

THE FATE OF A VIRULENT HEMOLYTIC STREPTOCOCCUS  
INJECTED INTO THE SKIN OF NORMAL AND  
IMMUNIZED RABBITS

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The fate of avirulent hemolytic streptococci after inoculation into the skin of normal and sensitized rabbits has been previously (1) described. When small numbers of relatively avirulent hemolytic streptococci were injected into the skin over the flank of normal rabbits they seldom diminished and usually increased in number during the first 5 hours after inoculation, but after 12 hours had usually disappeared. Streptococci reached the inguinal lymph nodes of normal animals in considerable number within 15 minutes; the number recovered was greatest within 2 hours after injection and gradually decreased so that few organisms were recovered from the lymph nodes after 7 hours. A smaller number reached the iliac lymph node. They were recovered in several instances from the blood and were occasionally found in the spleen.

When the same dosage of avirulent streptococci was injected into the skin of rabbits sensitized by repeated injection of avirulent hemolytic streptococci, the reaction at the site of inoculation was larger, more edematous and associated with more necrosis than in the normal animals. The avirulent streptococci multiplied for about 5 hours and were recovered in larger numbers from the sensitized than from the control animals. They persisted for a longer time in the skin of the sensitized (24 hours) than in that of the control rabbits. Streptococci were recovered from the inguinal lymph node in fewer instances and in much smaller numbers than from those of the controls. On no occasion did we recover streptococci from the iliac lymph node, blood, liver or spleen of sensitized animals. The multiplication in the skin of sensitized rabbits was favored by local injury with necrosis which

was more extensive than in the normal animals. In association with this more intense inflammatory reaction in the sensitized rabbits, the streptococci were fixed or held at the site of inoculation. It is evidently desirable to repeat the experiments with a hemolytic streptococcus virulent for rabbits.

The term sensitized was used in the above experiments because of the striking skin hypersensitivity produced by an avirulent hemolytic streptococcus which we have designated as strain B<sub>1</sub>. It will be seen that the virulent hemolytic streptococcus, that will be designated as strain H, produces a very different picture. There is at first a moderate amount of cutaneous hypersensitiveness that gradually diminishes with subsequent injections of the organism. A very conspicuous degree of immunity indicated by diminishing local reaction is later produced. It seems appropriate in the following experiments to describe our animals as immunized against the virulent streptococcus.

### *Methods*

*Culture.*—The virulent organism used in these experiments was a hemolytic streptococcus kindly given to us by Dr. F. P. Gay. It was originally isolated from a case of human empyema and has been used rather extensively by him during the past 15 years (2). He has designated it as *Streptococcus pyogenes* "H." The culture when received was in pleural fluid from a rabbit that had died with empyema. This pleural fluid was kept in the ice box, and cultures from it were designated as stock cultures. Subcultures were obtained by transferring 0.1 cc. of the pleural fluid to plain broth with a pH of 7.6 and incubating for 18 hours at 37°C. At first 1 cc. of a 1:100 dilution was fatal 7 days after intrapleural inoculation; this dosage was never fatal when injected intracutaneously. To increase the virulence of the organism, we followed successfully the method suggested by Gay (2). The stock culture was passed through the pleural cavity of several normal rabbits until 1 cc. of a 1:1,000,000 dilution proved fatal. Pleural fluid thus obtained was kept on ice and denoted the passage culture. In order to maintain a high virulence, the organism was passed through the pleural cavity of a normal rabbit once every 2 to 3 weeks. The virulence varied slightly from time to time, but was maintained at such a level that during the course of these experiments 1 cc. of a 1:10,000 dilution regularly caused the death of animals within 3 to 4 days. It is noteworthy that only one strain of streptococcus was used in these experiments.

*Immunization.*—Chinchilla rabbits ranging in weight from 1600 to 2400 gm. were used. They received intracutaneous injections of 0.1 cc. of 1:100 dilution

of an 18 hour stock broth culture of the H hemolytic streptococcus every week, usually for a period of 5 weeks. A few rabbits received one or two additional inoculations. In a few instances the initial dose was 0.1 cc. of a 1:10 dilution; however, this produced such large necrotic lesions that it was discontinued. The first injection produced uniformly large abscesses associated with much erythema, edema and a central yellow necrotic area. This lesion increased in size, usually for a period of from 4 to 5 days, and disappeared after from 5 to 6 weeks. In a few instances on the 5th or 6th day the erythema and edema surrounding the lesion spread for a considerable distance beyond it, resembling erysipelas, and then disappeared within 3 days.

The lesion produced by the second inoculation was usually slightly smaller than the first, there was less or occasionally no necrosis, and it usually disappeared in a somewhat shorter time. Subsequent inoculations regularly produced smaller red and edematous lesions with a somewhat firmer center; they reached a maximum size in 48 hours and rapidly subsided, disappearing entirely within from 3 to 4 days.

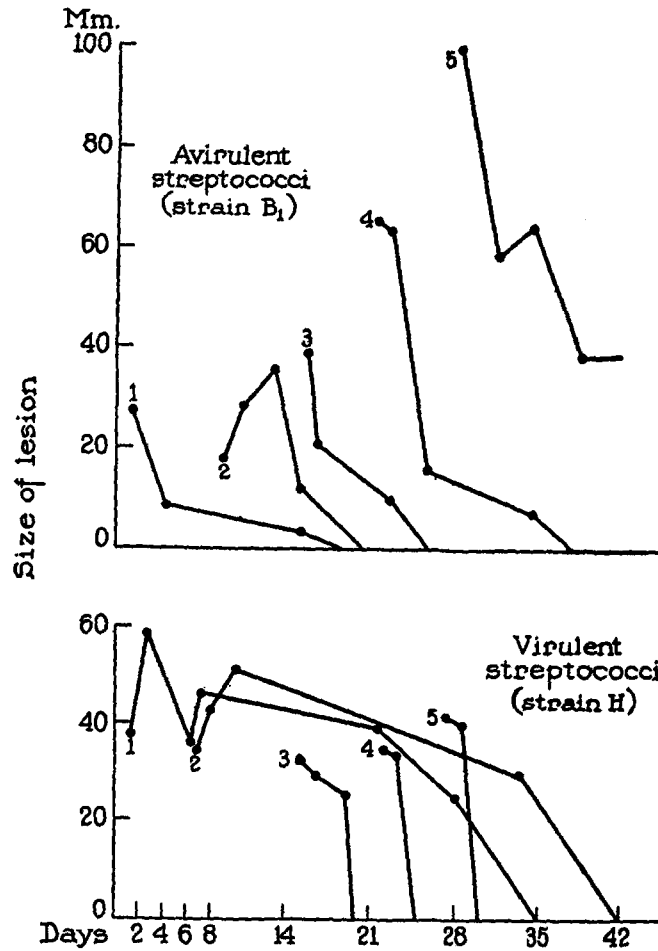
*Test for Hypersensitivity.*—Bottles (100 cc.) of broth with pH 7.6 were inoculated from the passage culture in pleural fluid and incubated for 48 hours at 37°C. The cultures were passed through a Seitz filter and the resulting filtrate tested for sterility. The filtrate was kept in the ice box and used as required. No deterioration was noted after 4 months in the ice box.

Skin hypersensitiveness to hemolytic streptococci was determined by an intracutaneous injection of 0.1 cc. of the streptococcus filtrate. Each rabbit was tested with filtrate before and at intervals during the period of immunization. The first injection was usually negative except for a slight diffuse erythema unaccompanied by edema in an occasional animal.

All immunized rabbits (Table I) developed some skin hypersensitivity to filtrate, although they usually reacted to filtrate more strongly about the 12th day than at the end of the period of immunization. The size of the edematous lesion produced with filtrate roughly paralleled that of lesions produced by simultaneous intracutaneous injections of living hemolytic streptococci.

A graphic comparison of the size and duration of the skin lesions produced by five injections of the virulent strain of hemolytic streptococci with those produced by the avirulent strain is given in Text-fig. 1. The two animals represent typical examples of similarly injected rabbits. With repeated injections of avirulent streptococci the lesions increase in size, becoming more erythematous and edematous; the later lesions also tend to persist longer. The first two injections of virulent streptococci, on the contrary, produce large ulcerative abscesses that heal after several weeks. Repeated injections usually

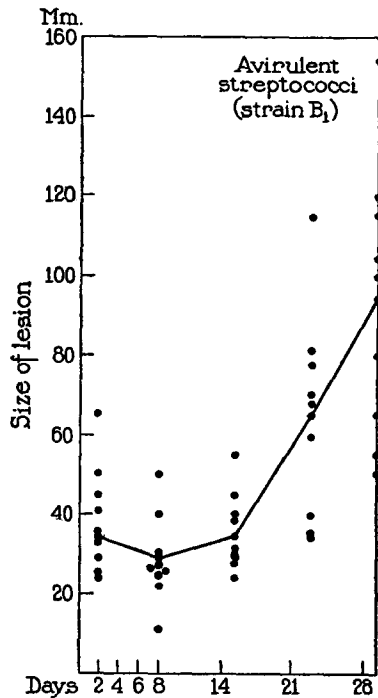
produce smaller erythematous and edematous lesions that disappear after 2 or 3 days.



TEXT-FIG. 1. Comparison of the size and duration of lesions produced in the skin of rabbits by intradermal injections of 0.1 cc. of a 1:10 dilution of a broth culture of avirulent, and 0.1 cc. of a 1:100 dilution of virulent hemolytic streptococci. The size of the lesion represents the sum of the two longest diameters.

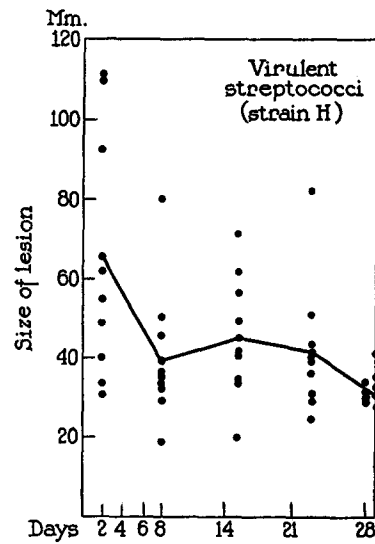
A comparison of the size of the lesions produced in nine comparable rabbits injected with avirulent and virulent hemolytic streptococci is given in Text-figs. 2 and 3. In the former instance, the increasing

size of the lesions is evident, whereas in the latter the lesions become much smaller and tend to be more uniform in size. The dosage of avirulent streptococci was 0.1 cc. of a 1:10 dilution, whereas that of the virulent streptococcus was smaller, being 0.1 cc. of a 1:100 dilution.



TEXT-FIG. 2

TEXT-FIG. 2. The size of lesions of nine rabbits, each receiving five subsequent injections of 0.1 cc. of a 1:10 dilution of a broth culture of avirulent hemolytic streptococci (strain B<sub>1</sub>).



TEXT-FIG. 3

TEXT-FIG. 3. The size of lesions of nine rabbits, each receiving five subsequent injections of 0.1 cc. of a 1:100 dilution of a broth culture of virulent hemolytic streptococci (strain H).

It was desirable to use the smaller dose of virulent organisms so that extensive lesions might be avoided. A few animals were injected with the larger dose of 0.1 cc. of a 1:10 dilution of virulent hemolytic streptococci and a similar, but even more striking, degree of immunity was evident.

## EXPERIMENTS

In each experiment a normal and an immune rabbit of about the same weight received an intracutaneous injection of 0.1 cc. of a 1:100 dilution of an 18 hour passage culture of highly virulent H hemolytic streptococci on the left flank about

TABLE I

*The Number of Streptococci Recovered from Normal and Immunized Rabbits after Intracutaneous Injection of the Organisms*

Experiment No.	Time interval	No. of organisms injected 0.1 cc. 1:100	No. of organisms recovered																		
			Normal rabbits					Immune rabbits													
			Skin	Lymph node		Blood	Spleen	Skin	Lymph node		Blood	Spleen									
				Inguinal	Iliac				Inguinal	Iliac											
	<i>min.</i>																				
1	15	130,000	201,000	0	—	0	0	0	0	61,000	0	—	0	0							
	<i>hrs.</i>																				
2	1	290,000	2,320,000	Few	0	0	0	0	0	1,023,000	Few	0	0	0							
3	2	350,000	2,221,000	4180	144	0	0	0	0	1,720,000	70	0	0	0							
4	4	160,000	10,800,000	75	0	0	0	0	0	560,000	0	0	0	0							
5	6	130,000	26,800,000	2700	4	0	0	50	0	17,000	0	0	0	0							
6	8	190,000	121,000,000	5550	16	Pos.	0	20	0	6,600,000	400	0	0	0							
7	12	290,000	150,000,000	11,450	0	0	0	0	0	2200	0	0	0	0							
8	21	350,000	23,200,000	1535	0	0	0	0	0	0	1 colony	0	0	0							
9	24	290,000	7,530,000	714	—	0	0	0	0	190,000	556	—	0	0							
	<i>days</i>																				
10	2	600,000	43,000,000	30,340	1072	Pos.	300	0	0	5000	9	0	0	0							
11	3	600,000	1,350,000	4600	0	0	0	0	0	0	0	0	0	0							
12	4	190,000	8,600,000	3200	1000	0	100	0	0	0	0	0	0	0							
13	5	130,000	3,610,000	43,950	52	Pos.	30	0	0	0	0	0	0	0							
14	7	130,000	230,000	360	0	0	0	0	0	0	0	0	0	0							
15	9	130,000	1,870,000	0	0	0	0	0	0	0	0	0	0	0							
16	13	190,000	93,000	0	0	0	0	0	0	0	0	0	0	0							
17	16	200,000	1,950,000	0	0	0	0	0	0	0	0	0	0	0							
18	19	200,000	0	0	0	0	0	0	0	0	0	0	0	0							
19	21	200,000	5000	0	0	0	0	0	0	0	0	0	0	0							

4 cm. cephalad to the left ilio-inguinal lymph node. The number of organisms injected was estimated by the procedure used in the former study (1).

Lesions at the site of inoculation in the control rabbits, evident after 4 hours, were erythematous and measured about 11 by 12 by 1 mm. They increased

during 48 hours, when they appeared as large, edematous, ulcerated lesions of which the largest measured 51 by 37 by 9 mm.

In the immunized rabbits pale, edematous, occasionally pink wheals appeared within 4 hours. The inflamed area did not increase in size; however, in some instances after 48 hours, it measured 25 by 25 by 2 mm., and had a small yellow center. In no instance did ulceration occur.

The skin lesions, inguinal lymph nodes, iliac lymph nodes and spleens were removed and cultured as previously described (1). A culture from the blood of the heart was made immediately before death from each rabbit. The number of organisms injected and the number recovered are given in Table I. We made no cultures from the immune animals after 4 days because no organisms had been recovered from this group from 2 to 4 days after inoculation.

The number of organisms injected varied from 130,000 to 600,000, with an average of 300,000 organisms for each injection.

*Number of Streptococci at the Site of Injection.*—The organism was recovered in large numbers from the normal animals in every instance. In the first twelve experiments, from 201,000 to 150,000,000 organisms (average about 35 million) were recovered from the skin of the normal rabbits, as contrasted with from 0 to 6,600,000 organisms (average about 800,000) from the twelve immunized rabbits. In normal rabbits multiplication began during the first 2 hours, proceeded rapidly for 12 hours and then diminished slightly up to 24 hours. Large numbers of streptococci were recovered from both inguinal and iliac lymph nodes, blood and spleen, during 5 days after injection, but none were recovered after the 7th day because the rabbits had become immune. Two of three animals injected at the same time, not included in Table I, died with bacteremia on the 7th day.

Streptococci were recovered from rabbits killed from 5 to 16 days after injection. On the 19th day the site of injection was found to be sterile, and a few organisms were recovered from a normal rabbit examined on the 21st day. Irregularities in the numbers recovered from normal rabbits may be due in part to individual differences in rabbits; it is probable that in some instances a great many organisms were lost in purulent discharge, whereas in others there was little or none.

The number of organisms recovered from the skin of the immune rabbits was less than the number injected in all save 4 instances, and in these there was some multiplication. The greatest multiplication

occurred in an animal killed 8 hours after injection (Table I); however, 121 million, or 18 times as many organisms, were recovered from the corresponding control rabbit. In one immune rabbit the organisms had entirely disappeared from the site of injection within 21 hours and none were recovered after 48 hours. Variations in the numbers recovered probably bear some relationship to variations in the degree of immunization produced in animals.

*Inguinal Lymph Node.*—There are conspicuous differences in the number of streptococci in the inguinal lymph nodes of the two groups (Tables I and II). In the first twelve experiments, an average of 5362 organisms was recovered from the inguinal lymph nodes of the normal,

TABLE II

*Number of Normal and Immunized Rabbits with Streptococci in Lymph Nodes, Blood and Spleen*

Culture taken from	Normal rabbits			Immunized rabbits		
	No. of rabbits	Showing hemolytic streptococci in culture		No. of rabbits	Showing hemolytic streptococci in culture	
		No.	per cent		No.	per cent
Inguinal lymph node	12	11*	91.6	12	6†	50
Iliac lymph node	10	5	50	10	0	—
Blood	12	2	16.6	12	0	—
Spleen	12	4	33	12	0	—

\* Streptococci averaged 5362 per lymph node.

† Streptococci averaged 86 per lymph node.

as contrasted with an average of 86 organisms in those of the immune rabbits. The number of organisms in the lymph nodes appears to be associated in a general way with multiplication at the site of inoculation and the greater the multiplication in the injected skin, the greater is the passage of organisms to the adjacent lymph node. Streptococci were recovered in relatively large numbers from all except one of twelve inguinal lymph nodes of normal rabbits. The inguinal lymph node removed after 15 minutes was sterile. Streptococci were recovered in smaller numbers and from only six of twelve inguinal lymph nodes of the immune rabbits.

*Iliac Lymph Node.*—The iliac lymph nodes were not found with



certainty in two instances and hence they were omitted from the enumeration in Tables I and II. We were able to culture an average of about 223 colonies of hemolytic streptococci per lymph node from five of ten normal rabbits. The other five were sterile. Streptococci were usually recovered from the iliac nodes when large numbers of organisms were present in the inguinal lymph node. The iliac lymph nodes of immune rabbits gave no growth.


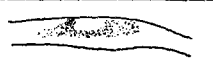
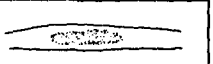
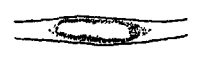
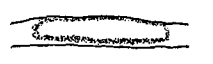
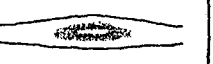
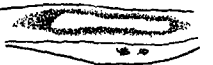



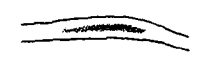



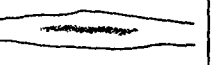
*Blood.*—Positive blood cultures were obtained from two of twelve normal rabbits killed within 4 days after inoculation and in these there was a great deal of multiplication of streptococci in the skin; they were numerous in the inguinal lymph nodes and were present in the iliac lymph nodes. Positive cultures were obtained from the blood only in animals with streptococci in the iliac lymph nodes. Cultures made of the blood of immune animals were in all instances sterile.

*Spleen.*—Organisms were grown from the spleen in four of the twelve normal animals killed within 4 days after inoculation. In all instances in which streptococci were found in the spleen, they had undergone conspicuous multiplication at the site of inoculation, were numerous in the inguinal lymph nodes and were recovered from iliac nodes. The spleen from immune rabbits was sterile in each instance.

*Histological Changes at the Site of Injection and in Adjacent Lymph Nodes in Normal and Immunized Rabbits*

A group of six rabbits received five intracutaneous injections of 0.1 cc. of a 1:100 dilution of living virulent hemolytic streptococci at weekly intervals. After the third injection of streptococci a test dose of filtrate produced edematous wheals. The average lesion for the entire group was about 22 mm. across and 2 mm. high. After the fifth injection, although there was some hypersensitivity to the filtrate, the reaction was considerably less. It was only slightly elevated above the skin surface and measured 12 cm. across. This group together with six normal rabbits received an intradermal injection of 0.1 cc. of a 1:100 dilution of an 18 hour broth culture of virulent streptococci (190,000). The site of injection was carefully marked with India ink and this remained visible until the sections were cut. An immunized and a control rabbit were killed simultaneously after intervals of 2, 4, 8, 12, 24 and 48 hours. The skin at the site of injec-

tion and the inguinal lymph nodes were placed in Zenker's fixative, sectioned and stained with Mallory's eosin and methylene blue. Text-fig. 4 gives diagrammatic cross sections of the skin lesions. The dark stippled area represents the extent of cellular reaction, and the clear central area the site at which the streptococci are seen. Descriptions of the skin lesions in two groups of animals follow.

Hours after injection	Skin lesions with hemolytic streptococci (0.001 cc.)		
	Virulent (strain H)		Avirulent (strain B <sub>1</sub> )
	Normal	Immunized	Normal
4			
8			
12			
24			
48			

TEXT-FIG. 4. Diagrammatic representation of the skin lesions (enlarged 1.5 $\times$ ) in normal and immunized rabbits. The lesions were removed at intervals after intracutaneous injections of 0.1 cc. of a 1:100 dilution of a broth culture of virulent hemolytic streptococci (strain H). Lesions from normal rabbits that received the same dose of avirulent hemolytic streptococci (strain B<sub>1</sub>) are included for comparison.

*After 2 Hours.—*

*R 1, Control.*—The site of inoculation is not visible. There is infiltration of polymorphonuclear leucocytes throughout the corium, together with a few lymphocytes and mononuclear cells. A few cocci are seen to be free between the collagen fibres and in very few instances one is found within the cytoplasm of a polymorphonuclear leucocyte.

*R 2, Immunized.*—There is no alteration in the skin that is visible to the naked eye. Microscopical changes are similar to those described above but there are fewer polymorphonuclears. A considerable number of them contain blue staining cocci. The organisms are recognized with much difficulty in intact cells, because they are obscured by the eosinophilic granules. On the contrary, in damaged cells with few or no granules they are recognized readily.

*After 4 Hours.—*

*R 3, Control.*—There was some edema and faint erythema at the site of inoculation. Many polymorphonuclear leucocytes are scattered throughout the section and some are in foci. A moderate number contain cocci singly and in pairs. Extracellular streptococci are readily discernible in considerable numbers.

*R 4, Immunized.*—The skin was slightly injected. Microscopically there are large numbers of streptococci occupying an area containing few cells still intact. This area is surrounded by a fairly extensive accumulation of polymorphonuclear leucocytes, a few lymphocytes and mononuclear leucocytes. A moderate number of polymorphonuclears adjacent to the streptococci show phagocytosed organisms. They are seen with difficulty in the cells with numerous granules and in areas where there is marked fragmentation and pyknosis of the nuclei.

*After 8 Hours.—*

*R 5, Control.*—The skin lesion measures 12 by 11 by 1 mm. and is intensely hemorrhagic with a central dark purple area. A red streak upon the skin corresponds to the lymphatic draining this area. There are large numbers of organisms in the section and an extensive infiltration of polymorphonuclear leucocytes. Streptococci are not limited to the site where polymorphonuclear leucocytes occur but are seen in considerable numbers in the tissue beyond the barrier of leucocytes and here they have apparently multiplied. A moderate number of polymorphonuclear cells contain single cocci.

*R 6, Immunized.*—There is a pale edematous wheal on the skin surface that measures 12 by 18 by 2 mm. A large number of streptococci, but definitely fewer than in the control animal, are scattered between the collagen fibrils within a dense zone of polymorphonuclear leucocytes that serves as a barrier. Cells adjacent to organisms are largely disintegrated whereas those at the periphery are intact. At the junction of well preserved polymorphonuclears and those that have lost their granules there is a considerable amount of phagocytosis.

*After 12 Hours.—*

*R 7, Control.*—The skin lesion measures 20 by 14 by 3 mm. with a yellow necrotic area in the central part. Streptococci are present in larger numbers than in any previous section. They occupy a large area with few cells in which the areolar tissue is edematous; this area is palely stained in contrast to the surrounding dense, fairly complete zone of polymorphonuclear leucocytes. There is a moderate amount of phagocytosis and cells that have ingested streptococci often have damaged nuclei and diminished granules.

*R 8, Immunized.*—The skin lesion measures 12 by 12 by 2 mm. with a very small yellow center (two other immunized rabbits, R 10 and 12, show no lesion at the site of inoculation at this time). A very dense collar of polymorphonuclear leucocytes surrounds a small clear area with few cells and relatively large numbers of streptococci. The nuclei of the innermost cells are greatly fragmented. The intact polymorphonuclear leucocytes show considerably more phagocytosis than do those in the control rabbit.

*After 24 Hours.—*

*R 9, Control.*—A superficial abscess is present on the skin; it measures 13 by 18 by 2 mm., with a definite yellow central part. There is extensive necrosis of the areolar tissue of the corium. Practically no cells but great numbers of streptococci in chains and clumps are present in this area. The necrotic tissue is surrounded by a wide dense zone of polymorphonuclear and mononuclear leucocytes and lymphocytes. A slight amount of phagocytosis is noted.

*R 10, Immunized.*—The site of injection shows slight redness that measures 6 by 6 mm.; there is no edema. In the section there is a dense collection of polymorphonuclear leucocytes associated with localized necrosis of tissue. There are many mononuclear macrophages, some of which have ingested polymorphonuclear leucocytes. Very few cocci are seen free in the tissue. A considerable number are contained within the cytoplasm of both the polymorphonuclears and macrophages.

*After 48 Hours.—*

*R 11, Control.*—The skin lesion is a large abscess that measures 35 by 25 by 4 mm.; superficial ulceration measures 1 cm. across. A section shows that the abscess involves all layers of the skin; the central part is necrotic. There are many polymorphonuclear and mononuclear leucocytes throughout the edematous tissue. A few small clusters of blue-staining cocci are seen free in the tissue. A relatively large number are contained within the polymorphonuclear leucocytes.

*R 12, Immunized.*—The skin lesion is quite erythematous and measures 12 by 12 by 2 mm. There is no gross evidence of necrosis. The section of skin contains a well localized abscess composed of necrotic cells and tissue surrounded by a zone of polymorphonuclear leucocytes, together with a large number of macrophages that have ingested polymorphonuclear leucocytes and contain a considerable amount of nuclear debris. A few scattered cocci are present, most of them within the cells.

### *Inguinal Lymph Nodes*

The changes in the lymph nodes parallel rather closely those of the corresponding skin lesions described above. The lymph nodes from the normal and immune rabbits do not differ conspicuously either in the gross or microscopically. However, several features are worthy of note.

*Control Rabbits.*—In the normal rabbits the lesion is associated with a greater polymorphonuclear infiltration up to 12 hours. Mononuclear leucocytes appear in fairly large numbers after 12 hours and a few scattered cocci are seen in practically every section. Within 24 hours small focal abscesses, quite definite after 48 hours, are appearing; after 4 days these abscesses become confluent. At this time suppurating thrombi occlude many of the lymphatics on the periphery of the node. After 7 days in an animal dying with a generalized bacteremia there are

somewhat similar changes, but mononuclear cells have entered these lymphatics and there are large clumps of bacteria in necrotic lymphatic tissue and in the cortical lymph sinus.

*Immune Rabbits.*—In the immunized rabbits inflammation is somewhat less intense and at 48 hours is definitely subsiding with a diminution in number of polymorphonuclears. The mononuclear leucocytes appear after 4 hours in moderately large numbers. It is difficult to compare the number of microorganisms seen in the two groups; however, in several of the immune animals they were found with more difficulty and were probably present in smaller numbers.

From observations described above, it is evident that in normal rabbits streptococci multiply at the site of inoculation in most instances, and rapidly cause the formation of a large local abscess. When the rabbit recovers the lesion heals and the organisms disappear, whereas if the animal dies with bacteremia streptococci are found in large numbers at the site of inoculation as well as in the adjacent lymph nodes. No deposition of fibrin was seen in the skin lesions; however, after 4 days many of the lymphatics are occluded with suppurating thrombi. In the immune group the site of injection remains smaller, never ulcerates, and the inflammation disappears within a relatively short time. There is more phagocytosis in the lesions of the immune than in those of the normal rabbits.

Comparable skin sections from rabbits injected with an avirulent hemolytic streptococcus are included in Text-fig. 4. It is readily seen that rabbits immunized with a virulent streptococcus have reacted to subsequent injections of the same organism in much the same way that normal animals have reacted to a first injection of an avirulent streptococcus.

#### DISCUSSION

In comparing the fate of virulent hemolytic streptococci injected into both normal and immunized rabbits with that of avirulent streptococci under the same conditions, only one strain of each has been used. Smaller doses of virulent than of avirulent streptococci have been used in most instances to produce immunization.

When small amounts (0.1 cc. of a 1:100 dilution) of virulent hemolytic streptococci were injected into the skin of normal rabbits they multiplied at the site of inoculation and were still present after 3 weeks (Table I). The organisms invaded the lymph nodes and were demon-

strable in large numbers up to 7 days. In several animals, they were recovered from the blood stream and spleen. Two animals died. When avirulent hemolytic streptococci were injected intracutaneously the result was different. The organisms multiplied for a short time in the skin and were practically all dead after 12 hours. They passed to the lymph nodes in small numbers for a short time after inoculation and a few were obtained from the heart's blood and spleen. When larger doses were given organisms were not recovered from the blood as with the more virulent organisms.

Although there was some slight multiplication of avirulent hemolytic streptococci in the skin of normal rabbits after intracutaneous injection (1), it never at any time approached the degree of multiplication of virulent hemolytic streptococci injected into the skin of normal rabbits. The numbers of streptococci recovered from the inguinal lymph nodes were also much greater following the injection of virulent organisms.

There are few observations on the fate of bacteria injected into the skin but numerous studies of inoculation into the blood stream (Buxton and Torrey (3), Bull (4)). Hopkins and Parker (5) found that a sublethal dose of hemolytic streptococci with a low virulence for rabbits disappeared from the blood stream in a few hours. After a lethal dose (5) over 90 per cent of the cocci were removed from the blood stream in the first few minutes; they were at a minimum at 2 to 3 hours, but after 4 to 6 hours they began to increase and persisted until the death of the animal. In our experiments, after intracutaneous injection there is seldom if ever any immediate local diminution, but multiplication begins during the first 2 hours.

Many workers have been able to immunize rabbits against streptococci, whereas others have been less successful.

Gay and Rhodes (6), using living cultures of the H streptococcus that Dr. Gay kindly furnished to us, were able to protect rabbits against experimental erysipelas produced by intradermal injections of the same strain of hemolytic streptococci. McLeod (7) on the other hand experienced great difficulty in immunizing rabbits against strains of streptococci of which the virulence may be enhanced by animal passage. Varying results have been obtained with heat-killed vaccines. Hopkins and Parker (5) were unable to protect rabbits against streptococci with repeated intravenous inoculations of heat-killed organisms over a period of 8 months.

Day (8) immunized white mice with intraperitoneal injections of heat-killed

vaccines prepared from both fully virulent and less virulent streptococci of the same strain. The mice then received intraperitoneal injections of living virulent streptococci. It was found that the mice immunized with the vaccine prepared from the fully virulent streptococcus were better protected than those immunized with the vaccine prepared from the less virulent streptococcus. He concluded that unless the virulence was of a high degree vaccines failed to give protection even to streptococci of submaximal virulence and might even induce susceptibility.

Downie (9) attempted to immunize rabbits with the toxin prepared from filtrates and with heat-killed cocci of a moderately virulent (Dochez) strain and with a highly virulent (Davis) strain. He found that the toxin of the moderately virulent strain immunized against a fatal infection after intradermal or intravenous injection of the same strain, whereas the coccus-immunized rabbits and the controls died. The filtrate of a highly virulent organism afforded little protection when given either intradermally or intravenously against an homologous strain, whereas the heat-killed cocci protected against an homologous strain (Davis) but not against a less virulent (Dochez) one. This group of experiments demonstrates some of the difficulties in the interpretation of streptococcus immunity. Those who have immunized with the living organisms have had more success.

The experiments that have been described show that rabbits inoculated with a moderately virulent H streptococcus are well immunized against the same strain of streptococcus even after the virulence has been considerably increased by animal passage. Injections controlled subsequent inoculations of streptococci with increasing efficiency, demonstrated by both gross and microscopic changes and by bacteriological examinations (Tables I and II). These animals were not tested with increasing doses of streptococci to determine the degree of acquired immunity, and observations concerning immunity are on a qualitative rather than a quantitative basis. The observation of this conspicuous immunity produced in association with a diminishing and slight degree of hypersensitivity, is one of many observations of a high degree of immunity with scant if any hypersensitiveness. The rabbits immunized with virulent streptococci did, however, have some cutaneous hypersensitivity to the streptococcus filtrate, though this was at a maximum before the end of immunization, so that these experiments give no information concerning the relationship between hypersensitivity and immunity.

The histological sections of lesions from normal and immunized rabbits show more phagocytosis in the immune than in the normal rabbits. The early experiments of Denys and Leclef (10) on the

phagocytosis of streptococci showed that there was more phagocytosis by the polymorphonuclear leucocytes in vaccinated rabbits than in normal rabbits. Corresponding observations were made *in vitro*.

It is noteworthy that skin lesions following the injection of avirulent hemolytic streptococci into normal rabbits (1) are similar in gross and histological characters to lesions produced by a virulent hemolytic streptococcus in rabbits that had received repeated injections of virulent streptococci. In other words, a virulent hemolytic streptococcus produced much the same type of reaction in immunized rabbits that an avirulent streptococcus produced in normal rabbits. The fate of the streptococci determined by counting of colonies was found to be similar in the two instances.

The relationship of virulence to the production of immunity is still obscure, although there are several observations indicating that increased virulence promotes immunization. The ability of virulent hemolytic streptococci to multiply in the body and invade the blood stream may help to explain why animals injected with virulent organisms develop a high degree of immunity.

#### SUMMARY AND CONCLUSIONS

1. Hemolytic streptococci, highly virulent for rabbits, when injected into the skin of normal animals increased greatly in number at the site of injection during the first 12 hours, diminished somewhat after 24 hours but still persisted after 21 days. They produced large abscesses within 24 hours, there was ulceration, and healing occurred after about 5 weeks. Histological observations confirmed the bacteriological evidence that the streptococci underwent great multiplication at the site of their injection.

2. Virulent hemolytic streptococci injected into the skin of normal rabbits appeared in small numbers within 1 hour in the lymph nodes. As multiplication proceeded in the skin, hemolytic streptococci were found in large numbers from 1 hour to 7 days after inoculation in the inguinal lymph nodes.

3. Hemolytic streptococci were recovered from deeper lymph nodes, that is, from the iliac nodes, but only in animals of which the inguinal lymph nodes contained bacteria in relatively large numbers.

4. Virulent hemolytic streptococci injected into the skin of normal



rabbits in some instances entered the blood stream in considerable number, and occasionally caused death with bacteremia. Streptococci were recovered more frequently from the spleen and were present in this organ only when they had been recovered from the deep (iliac) lymph nodes.

5. When virulent hemolytic streptococci were injected into the skin of immunized rabbits, in a few instances they increased in number for a short time, but usually diminished rapidly and had entirely disappeared in 48 hours. The gross lesions were smaller than in normal rabbits. There was more phagocytosis, and redness and edema had disappeared after 48 hours.

6. When virulent streptococci were injected into the skin of immune rabbits they passed to the regional lymph nodes in relatively smaller numbers than in the previously normal controls and appeared in these nodes in considerable numbers only in animals in which there had been conspicuous multiplication at the site of inoculation. No streptococci could be found in the iliac lymph node, blood or spleen.

7. Virulent streptococci injected into the skin of normal animals multiply actively, resist phagocytosis, invade the tissues widely, enter adjacent and distant lymph nodes and in some instances are distributed by the blood stream to internal organs. After immunization associated with some sensitization, virulent streptococci are more readily ingested by phagocytes, remain sharply localized, are rapidly destroyed, fail to pass the nearest lymph nodes and do not enter the blood stream.

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