REACTIONS OF RABBITS TO NON-HEMOLYTIC STREPTOCOCCI.

I. General Tuberculin-Like Hypersensitiveness, Allergy, or Hyperergy Following the Secondary Reaction.

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INTRODUCTION.

In previous communications (1) we reported the phenomenon of secondary reaction to certain strains of green streptococci. Briefly this consists of an inflammatory reaction which appears about the 8th to 10th day after intracutaneous inoculation of rabbits with these microorganisms and at a time after the primary reaction has receded. Because we were unable to obtain the reaction in any species of animal except the rabbit it was thought that it might be a hypersensitive reaction of the nature of the Arthus phenomenon; but a number of experiments designed to test this hypothesis failed to prove it (2). As many features of the secondary reaction recalled certain points in the type of allergy seen in tuberculous infection attempts were made to determine whether it might not represent an allergic reaction of this type. The object of this communication is to present the detailed evidence which indicates that after the development of the secondary reaction the animals have a type of hypersensitiveness which closely resembles so called tuberculin allergy.

Methods.

The tests used to prove that an animal was hypersensitive have been three: ophthalmic, cutaneous and lethal. The first two of these have proven the most useful because they could be repeatedly applied to the same animal and thus variations in its allergic state be roughly estimated.

Ophthalmic Reaction.—The reactivity of the eye was tested as follows: The eye was anesthetized by instilling a drop of 10 per cent cocaine into the conjunctival sac. After 5 minutes the upper quadrant of the cornea was lightly scarified with the point of a very sharp iridectomy knife so that two or three very superficial abrasions passed from the center of the cornea, upwards to within 2 or 3 mm. of the sclerocorneal junction; but care was taken not to touch the sclera. Then a drop of bacterial sediment was placed in the conjunctival sac with a pipette and the lid rubbed over the surface of the eyeball a few times. The bacterial sediment was a centrifugate of an 18-hour blood broth culture.

In a normal rabbit such an inoculation was followed by very little reaction; usually nothing could be seen except a very slight gray streak at the site of scarification. Occasionally there was slight injection of the ocular conjunctiva for a few millimeters above the sclerocorneal junction, which sometimes persisted for 48 hours, but as a rule, when present, disappeared after 1 day.

In an animal in which a secondary reaction had been induced by several fair sized intracutaneous inoculations of green streptococci an entirely different reaction to corneal inoculation often occurred. The day following inoculation there was a distinct injection of the scleral conjunctiva in the upper quadrant; this increased in intensity for 2 to 4 days, and at times was so marked that the white appearance of the sclera was practically obliterated. In marked reactions there was distinct congestion of the palpebral conjunctiva of the upper lid, and with the most intense reactions distinct edema of this conjunctiva. Simultaneously turbidity of the upper quadrant of the cornea often appeared. This at first was seen along the lines of scarification but later usually involved the whole quadrant; in very severe reactions it involved the whole upper half and even extended to the lower half of the cornea. Usually, however, when well developed it was most intense near the sclerocorneal junction on either side of the scarification, and diminished in intensity towards the center of the cornea. At times corneal turbidity did not appear for 2 or 3 days. The turbidity persisted for varying periods according to the degree of hypersensitiveness of the animal. When slight it was present for only 2 or 3 days; when marked it lasted for 10 to 15 days. Diminution in corneal turbidity was usually preceded by decrease in the conjunctival congestion. Then gradually the opacity of the cornea diminished until macroscopically only a faint scar remained at the site of instrumental traumatization. Before this occurred, however, a distinct vascular reaction was observed. A day or two after the appearance of turbidity loops of small blood vessels appeared at the upper pole of the cornea and gradually grew downwards towards its center, most marked along the line of scarification. This pannus was usually grossly visible for 1 to 3 days after corneal turbidity had diminished. If the rabbit remained hypersensitive and 1 or 2 months later the cornea was similarly scarified in another quadrant and reinoculated these loops of vessels in the upper quadrant became more quickly visible than following the first inoculation; they also appeared earlier than the pannus at the site of the second corneal inoculation. This demonstrates that although the vessels could no longer be

seen with the unaided eye they probably remained latent but ready to function when injury was inflicted on the eye later. Similar vascular latency was described by Lewis and Montgomery (3) in tuberculous infection of rabbits' eyes. Some injection of the upper portion of the iris was sometimes seen early, but if reaction of the iris was a feature of the process it was masked by the clouding of the overlaying cornea. Gross outpouring of exudative cells into the aqueous humor to the extent that it became turbid practically never occurred.

In the presence of the most severe reactions there was some mucopurulent secretion in the conjunctival sac, occasionally so abundant that it glued the lids together. Films of this exudate showed an admixture of pus cells and a few desquamated epithelial cells of conjunctival origin; but bacteria were never recognizable. Cultures of the conjunctival secretion and mucopurulent exudate uniformly failed to demonstrate viable streptococci.

These reactions resembled those severe lesions induced in tuberculous animals by the instillation of tuberculin into the conjunctival sac, and recalled some of the severe reactions seen in tuberculous patients at the time when Calmette's reaction was widely used. The interesting point is that green streptococci are so innocuous, relatively speaking, when applied in this manner to a normal rabbit's eye, yet set up such severe inflammatory reactions in the eyes of animals which have been hypersensitive. Naturally the intensity of the response varied within fairly wide limits, doubtless due to varying degrees of the general state of hypersensitiveness of the animals. Detailed evidence concerning this point will be given later. It may be stated here, however, that control hypersensitive animals tested in the same manner with plain broth never gave a positive eye reaction.

Lethal Reaction.—It was early noted that after a rabbit had shown a secondary reaction attempts to immunize it by intravenous injection of homologous streptococci in doses well tolerated by normal rabbits often resulted fatally. The deaths occurred between 18 and 48 hours following inoculation. Autopsy revealed a fairly constant gross alteration in certain organs: the lymph nodes were much enlarged and showed both over their surfaces and throughout their substance many hemorrhagic areas from pin-point to 1 or 2 mm. in diameter. The lymphatic tissue of the intestines was swollen and often showed hemorrhages. The thymus was enlarged from a normal of about 1 gm. to 3 or 4 gm. in weight, had a reddish gray edematous appearance and was filled with red punctate areas similar to those seen in the lymph nodes. The

marrow in the shafts of the long bones had a similar hemorrhagic appearance. While it was evident that the tissues of the hematopoietic system suffered severely in this reaction, the spleen, as a rule, did not show much gross evidence of damage. Although at times small focal hemorrhages were seen in the pericardium, endocardium and myocardium, visceral hemorrhages were rare compared with the frequency of those in the lymphatic tissues.

After the lethal test was applied more systematically it was noted that animals would sometimes appear very sick for 24 or 48 hours and then recover. If these animals were chloroformed on the 3rd or 4th day, similar, but less intense, gross pathological changes were seen. Normal rabbits and most animals that had not shown secondary reactions, on the other hand, manifested little, if any, clinical reaction to the usual test dose of streptococci, i.e., the centrifugate of 4 cc. of broth culture per kilo body weight given intravenously, and if sacrificed the 3rd or 4th day by chloroforming, had little if any gross alteration of their lymphatic system. Occasionally animals which had previously failed to show secondary reactions at the site of their primary inoculations, succumbed to intravenous inoculation and showed characteristic postmortem alterations. The correlation of this lethal reaction with secondary reactions will be shown later.

Cutaneous Reaction.—Still another evidence of the altered reactivity of rabbits was furnished by reinoculating the skin with small doses of bacteria, first, doses that gave reactions in normal animals, and second, those that failed to elicit any gross reactions in normal rabbits. In the first instance it was observed that rabbits gave much more marked reactions at the time of second inoculation than originally, and also more intense lesions than normals simultaneously inoculated with comparable doses of the same culture. This is well brought out in the following experiment.[1]

[1] The choosing of proper rabbits and preparation of their skin for testing was carried out in the following manner.

It was necessary to select the animals carefully because certain of them had pigmented skins and also areas in which the hair was coarse. This selection was easily accomplished by grasping the rabbit with the two hands and blowing gently from the thigh forward to the shoulder. In this manner the areas of pigmented skin were noted. These latter areas were found to be covered by a coarser hair.

Experiment 1.—A series of rabbits were inoculated intracutaneously with the sediment of 24-hour blood broth culture of Strain V92/0/10. Group A received 4 injections of 5 cc. each, Group B, 2 of 5 cc. each and Group C, 1 of 5 cc., and

TABLE I.

Average of Sum of Diameters of Lesions Following Reinoculation.

	Animals with previous secondary reaction				Animals without previous secondary reaction			
	Number of animals	Amount of inoculum			Number of animals	Amount of inoculum		
		10^{-1} cc.	10^{-2} cc.	10^{-3} cc.		10^{-1} cc.	10^{-2} cc.	10^{-3} cc.
Group A	5	67	37	28	1	62	37	25
" B	5	71	34	22	—	—	—	—
" C	5	54	32	20	1	44	24	18
Controls	3	39	20	9	—	—	—	—

Figures indicate average sum of diameters in millimeters.

all were observed daily for secondary reactions. 16 days later all were inoculated in 3 areas with the sediment of 10^{-1} cc., 10^{-2} cc. and 10^{-3} cc. of homologous culture respectively. At the same time 3 normal controls were inoculated similarly.

For the actual removal of the hair one required *impure* barium sulfide; it would not work if chemically pure. We have used Mallinckrodt's Barium Sulphide, Gray, Approx. 65 per cent. After wetting the hair thoroughly with water a fairly heavy coating of this powder was dusted on to the side of the rabbit. A heavy cotton swab on a stiff wire or stick was made wet and rubbed gently over the powdered area for about 1 to 1½ minutes; in this way a thick paste was made in the fur. As soon as it was noted that the hair was coming away the rabbit was held under running water until the paste was carefully and completely washed off by gently rubbing with the swab during the washing. If the operation had been properly performed the hair usually came away cleanly. If all the areas were not depilated the process was repeated over these areas, but it was found that there was danger of burning if the reagent was allowed to touch the bare skin. When burning resulted it was usually due to too vigorous rubbing or to allowing the paste to remain on the skin too long. It was also necessary to exercise care in removing all the barium sulfide from the hair at the margins of the denuded area. Following the washing this area was dried by gently patting with a towel and the animal placed back in the cage. It was found better to make the depilation 1 to 3 days before using the animals, for at the end of this period any burning of the skin could be detected and the areas avoided when applying the skin tests.

The maximum reaction occurred 48 hours after inoculation. Table I gives the average of the sum of the diameters of the lesions, measured at right angles to each other, recorded in the different groups at this time. One rabbit in each of Groups A and C had previously failed to show secondary reactions; and in these two the lesions resulting at the time of the second inoculation were slightly smaller than the average for their corresponding group. The figures in this table indicate clearly that the skin reactivity of the previously inoculated rabbits was from one and one-half to two times as intense as that of the controls. The animals of Groups A and B, which had received 4 and 2 lesions respectively, were more highly sensitive than those of Group C. Ophthalmic and lethal tests also demonstrated a similar high degree of sensitiveness of these two groups, compared with Group C, which in turn was more sensitive than the normal controls. In thickness and duration the lesions also showed striking differences. At the site of 10^{-3} cc. inoculations the most sensitive animals had indurated nodules from 2 to 2.5 mm. in height; the less sensitive animals had smaller lesions from 1 to 1.5 mm., and the controls had soft lesions 1 mm. or less. The average duration of these smallest lesions in the sensitive group was 8 days compared with 3 days in the controls. In still another respect the hypersensitive animals showed differences at the site of skin inoculation, viz. in color of the lesions, especially on the 2nd or 3rd day. Frequently the smaller lesions at this time had a dull red or dull pinkish red hue which did not disappear so readily on pressure as did the brighter red color seen in comparable lesions in normal animals. The color in these hypersensitive lesions reminds one somewhat of that seen in chronic granulomata such as lupus nodules or nodular syphilides.

In measuring the reactivity of the skin of rabbits with various doses of blood broth cultures of green streptococci it was observed that most normal rabbits gave no reaction with 10^{-4} cc. when this amount of culture was diluted with normal salt solution or plain broth and injected intracutaneously in quantities of 0.05 cc. On the other hand, animals previously injected intracutaneously with larger doses of culture showed lesions when tested later with these small quantities. Often the lesions in various animals had similar diameters, but those in the more sensitive animals were more raised, more indurated, duller red in color and persisted longer. It seemed, therefore, advisable to attempt to express the degree of reaction by the volume of the reacting macule, papule or nodule. The height of the lesion was estimated from measurements with special calipers. As the lesions were practically round, a rough estimation of their volume could be made by applying the formula $v = \frac{1}{6} \pi h(h^2 + 3a^2)$ where v = volume, h = height of lesion and $a = \frac{1}{2}$ of the average diameter. As a rule a was estimated

as $\frac{1}{4}$ of the sum of the two longest diameters taken at right angles to one another. From curves constructed from this formula for lesions of various sizes the volumes of measured lesions could be rapidly computed. It is recognized that a large source of error is inherent in this method; first because of the amount of reaction that may occur in the deeper layers of the skin without producing a comparable thickness of the papule; second, on curved surfaces such as presented by the contour of the rabbit's body it is difficult to estimate the height of the lesion accurately; and third, because different lesions on the same animal resulting from the same sized inoculum may vary in size,

TABLE II.

Correlation of Secondary Reactions, Ophthalmic Reactions and Test Lesions with 10^{-4} Cc.

Group	A	B	C	D	E (sick)
Secondary reactions	+	+	—	—	—
Ophthalmic reactions	± to +++	—	±	—	—
Number of animals	8	21	1	5	4
Average size of 10^{-4} cc. lesions, c. mm	109	61	215	47	12
Above average	4	10	—	2	3
Variation in size, c. mm	110–180	65–120	—	75–92	13–20
Average	1	1	—	—	—
Below average	3	10	—	3	1
Variation in size, c. mm	54–72	25–48	—	23	0

— signifies negative reaction.

± to + + + signifies strength of reaction.

either because of differences of reactivity of skin of different textures and anatomical structure, or because the inoculum is not always introduced at the same depth. But in spite of these sources of error we have found this method of estimating the degree of sensitiveness to be of distinct value. The correlation of the intensity and frequency of the three reactions and their variation among rabbits is shown in Experiment 2.

Experiment 2.—Each of thirty-nine rabbits was inoculated intracutaneously with the sediment of 18-hour blood broth culture of Strain V110A/0/8 in 4 areas as follows: 2 with 5 cc., 1 with 10^{-1} cc. and 1 with 10^{-2} cc., and observed daily for

secondary reactions. 9 days later each animal was given a subcutaneous focus of agar inoculated with the sediment from 5 cc. of a similar culture. 38 days after the first inoculation the skins of all were tested with 10^{-4} cc. of homologous culture and the eye test was applied in the usual way. The results are shown in Table II.

It will be observed that the animals with positive ophthalmic reactions gave, on the average, the largest skin reactions. While twenty-nine animals had given secondary reactions about a month previous to the time of testing the skin and eye, only nine gave positive eye tests

TABLE III.

Correlation of Secondary Reaction, Ophthalmic Reaction and Skin Test Lesion with 10^{-4} Cc.

Group	A	B	C	D	E	F	G	H
Secondary reaction	+	+	±	—	±	+	±	—
Ophthalmic reaction	+	±	+	+	±	—	—	—
Number of animals	12	1	2	2	2	2	2	4
Average size of 10^{-4} cc. lesions, c. mm	88	65	55	29	20	22	33	22
Above average	4	...	1	1	1	1	1	2
Variation in size, c. mm	125–227	...	65	40	22	30	48	28–40
Average	...	...	...	...	...	...	...	...
Below average	8	...	1	1	1	1	1	2
Variation in size, c. mm	18–85	...	45	18	18	15	18	10–12

+ signifies distinctly positive secondary reaction.

± " doubtfully " " "

— " no secondary reaction.

... " no animals in this category.

on the 38th day. It was subsequently determined that the culture had deteriorated, which offered one possible explanation of this discrepancy. However, with the exception of the one animal in Group C, the rabbits with both positive secondary and ophthalmic reactions gave the average largest skin lesions, and those failing to give these reactions had the smallest; while those with a previously positive secondary reaction and later a negative ophthalmic reaction were midway between. The reactions in the four rabbits of Group E are noteworthy. These animals were very sick from snuffles or subcutaneous abscesses and gave

very poor reactions to all the tests.   Their lesions probably represented the well known failure of cachectic individuals to react to infection in the normal manner.

The correlation of secondary reactions with ophthalmic reactions and skin reactivity to 10^{-4} cc. inoculation made at an earlier period than in Experiment 2 is shown in Experiment 3.

Experiment 3.—Twenty-seven rabbits were each inoculated intracutaneously in 4 areas with the sediment of 5 cc., 5 cc., 10^{-1} cc. and 10^{-4} cc. in 0.1 cc. amounts respectively; the inoculum was an 18-hour blood broth culture of Strain V110A/0/6 recently taken out of frozen and dried stock.   None of the animals showed appreciable lesions at the site of the 10^{-4} cc. inoculation.   On the 11th day the skin reactivity was tested with 10^{-4} cc. and the eye was tested with the same culture in the usual manner.   The results of the three reactions are summarized in Table III.

Here again it will be seen that animals in Group A with both positive secondary and ophthalmic reactions gave on the average distinctly more voluminous skin reactions.   One of this group had a lesion of only 18 c.mm., one a lesion of 40 c.mm., but in all of the others the lesions were 45 c.mm. or larger.   Most of the other groups were too small to permit of valuable generalization; but among the twelve animals comprised in Groups D to H the average of the lesions was below 35 c.mm. and only one lesion as large as 48 c.mm. was found.   Because these animals were to be used in other work it was not feasible to apply the lethal test.

An important point to be made from the results of both Experiments 2 and 3 is that an increased reactivity of the skin of rabbits follows previous intracutaneous inoculation whether or not a secondary reaction develops at the site of the primary inoculation.   But in those animals which have recently shown a secondary reaction the reactivity of the skin to reinoculation as a rule is much greater than in those rabbits which had failed to show secondary reactions.

It is impossible to determine the degree of sensitiveness in a group of animals by applying all of the tests simultaneously, because a certain amount of time must be permitted to elapse in order to study the secondary reaction, the response to cutaneous reinoculation and the ophthalmic reaction before the lethal test can be applied.   Animals vary considerably in the time at which they show a secondary reaction, and also in the degree of the reaction when it is present.   All degrees of

intensity of ophthalmic reactions are also seen. A summary of the results obtained in 51 rabbits in three different experiments is shown in Table IV. All animals were sensitized with Strain V92/0/10. Six had 4 inoculations of sediment from 5 cc. each; 22 had 2 inoculations of 5 cc. each; 17 had 1 inoculation of 5 cc. and 1 of 10^{-1} cc.; and 6 had 1 of 5 cc. The ophthalmic test was applied on the 14th or 15th day and the lethal test on the 19th day in 17 animals, and on the 22nd day in the remainder. In this lot of animals no positive ophthalmic reactions were observed in those which had failed previously to show a secondary reaction. Among the total 42 animals comprised in Groups

TABLE IV.

Correlation of Occurrence of Secondary Reaction, Ophthalmic Reaction and Lethal Reaction.

Group	A		B		C	
Secondary reaction	+	+	+	+	−	−
Ophthalmic reaction	+	+	−	−	−	−
Lethal reaction	+	−	+	−	+	−
Number of animals	15	12	10	5	1	8
Percentage in group	55%	45%	66%	33%	11%	89%

+ signifies positive reaction.

− signifies negative reaction.

A and B, 25, or 60 per cent, died following intravenous inoculation, a striking contrast to the results in Group C. While a comparison of Groups A and B would indicate that rabbits with a positive ophthalmic reaction were less liable to succumb to intravenous inoculation than those without, this conclusion is probably not justified because of the differences in elapsed periods following the primary inoculation.

It is obvious that the development of a hypersensitive state as indicated by the occurrence of a secondary reaction cannot be detected unless the site of the primary injection can be observed and measured daily. The demonstration of ophthalmic hypersensitiveness and of the value of the lethal test, in animals made hypersensitive by intradermal inoculation, permitted us to apply these two tests to animals which were inoculated by various other routes. Experiments 5 and 6 give the results of such a study.

Experiment 5.—The animals were sensitized with the sediment of 10 cc. of 18-hour blood broth culture of Strain V92/0/11 which was concentrated by centrifugation and discarding the supernatant broth. The various sites of inoculation are indicated in Table V. In Group A the sediment of 5 cc. contained in 0.5 cc. solution was injected into each knee joint after we had assured ourselves that the needle was properly placed by first injecting and withdrawing normal salt solution. The subcutaneous injections were made over the large lumbar muscles; the intramuscular injections into these muscles. The intrapleural inoculum consisted of the sediment of 10 cc. of culture and was injected into the right pleural cavity of each animal; the intraperitoneal inoculum also consisted of the sediment of a similar amount. The paranasal sinuses were inoculated by etherizing the rabbits, boring a small hole through the thin plate of bone covering the sinuses, inserting a short needle through the trephine opening and scarifying the lining mucosa somewhat; then the sediment of 5 cc. of culture in 0.1 cc. volume was injected into the sinus on each side. The intratesticular inoculations were 0.1 cc. in volume. The vaginal inoculation was made by placing a purse string suture about the introitus, inserting a blunt needle and tightening the suture; 1 cc. of concentrated culture representing 10 cc. of growth was then injected and the needle withdrawn, and the suture tied. This suture was external to the urethra. The next day, both because the vulvæ were edematous and because the animals were unable to void, the sutures were cut and considerable amount of purulent exudate was discharged.

It was possible to measure the knees, subcutaneous lesions and testicles daily. The size and character of the lesions in the lumbar muscles could also be followed; hence in these four areas an increase in the intensity of the local reaction, about the usual time of occurrence of secondary reactions, could be estimated. But where the inoculations were made into body cavities no observations of this nature were possible.

Ophthalmic tests were applied to the right eye on the 14th day after the primary inoculation and to the left eye on the 20th day. The lethal test on the 24th consisted of 4 cc. of 18-hour blood broth culture per kilo body weight. 2 or 3 days later all animals which had not died as a result of this treatment were chloroformed and autopsied. It was thus possible to estimate roughly the sensitiveness of the tissues even though the animals had not been fatally infected.

It was evident from clinical observation that there often occurred an enlargement and more marked induration of the local inoculated areas about the 7th to 12th days, an indication that the reactivity of the tissues was undergoing an alteration at this time. Ophthalmic tests also indicated that animals in all groups were sensitized as a result of these inoculations. Twenty-one out of the thirty-six animals in the entire group gave a positive ophthalmic reaction; but comparison of

TABLE V.

Effect of Different Modes of Inoculation—into Tissues.

Group	Rabbit	Site of inoculation	Secondary reaction	Ophthalmic reaction 14th day	Ophthalmic reaction 20th day	Lethal reaction 24th day	Thymus	Lymph nodes
A	Q571	Knee	±	+±D	++	—	—	—
	Q572		—	+	++	+	+++	+++
	Q573		+	++	++	—	—	—
	Q574		+++	—	—	—	—	—
	Q575		—	—	—	—	±	—
B	Q580	Subcutaneous	+++	—	—	—	—	—
	Q581		++	—	—	—	±	—
	Q582		++	++D	—	—	—	—
	Q583		+++	++D	++D	—	+	+
	Q584		—	—	—	—	±	±
C	Q585	Intramuscular	—	++	++	—	—	—
	Q586		?	—	—	—	—	—
	Q587		++	+D?	—	—	—	—
	Q588		—	+	++	—	±	—
	Q589		++	±D?	—	+	++	++
D	Q590	Intraperi-toneal	0	±	—	—	—	—
	Q591		0	+±	—	—	—	—
	Q592		0	—	—	—	—	—
	Q593		0	+	+	+	+++	++
	Q594		0	±	—	—	±	—
E	Q595	Intrapleural	0	—	—	—	—	—
	Q596		0	+	±	—	—	—
	Q597		0	++	±	—	+	—
	Q598		0	—	—	—	±	—
	Q599		0	+±	—	—	±	—
	Q600		0	—	—	—	±	—
F	Q601	Paranasal sinus	0	+	+	—	+	—
	Q602		0	—	+±	—	+	—
	Q603		0	—	—	—	±	—
	Q604		0	+	+±	—	+	—
G	Q605	Intratesticular	?	++±	—	—	+	—
	Q606		—	—	—	—	—	—
	Q607		—	—	—	—	—	—
	Q608		+	—	—	—	—	—
H	Q577	Vaginal	0	—	—	—	—	—
	Q578		0	++	+	—	—	—

± to +++ signifies various degrees of intensity of reaction.

— signifies negative reaction.

0 " no observation.

D " reactions delayed in time of appearance.

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the corneal sensitiveness on the 14th and 20th days showed that at the time of first testing twenty of the animals showed positive reactions contrasted with only thirteen at the second testing; and in addition a number of the reactions at the time of later testing were distinctly weaker than on the 14th day. This indicates that in general the degree of sensitiveness was decreasing. It was previously reported by us (1) that the green streptococci are rapidly killed off in the intracutaneous foci. In this experiment one subcutaneous focus excised on the 11th day was sterile; and all foci cultured at autopsy failed to show living organisms. It thus seems probable that the stimulus to sensitization, which probably occurs as a result of reaction between tissues and bacteria, is not continuous because a new supply of bacteria is not available for its production. Likewise a diminution in the degree of hypersensitiveness probably explains the small number of lethal reactions, for this test was not applied until the 24th day after the primary inoculations.

A study of the various groups reveals some results of further interest. With the ophthalmic reaction as a guide, Group G with intratesticular inoculations and Group B, subcutaneously inoculated, showed the least sensitization, for in both the eye reactions were much delayed, and occurred relatively infrequently.

While a high proportion of the animals in Groups D and E, inoculated intraperitoneally and intrapleurally respectively, showed positive eye reactions on the 14th day, they were weak or delayed in three, and in all except Rabbit Q593 had become much weaker or negative when tested on the 20th day. This animal succumbed to intravenous inoculation 4 days later, an indication that its hypersensitiveness was continuing on a high level.

Group C, with intramuscular foci, had two members in which the eye reaction was weak and delayed on the 14th day, but also had two others in which the corneal sensitiveness persisted and was as strong or stronger on the 20th day. It is noteworthy that in only two other groups was this the case. In both the intraarticularly inoculated animals, Group A, and in those with paranasal sinus inoculations, Group F, the degree of corneal sensitization persisted and was as strong or stronger by the 20th day. It is interesting to note that infection of the mucosa of the paranasal sinuses without gross abscess formation,

as revealed post mortem, should lead to such rapid and continuing hypersensitiveness. A similar persisting state from intraarticular inoculation, is also interesting in view of the fact that in only one of these animals, A574, was there a frank abscess in the joint. In all others the synovia had a gelatinous appearance.

In the one rabbit with vaginal inoculation showing positive ophthalmic reaction there was an abscess in the vaginal wall.

TABLE VI.

Effect of Different Modes of Inoculation—Intravenous.

Group	Rabbit	Intravenous inoculation	Ophthalmic reaction		Lethal reaction 19th day	Thymus	Lymph nodes
			13th day	16th day			
		cc.					
A	Q388	10	X	—	—	—	—
	Q389	10	X	—	—	—	—
	Q390	10	X	—	—	—	—
B	Q382	10	—	—	—	—	—
	Q383	10	—	±?	—	—	—
	Q384	10	—	—	—	—	—
					10th day		
C	Q320	5, 10	X	X	—	—	—
	Q321	5, 10	X	X		—	—
	Q322	5, 10	X	X	—	—	—

— signifies negative reaction.

X   "   not inoculated at this time.

If now we contrast these results with those in a series of rabbits inoculated only intravenously, as in Experiment 6, the differences are most striking. The mode of inoculations and results are summarized in Table VI.

Experiment 6.—The animals of Groups A and B were inoculated intravenously with the sediment of 10 cc. of 20-hour blood broth culture of Strain V92/0/10; those of Group C with sediment of 5 cc. As we had occasionally observed deaths following early intravenous inoculation of intracutaneously sensitized animals the rabbits in Group C were reinoculated 3 days later with sediment of 10 cc. of culture. 2 days later Q321 died; but post mortem was found to have a severe *B. lepisepticum* infection; no gross lesions, however, were found in the thymus,

lymph nodes or bone marrow. The other two rabbits of Group C were again inoculated intravenously on the 10th day with sediment of 7.5 cc. of culture (4 cc. per kilo body weight) without any untoward symptoms, and with negative postmortem findings 3 days later.

The right eyes of Group B animals tested on the 13th day all failed to react. The left eyes of all surviving animals were tested on the 16th day with negative reactions in each instance except one where there was slight corneal turbidity, lasting only 2 days. Reactions of this degree sometimes occur in normal animals; hence it is safe to conclude that there was no corneal sensitization in any of the animals. All were inoculated intravenously with sediment of 10 cc. of homologous culture on the 19th day without showing any evidence of sickness afterwards. 3 days later, at autopsy, none showed any of the characteristic changes in the hematopoietic organs; Q390 showed a mild arthritis of the left knee and right shoulder. Thus lethal tests on the 4th, 10th and 19th days and ophthalmic tests on the 13th and 16th days failed to reveal any evidence of hypersensitiveness in animals in which the first inoculation was by the intravenous route and was of the same size as induced hypersensitiveness in all groups of animals in Experiment 5. Controls of the cultures used to make these tests proved that they were active in hypersensitive animals. Similar results following primary intravenous inoculations have been obtained with other strains of non-hemolytic streptococci.

It seems, therefore, that the condition necessary for the production of an early hypersensitive state, such as occurs in rabbits with the appearance of secondary reactions after intracutaneous inoculation with suitable non-hemolytic streptococci, is the production of an inflammatory focus some place in the body. Altered reaction doubtless occurs in an animal regardless of the microorganism introduced or the route through which it is introduced. There are, moreover, wide variations in the capacity of different animals to react to inoculation into similar tissues, and also distinct differences in different strains in their capacity to sensitize a group of animals. This has been already mentioned in our earlier publications, but the importance of recognizing variation in both the power of the microorganism to sensitize and the capacity of the animal to react is more clearly demonstrated in another place (4). The present discussion has rather to do with the influence of primary reaction in various tissues. When non-hemolytic streptococci of low virulence are introduced into the blood stream of normal animals they rapidly disappear and, in the doses here used, do not, as a rule, set up gross focal lesions in the tissues of the body. Their influence is probably exerted over a relatively wide area as

represented by the ramifications of the circulatory system. Doubtless certain organs bear the brunt of the attack more than others, but all are usually able to dispose of these streptococci without undergoing severe damage. When, on the other hand, these microorganisms are introduced directly into the tissues there is a local reaction, with a certain amount of tissue destruction, and before the body has been able to dispose of them effectively the inflammatory material has been produced either in sufficient quantity or of a quality to alter the bodily response in the direction of overreaction to a second inoculation at a distant site. It is interesting to note that such relatively slight reactions in the paranasal sinuses should lead to more marked sensitization than followed more marked reactions in the testicles, and also should be followed by corneal sensitization of longer duration than occurred after intrapleural or intraperitoneal inoculation. In view of the suspected rôle of streptococci in chronic arthritis the fact that general sensitization can be easily effected from intraarticular inoculation is also noteworthy.

DISCUSSION.

From the experiments presented in this and other communications it is obvious that the production of a lesion in rabbits by inoculation with non-hemolytic streptococci into some tissue is followed by a state of hypersensitiveness. This state is made evident by increased reactivity of the skin to reinoculation with small doses of the streptococci, by the marked reactivity of the scarified cornea—a non-vascular tissue —to instillation of the streptococci into the conjunctival sac and by the death of many of the animals following intraveneous inoculation with cultures in amounts well tolerated by normal rabbits. The lesions found in the lymphatic and hematopoietic organs of these animals are grossly very similar to those described originally by Koch (5) in tuberculors animals following inoculations with large doses of tuberculin. We have observed similar lesions in normal rabbits following intravenous injections of more virulent hemolytic streptococci, and have noted ophthalmic reactions similar to those seen in hypersensitive rabbits following primary inoculation of the cornea of normal rabbits with living hemolytic streptococci. Thus, the condition of the hypersensitive rabbit has been altered in such a manner that the relatively

avirulent non-hemolytic streptococci set up immediate reactions grossly comparable to those which follow infection of normal animals with virulent hemolytic streptococci.

The death of these animals after intravenous inoculation does not occur immediately or within a period of 1 or 2 hours, nor is it accompanied by symptoms of acute shock such as were originally described by Arthus (6) in rabbits sensitized to various coagulable proteins. Neither did Auer (7) or Opie (8) describe lesions of this type in rabbits fatally shocked with horse serum or egg white. In a few animals intracutaneously inoculated with egg albumin we have not observed corneal hypersensitiveness comparable with that of the streptococcus-hypersensitive rabbits; nor have we found in the literature a description of this type of eye sensitiveness to non-bacterial coagulable proteins.

It has already been shown (2) that the secondary reaction bears no definite relationship to the presence of antibacterial immune bodies in the sera of the infected rabbits. In this respect the hypersensitiveness to non-hemolytic streptococci differs from that of rabbits immunized against non-bacterial coagulable proteins, where regardless of method of immunization a heightened antibody content of the serum is accompanied by an increased intensity of the Arthus phenomenon.

In all respects, therefore, where analogies can be applied, it seems that the hypersensitiveness of rabbits following the production of lesions with certain non-hemolytic streptococci, is comparable to that form described as tuberculin type of allergy or the hypersensitiveness of infection (Coca (9)). Zinsser (10) has clearly indicated how the two types of hypersensitiveness, or allergy, differ with respect to the presence of immune bodies; and later he expressed the opinion that the bacterial sensitization resulted from a peculiar form of autolysis of bacteria which probably occurs most readily in inflammatory foci. Roessle (11) also directed attention to the differences between the tissue reactions and incitants in the two types of hypersensitiveness.

It is, furthermore, perfectly evident that this type of hypersensitiveness or allergy is not peculair to non-hemolytic streptococci, for in such chronic diseases as tuberculosis, syphilis, glanders, blastomycosis and trichophytosis, to mention only a few, skin reactions to the respective bacterial extracts are well known. Studies in typhoid reactions by

Gay (12) and his coworkers, and in reactivity to pneumococcus extracts or nucleoproteins after pneumococcus infections, by Mackenzie and Woo (13), and by Zinsser and Grinnell (14), show that acute infections also induce a similar type of bacterial allergy. Hanger's (15) studies of the reactions of rabbits to *B. lepisepticum* filtrates also indicate that rabbits spontaneously infected with these microorganisms develop a peculiar type of allergy towards them.

In the field of streptococcus infections, especially as represented by recent developments in the study of scarlet fever and erysipelas, the accumulating evidence all indicates that bacterial allergy plays an important rôle in certain phases, at least, of these diseases. This work summarized by Zinsser (16) is well illustrated by the studies of Dochez and Stevens (17), Birkhaug (18), Zinsser and Grinnell (19) and Mackie and McLachlan (20); Kirchner (21) showed moreover that there was increasing hypersensitiveness of such an avascular tissue as the cornea to the so called scarlatinal toxin.

But it should be pointed out that in these studies although the primary inoculation in many instances was with cultures of the respective streptococci, in most instances the substance applied in testing was a broth filtrate or some bacterial fraction. In most of our studies we have used whole living non-hemolytic streptococci, though in a few instances we have tested hypersensitiveness with nucleoproteins, and filtrates. Mackenzie and Woo used nucleoproteins, and Zinsser and Grinnell used *Streptococcus scarlatinæ* nucleoproteins as well as filtrates as testing substances. Still more recently Julianelle and Avery (22) have carried out more extensive series of experiments with pneumococcus infections in rabbits with results very similar to those we have obtained with non-hemolytic streptococci.

Because of the relatively low primary virulence of non-hemolytic streptococci we have thought it best to study the type of reaction following both primary inoculation and reinoculation with living microorganisms. The low invasive capacity of these streptococci and the rapidity with which they are annihilated in the rabbit's body made it especially desirable to determine just how the animal reacted towards the whole living microorganism during the various phases of immunity and resistance. While tubercle bacilli, *Spirochæta pallida* or the various fungi found in trichophytosis have a comparable low grade of

primary virulence, the fact that they remain viable in the body of an animal and hence give rise to new foci for long periods creates a different set of conditions than follows inoculation with non-hemolytic streptococci. Primary inoculation with such microorganisms as capsulated pneumococci, hemolytic streptococci or staphylococci which have a higher invasive capacity, greater virulence and the ability to elaborate more markedly toxic substances, gives still other and more complicated conditions. Dold (23), though aware of the phenomenon of secondary reaction, did not record its occurrence in rabbits inoculated intracutaneously with a large variety of common pathogenic bacteria. Hence it appears that with this reaction of rabbits towards non-hemolytic streptococci we have available a set of factors quite favorable for analysis of certain phases of allergy and immunity.

The evidence brought out in Experiments 5 and 6 that foci some place in the body are necessary for the development of this type of allergy is most noteworthy. It is not merely a question of introducing the bacteria into the body but the fact that focal lesions are set up that seems to be important, for hypersensitiveness did not follow intravenous inoculation. Rapid destruction of the microorganisms without production of large focal lesions probably offers a suitable explanation of this phenomenon. If staphylococci, hemolytic streptococci or most strains of pneumococci, had been injected intravenously, either marked purulent focal lesions would have resulted, or the animal would have died before the allergic state could have been detected. Following intravenous injection with living tubercle bacilli there would have been produced multiple tubercles, foci of sufficient intensity to induce a condition of tuberculin allergy, and the persistence of tubercles would favor a continuance of the allergic state. In this connection, however, it is interesting to note that Petroff (24) observed that it was possible to induce a state of tuberculin allergy following inoculation of animals with killed tubercle bacilli by practically every route except intravenously. It is possible that the mode and duration of action of killed tubercle bacilli in respect of the inducing of allergy are much the same as those of non-hemolytic streptococci. The description by Calmette and Guérin (25) of secondary reactions at the site of inoculation of calves with attenuated tubercle bacilli B C G furnishes a striking parallel to our observations in rabbits. Wallgren (26), moreover, has very recently

reported the occurrence of secondary reactions in young children at the site of inoculation with Calmette's B C G bacilli, and found that cutaneous allergy to tuberculin only occurred in those children who had developed these secondary reactions.

We, therefore, seem to be in a position to give a reasonable explanation of the secondary reaction. Following the intracutaneous inoculation of rabbits with any strain of streptococcus there develops a state of tuberculin-like hypersensitiveness; but certain strains possess the capacity of stimulating the hypersensitiveness to a higher level than others, and certain rabbits are more capable of reacting, as is made evident by retesting the animals in different ways. Following the primary intradermal reaction there persists at these primary foci a certain amount of residual antigenic substance. When a sufficiently high degree of general hypersensitiveness develops the cells in the immediate vicinity of the primary lesion are in a condition to react with small amounts of suitable bacterial substances whether they are either freshly introduced or residual. The secondary reaction is, therefore, apparently an evidence of this reaction with some residual antigen persisting at the site of primary inoculation in an animal which has developed a general state of hypersensitiveness. The peculiar feature is that some strains should possess these stimulating or reacting substances to such a degree, while others are strains apparently lacking in them.

In the studies of Dochez and Stevens and of Birkhaug concerning the two phases of sensitization of rabbits towards toxic filtrates various routes of inoculation were employed. The first observers injected toxic filtrates intracutaneously, and later intravenously; the last experimenter injected living indifferent streptococci intraperitoneally. While Dochez and Stevens observed that the second phase of allergy followed prolonged intravenous inoculation, Birkhaug reported that skin desensitization followed intravenous injection of toxic filtrates. Therefore, there appears to be some contradictory evidence concerning the results following different routes of inoculation. But it should be noted that different types of streptococci were used by these two sets of workers. Nevertheless the influence of the types of lesions resulting from the different routes of inoculation has in general not been as carefully considered as it probably deserves. If our observations

concerning the effect of production of focal lesions compared with that of intravenous inoculation without focal lesions are substantiated with other bacteria, it may be necessary to revaluate certain of the factors in the production of tuberculin-like bacterial allergy.

CONCLUSIONS.

1. Accompanying and following the evolution of a secondary reaction in the skin of rabbits after inoculation with suitable doses of certain non-hemolytic streptococci there quickly develops a general state of hypersensitiveness or allergy towards these streptococci.

2. This state is made evident by ophthalmic reactions following corneal inoculations, by much increased reactivity of the skin following intracutaneous reinoculations, and by lethal reactions, resembling tuberculin shock, following intravenous inoculations.

3. In a given hypersensitive rabbit there is a rough parallelism in the intensities of these different kinds of reactions.

4. This type of hypersensitiveness or bacterial allergy does not follow primary intravenous inoculation of rabbits with comparable doses of the streptococci employed.

5. As the development of this type of hypersensitiveness or bacterial allergy seems to accompany the production of focal lesions of a certain intensity, it is probable that in these foci are produced the substances or conditions which lead to this type of bacterial allergy.

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