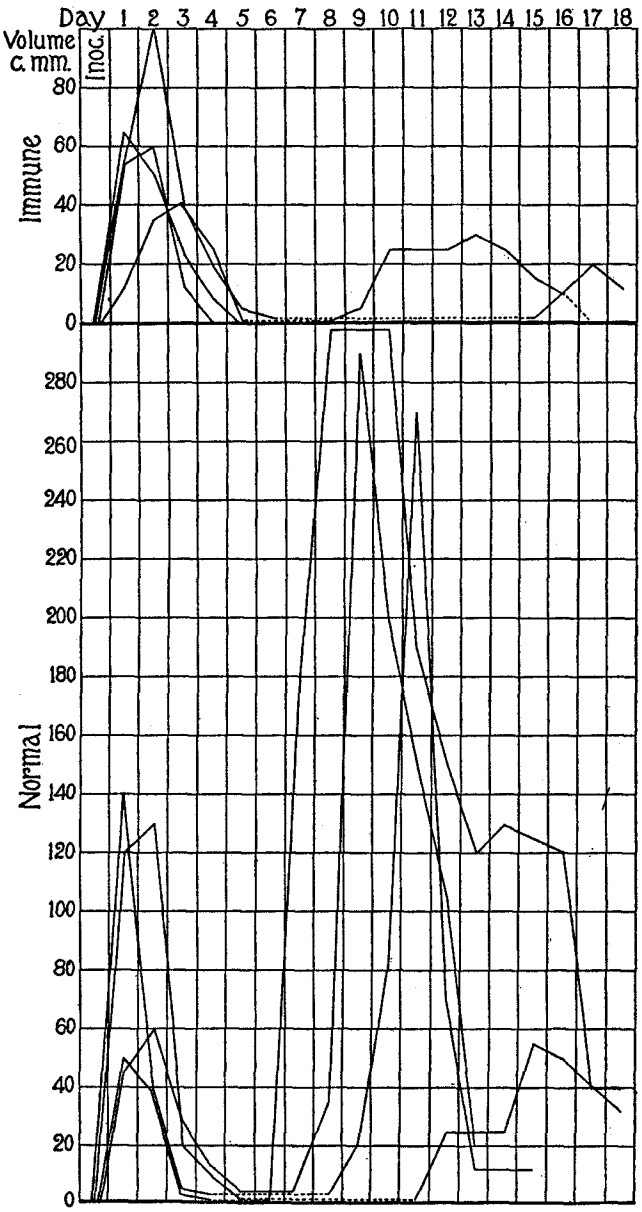

CHART 4. Comparison of volumes of lesions of rabbits immunized with nucleoprotein of green streptococci and normal rabbits following intracutaneous inoculation with 10^{-2} cc. of blood broth culture of Streptococcus V92.

periment was that the nucleoprotein fraction carried the immunizing substance.

As a final experiment the effect of prolonged intravenous immunization was studied as shown in Experiment 5.

Experiment 5.—Twelve rabbits were inoculated intravenously over a period of 9 weeks. In the beginning 0.5 cc. of whole blood broth culture of Streptococcus V110A was employed. This was gradually increased to 2 cc. after 10 days; but the rabbits began to lose weight so rapidly that immunization was discontinued for a week, then recommenced with 1 cc. of heat killed culture. This, in turn, was gradually increased until 5 cc. doses were given; subsequently immunization with living culture was resumed without depressing the rabbits. During the succeeding period of 4 weeks 9 inoculations were given by gradually increasing the dose from 1 cc. to 4 cc. of living blood broth culture. At the end of this period the sera of all the rabbits showed a precipitin titer of 1:4000 to 1:8000 when tested against nucleoprotein prepared from Streptococcus V110A. The agglutinin titer of the sera could not be determined because of spontaneous agglutination of the cultures.

After this course of 9 weeks' immunization the skin reactivity of all the rabbits together with that of 4 normal controls was tested with Streptococcus V110A/0/5 in 6 doses of from 10^{-1} cc. to 10^{-6} cc. None of the animals showed reactions at the sites of the 10^{-4} cc., 10^{-5} cc., and 10^{-6} cc. inoculations. The maximum and average sizes of the lesions in the 3 larger lesions in all of the animals are shown in Table III; also the volumes of the 10^{-2} cc. and 10^{-3} cc. lesions are given.

While quantitative variations in the response of the different animals occurred, there was almost a uniform tendency for the immunized animals to show smaller lesions than the controls. The ratio of the averages in the immune group compared with the normals at the sites of the different inocula was 1^{-1} cc. 2:3; 10^{-2} cc. 1:2; 10^{-3} cc. 2:3. When volumes of lesions are compared, just as in previous experiments, the differences are even more striking. In the 10^{-2} cc. lesions the average ratio was 1:3, and in the 10^{-3} cc. lesions 4:9. The daily notes of the consistency of the lesions also brought out striking differences. The immune animals had very hard shotty lesions, particularly at the sites of 10^{-2} cc. inoculations, while the normal animals showed some edema with only moderately infiltrated lesions from the 1st to the 3rd days. These differences were so striking that the immune animals could be readily differentiated from the controls by simple comparison of the size and character of the corresponding lesions.

None of the immune animals showed secondary reactions in any of

their lesions, while 3 out of the 4 controls developed secondary reactions. It may be objected that the differences in the size of the 2 groups tend to invalidate the comparisons, but all of 4 other normal rabbits inoculated with a corresponding culture 2 days later developed primary lesions and secondary reactions similar to these 4 controls; so it may

TABLE III.

Comparison of Skin Reactivity of Rabbits Immunized Intravenously Over a Long Period with Normal Controls.

	Rabbit No.	Sum of diameters of lesions			Volume of lesions	
		Size of inoculum			Size of inoculum	
		10^{-1} cc.	10^{-2} cc.	10^{-3} cc.	10^{-2} cc.	10^{-3} cc.
		mm.	*mm.*	*mm.*	*c.mm.*	*c.mm.*
Immunized intravenously	1119	40	17	12	98	15
	1120	35	17	v. and p.*	90	v. and p.*
	1122	42	21	13	117	17
	1123	34	19	12	108	6
	1124	24	16	9	50	2
	1125	40	25	15	165	22
	1131	38	16	12	10	14
	1135	47	18	10	54	2
	1136	53	28	20	188	60
	1138	38	20	14	106	20
	1139	33	20	15	100	12
	1140	42	28	15	157	22
Average.....		39	20	12	110	16
Normal	1253	70	39	20	517	55
	1254	52	27	15	150	10
	1255	53	34	18	315	48
	1256	78	38	17	423	45
Average.....		63	37	17	334	37

* Non-measurable, visible and palpable.

be safely concluded that these controls represented the average response of normal animals at this period. This experiment, therefore, indicates that prolonged intravenous immunization causes a group of animals to react to subsequent intracutaneous inoculation in a more uniform manner than does short immunization such as was carried out with living streptococci in the earlier experiments, or with streptococcus nucleoprotein as in Experiment 4.

DISCUSSION.

It is common knowledge that previous inoculation alters the reaction of an animal towards an infectious agent; in fact upon this knowledge are based our efforts towards the prevention and cure of infectious diseases where the various techniques of immunology are employed. In certain conditions the outstanding defensive agents are easily demonstrable in the body fluids in the form of antitoxins, opsonins or similar antibodies. In other diseases, where the invasive microorganisms have relatively low virulence, or elaborate comparatively little exotoxin, the defensive mechanism seems to reside in the cells rather than in the humors of the body. But in most infections both humoral and cellular factors apparently play a rôle; and in proportion as one or the other predominates, so, in part at least, may differences in the focal manifestations of infection and resistance be explained.

Following Pirquet's (4) observations and introduction of the term allergy, studies of the local manifestations of reinfection have assumed a constantly increasing importance, until at the present day an enormous literature has grown up about this word. It seems to be quite generally accepted that the phenomenon of allergy represents a very intense local effort on the part of the animal to limit the activity of infectious agents or foreign substances to the site where they gain entrance into the animal's body. It is also obvious that local reactions are quite different following the introduction of such agents as vaccinia, tubercle bacilli, egg albumin or primin into suitably allergized animals or men. Re-inoculation with vaccine virus results in smaller local injury than occurs at the time of first inoculation, while reinjection with egg albumin leads to more marked local reaction, which increases in intensity with increase of circulating antibodies (5). Re-inoculation with tubercle bacilli, on the other hand, is followed by destructive localized tissue reaction, the Koch phenomenon, without any corresponding increase of antibodies in the blood serum. Repeated treatment of the skin with primin, a nitrogen free ether extract of primrose results in a type of allergy made manifest by an eczematous inflammation of the skin (6). In these four examples of allergy the differences in local response may be explained in part by differences in the nature of the antigen or inciting agent. Following the introduction of tubercle bacilli into the body tubercles are formed

with an accompanying general state of tuberculin allergy regardless of the presence of demonstrable circulating antibodies. Egg albumin, on the other hand, may be introduced intravenously into an animal without producing gross focal injury; nevertheless with the development of antibodies a state of allergy ensues. It is therefore apparent, as pointed out by Zinsser (7), Doerr (8), Coca (9), and others, that the allergy induced by bacteria and by coagulable proteins is different. In practically all instances in which a tuberculin-like allergy is induced this follows some focal tissue reaction resulting from injury by the respective micro-organism.

But, in so far as we are aware, no comparison has previously been made of the type of tissue reaction which follows the introduction of bacteria into the body so that in the one instance gross lesions are produced and in the other no macroscopic lesions are formed. One difficulty attending such experimentation has been due to the fact that most bacteria, previously used in the study of allergy, induced focal lesions regardless of the route employed for inoculation. But with non-hemolytic streptococci, such as we have employed, these conditions could be more easily controlled. Strains with which it was possible to induce a condition of tuberculin-like allergy when they were injected into the tissues, could also be used to decrease the animal's tissue reactivity when they were injected intravenously in suitable doses. Ordinarily streptococci of this type quickly disappear from the blood stream without producing gross lesions; but in some instances, especially when large doses are employed, the animals develop endocarditis or arthritis. In one rabbit of Experiment 3, in which an arthritis of the wrist had followed the early intravenous inoculation, the skin test inoculation was followed by lesions of the hypersensitive rather than of the immune type. This isolated example taken in conjunction with other experiments previously reported (2) supports the viewpoint that focal lesions are probably necessary for the development of hypersensitiveness of infection.

Another point worthy of note is that intravenously immunized rabbits when inoculated intracutaneously with suitable doses show lesions of a different character than do either normal or hypersensitive animals, lesions that have little if any edema and are hard and firm after 24 to 48 hours. In fact these lesions show much less change in size

after 2 days than do the lesions in other types of animals. The larger inocula often are followed in 4 or 5 days by globular sac-like areas persisting for weeks, and smaller inocula produce small hard persistent nodules. In other words, the entire and complete reaction seems to take place much more quickly in the immune than in other states of allergy. The microscopic comparison of the different types of lesions will be reported in a later communication.

Finally, for purposes of completeness it should be noted that certain rabbits give reactions following intracutaneous inoculation which differ from any of those previously described by us. These reactions are usually less marked than those of normal animals, are soft, have very little color, fade rapidly, and do not show secondary reactions. They occur in rabbits which appear sick, either due to an overwhelming infection from streptococci, or from any other cause. These animals are usually emaciated and have a skin which is wrinkled, gray and lacking in tone. We have designated these reactions "cachectic," and after a little experience have learned to recognize them within 2 or 3 days following intracutaneous inoculation. Obviously the inclusion of such animals in any group is to be avoided for they vitiate statistical comparisons.

Within recent years it has become evident to many observers that different states of resistance towards a given bacterium could be indicated by differences in cutaneous reactivity. For example, a patient showing well marked positive reaction to intracutaneous injections of tuberculin may lose this skin reactivity during an attack of measles or other infectious disease, or if he becomes cachectic from a neoplasm or even from an overwhelming tuberculous infection. This has been designated as negative anergy, and corresponds with what we call cachectic reaction. It has also been observed that an animal may show decreasing skin reactivity to a certain fixed dose of a testing bacterial extract while it is becoming more resistant to a general infection with the same bacterium (10). This state has been designated as positive anergy, and probably corresponds with our designation, immune reaction. It seems probable, then, that the term allergy will have to be qualified in some manner, if it is to be continued as a means of expressing the idea of increased resistance accompanied by over-reaction of the tissues. For this reason we have at times used the term *hyperergy* which has been employed by a number of German

pathologists (11, 12) to indicate a hyper-reactivity of the tissues, and the terms immune or cachectic to indicate gross decreases in reactivity according to the manner in which this decreased tissue reactivity is induced.

SUMMARY.

Rabbits immunized intravenously with living culture or nucleoproteins of non-hemolytic streptococci react to subsequent intracutaneous inoculations with homologous streptococci with smaller and harder lesions than are shown by normal animals similarly inoculated; and they do not develop the general manifestations of hypersensitiveness such as are shown by animals previously inoculated into the tissues with the same cultures.

A rabbit may react to intracutaneous inoculation with non-hemolytic streptococci in one of four ways, depending on whether it is normal, hypersensitive, immune or cachectic. Most normal animals show a secondary reaction about 10 days after inoculation with suitable strains of non-hemolytic streptococci; hypersensitive, allergic, or hyperergic animals show much larger lesions than do normals with the corresponding doses of the same streptococci, and practically never show secondary reactions; immune animals show smaller and harder early lesions and usually do not have secondary reactions if they are fairly well immunized. Cachectic animals show very soft and rapidly fading primary reactions and no secondary reactions.

BIBLIOGRAPHY.

1. Andrewes, C. H., Derick, C. L., and Swift, H. F., *J. Exp. Med.*, 1926, xliv, 35.
2. Derick, C. L., and Swift, H. F., *J. Exp. Med.*, 1929, xlix, 615.
3. Lancefield, R. C., *J. Exp. Med.*, 1925, xlii, 397.
4. Pirquet, C., Allergy, Berlin, 1910.
5. Opie, E. L., *J. Immunol.*, 1924, ix, 231.
6. Bloch, Br., and Karrer, P., *Beiblatt z. Vierteljahrsschrift d. Naturforschenden Gesellschaft in Zurich*, 1927, lxxii, 1.
7. Zinsser, H., *J. Exp. Med.*, 1921, xxxiv, 495.
8. Doerr, R., *Ergebn. d. Hyg. Bakteriol. Immunitätsforschung u. exper. Therap.*, 1922, v, 71.
9. Coca, A. F., and Cooke, R. A., *J. Immunol.*, 1923, viii, 163.
10. Ramel, E., *Rev. méd de la Suisse Rom.*, 1925, xlv, 257.
11. Roessle, R., *Verhandl. d. deutsch. path. Gesellsch.*, 1923, xix, 19.
12. Gerlach, W., *Virchows Arch. f. path. Anat.*, 1923, ccxlvii, 294.