RELATION BETWEEN THE VIRULENCE OF STREPTO-COCCI AND HEMOLYSIN.

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Since the original observations on streptolysin by Marmorek (1) in 1902, there has been much discussion concerning the relation between the hemolytic property and the pathogenicity of streptococci. Clinical and laboratory studies have been made, but, due to the complexity of the problem, the opinions arrived at have been contradictory. In 1914, M'Leod (2) reviewed the literature, and although he believed that there was an intimate connection between hemolytic power and virulence, he considered that there had been no solution of the problem which had been generally accepted. To attempt a solution of the problem purely on a clinical basis is obviously impossible on account of the great variations in susceptibility of individuals to infection. This is undoubtedly the reason that observations depending on the course of any infection as an indication of pathogenicity have led to such indefinite and opposed conclusions. If it is possible to establish definite facts in the laboratory under constant environment suitable for the growth of the bacteria, the same methods can probably be applied to the clinical phase of the problem.

The ideal laboratory experiment has been indicated by M'Leod (3). Briefly, it consists in testing the hemolytic power of the streptococcus *in vivo*; there are, however, no trustworthy methods with which this procedure can be accurately carried out. The obvious substitute is to observe the hemolytic titer of virulent strains in the serum of the animal for which they are pathogenic. We attempted to use rabbits in this manner but had great difficulty in obtaining strains which were

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of constant virulence, and, furthermore, it was found that the hemolytic titers of cultures growing in rabbit serum varied considerably. In fact the only medium which gave consistent maximum titers was 20 per cent horse serum broth. Owing to these difficulties we have attempted first to determine the relation between pathogenic and non-pathogenic strains in respect to their lytic power, when they are grown in the serum of an animal for which their virulence has not been especially increased.

Method.

Media.—The character of the media employed is undoubtedly the most important essential for the production of strong hemolysin. Beef infusion broth to which horse serum had been added to 20 per cent of the volume was found to be the most satisfactory for the comparison of various strains. If the broth is made with 2 per cent peptone the titers are quite constant in different experiments with the same streptococcus. To ensure uniformity all the media used in this series were made at the same time from the same lot of beef infusion. They were titrated so that the pH was 7.6 after sterilization, and were distributed in quantities of 80 cc. in 250 cc. Pyrex flasks. The horse serum was obtained from the same horse in each instance. While it was still fresh 20 cc. were added to each flask. The contents of the flasks were then inactivated at 56°C. on 3 successive days and stored on ice until used. In this way it was possible to grow the cultures under conditions in which the available protein substances and the antihemolysins were constant.

Seeding and Bacterial Counts.—The second factor which may vary sufficiently to destroy the accuracy of the results is the nature of the seeding. According to De Kruif and Ireland (4) young cultures produce streptolysin more rapidly than those which have ceased to grow actively. Since it had been previously found (5) that, under the conditions which we employed, the period of active growth ceased after about 16 hours, the flasks were seeded with horse serum broth cultures from 14 to 18 hours old. The seeding was controlled by counting a dilution of bacteria in a weak aqueous solution of methylene blue. A Helber counting chamber with a thin cover-glass was used so that the count could be made with either a high power dry lens

or with an oil immersion lens. The chains of bacteria were counted and the average number of individuals in each group was estimated by counting the cocci in a film made from the dilution pipette. It was possible to approximate the number of cocci in each cubic centimeter of the culture used for seeding by this method. On account of the inaccuracy of the bacterial counts each experiment was done in duplicate or triplicate by seeding several flasks so that they contained approximately 1, 5, and 10 million per cc. The flasks were counted a second time before incubation by the dilution and plating method described in a previous article (5), and the growth of the cultures was followed at intervals in a similar manner. It was impossible to count the streptococci with any great degree of accuracy, but by seeding several flasks with varying numbers of cocci, a comparable growth was obtained in at least two. After several experiments it was found that the counts were unnecessary because in the tests in which the flasks were seeded within a fairly close range, the minimum hemolytic dose was the same in each flask of the triplicate experiments.

Hemolysin Titers.—Hemolysis was determined with the supernatant fluid obtained after centrifuging a portion of the culture at high speed for 10 minutes. The supernatant fluid was set up in a series of tubes with 1 cc. of 5 per cent mouse cells washed and suspended in isotonic salt solution. The smallest quantity of fluid necessary to hemolyze these cells completely without leaving sediment was called the titer of the culture. These determinations were made throughout the experiment, but more frequently during the time that streptolysin production was at a maximum. Since it is impossible to compare titers with different suspensions of red cells, a sufficient quantity was prepared to complete a single experiment. The character of the red cells does not change sufficiently when they are suspended in isotonic salt solution to vitiate results obtained within a period of 16 to 18 hours after the cells are placed in suspension. The titers obtained by these methods were practically constant for each strain.

EXPERIMENTAL.

Five strains of beta type streptococci obtained from acute human infections, in some instances from the blood stream and in others from pleural exudates, were used. They conformed to the *Strepto-*

coccus pyogenes of Holman (6) and gave a final hydrogen ion concentration of pH 4.9 to 5.2 in glucose broth. Before these strains were used for the present experiments they were stored on blood agar and transplanted at frequent intervals during a period of several months, in order that they might lose their original virulence for animals.

At the beginning of the experimental work the invasive power of each strain was determined on mice of approximately the same weight. They were then passed through mice by intraperitoneal injection and obtained in pure culture from the heart's blood. The doses were regulated so that the animals died within 24 hours. After each passage the strain was transferred to rabbit blood agar in the second subculture. When each streptococcus was sufficiently invasive, the avirulent and virulent forms were transplanted from the blood agar tubes into horse serum broth, then, after an interval of 14 to 18 hours, the trial flasks of bouillon were seeded with the necessary quantities of these cultures. In this way the streptolysin production was determined with actively growing cocci which were accustomed to the media in which the test was made. After the hemolysin tests, the virulence of the streptococci was again determined on mice with the corresponding subcultures from the stock media.

Experiment 1.—Strain J, which had been in stock culture for 2 months was passed through twenty mice. At the end of the passages the original culture and the virulent mouse strain were grown in horse serum broth and seeded into 100 cc. flasks of media. The seedings were made so that there were three flasks for each strain containing approximately 1, 5, and 10 million cocci per cc. The bacterial counts (Text-fig. 1) and the hemolysins were determined at frequent intervals until after the maximum streptolysin production had occurred. The pathogenicity for mice was determined immediately after the experiment, with a 24 hour plain broth culture of Strains J(0)5 and J(20)5.[1] The results of the tests are shown in Tables I and II.

Experiments 2 to 5.—The previous experiment was repeated with Strains K, M, R, and S. Only two seedings were used for each strain, however, one of 1 and one of 10 million cocci per cc. Table III summarizes the maximum titers, the hour at which they occurred during the growth of the culture, and the pathogenicity of the various strains for mice.

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[1] The strains have been indicated by letters, the number of passages by a figure in parentheses, and the subculture by the second numeral.

TABLE I.

Streptolysin Production by Strain J.

| Interval.	1 million.*		5 million.		10 million.	
	Count.	Titer.†	Count.	Titer.	Count.	Titer.
Strain J(0)5, not passed through animals.						
hrs.		*cc.*		*cc.*		*cc.*
0	$1.1\ (10^6)$‡	0.0	$5.5\ (10^6)$	0.0	$1.1\ (10^7)$	0.0
3½	$5.0\ (10^7)$	0.0	$4.7\ (10^8)$	0.0	$6.0\ (10^8)$	0.6
6		0.02		0.04		0.04
7½	$1.0\ (10^9)$		$1.3\ (10^9)$		$1.0\ (10^9)$	
8		0.06		0.08		0.08
10		0.06		0.2		0.15
12	$8.0\ (10^8)$		$7.5\ (10^8)$		$2.0\ (10^9)$	
Strain J(20)5, after passage through animals.						
0	$2.4\ (10^6)$	0.0	$1.2\ (10^7)$	0.0	$2.4\ (10^7)$	0.0
4	$1.5\ (10^7)$	0.0	$7.3\ (10^7)$	0.0	$5.6\ (10^8)$	0.0
5½		0.4		0.06		0.08
7		0.4		0.04		0.04
8	$6.5\ (10^8)$		$1.6\ (10^9)$		$7.8\ (10^8)$	
9		0.04		0.06		0.06
11	$9.8\ (10^8)$	0.04	$7.0\ (10^8)$		$8.1\ (10^8)$	
12		0.06				

* The counts designated in millions were calculated from the direct counts used for seeding. The others are expressed in powers of ten.

† The titers are the smallest amounts of the supernatant fluid which completely hemolyzed 1 cc. of the cell suspension after 1 hour at 37°C.

‡ All counts in the columns were made from dilutions and plates.

TABLE II.

Pathogenicity of Strain J for Mice.

Strain.	Quantity of culture.	Length of time animal survived.
	cc.	*hrs.*
No. J(0)5, not passed through animals.	1.4	18
	1.2	18
	1.0	(Recovered.)
	0.8	"
	0.6	"
No. J(20)5, after passage through animals.	0.2	17
	0.1	17
	0.08	17
	0.06	18
	0.02	(Recovered.)
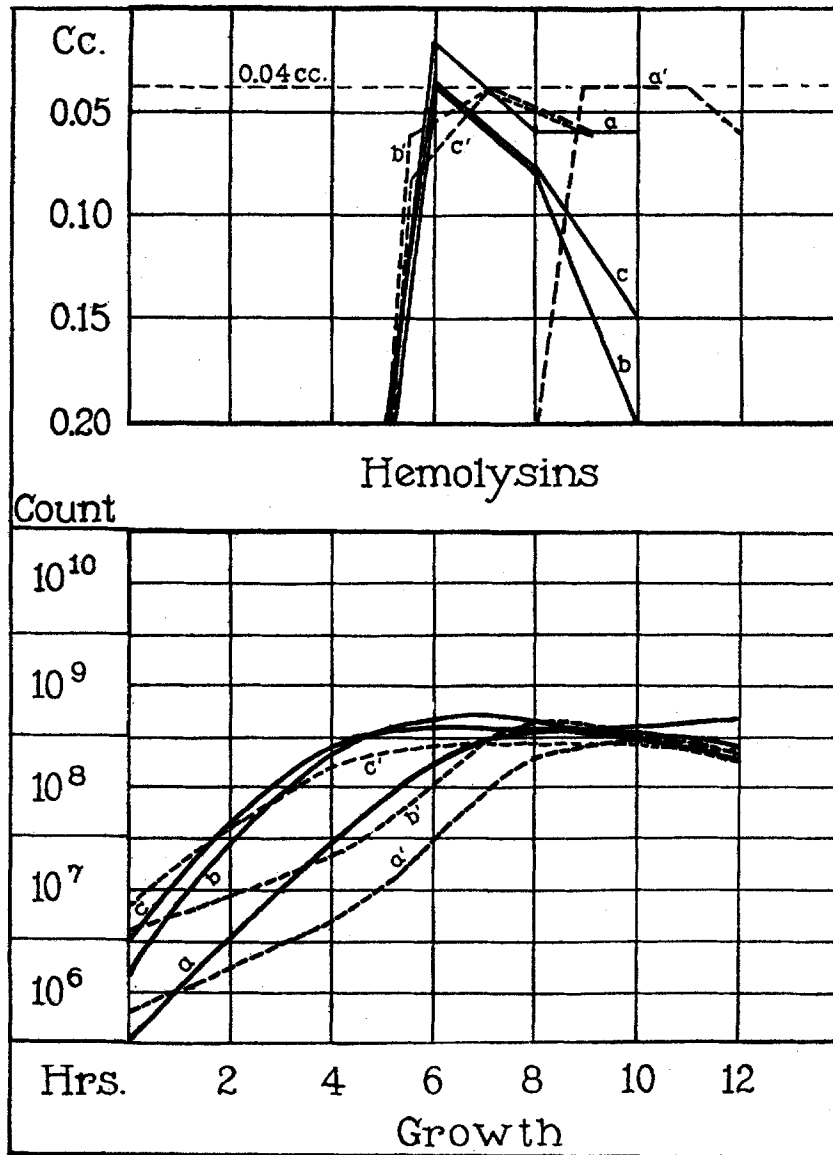
TEXT-FIG. 1. Comparison of the growth and of hemolysin production by Strain J before and after mouse passage. The counts are charted logarithmically. Curves *a* and *a'* give the results obtained with original seedings of 1 million, Curves *b* and *b'* with original seedings of 5 million, and Curves *c* and *c'* with original seedings of 10 million.

TABLE III.

Streptolysin Production and Pathogenicity for Mice of Strains J, K, M, R, and S.

Strain No.	Seeding.	Titer.	Time at which maximum titer occurred.	Pathogenicity.*
	million†	*cc.*	*hrs.*	*cc.*
J(0)5	1	0.02	6	1.2
	5	0.04	6	
	10	0.04	6	
" (20)5	1	0.04	9	0.06
	5	0.04	7	
	10	0.04	7	
K(0)2	1	0.04	7½	1.0
	10	0.04	7½	
" (17)2	1	0.04	12	0.1
	10	0.04	12	
M(0)4	1	0.04	10	‡
	10	0.02	8	
" (19)4	1	0.02	8	0.5
	10	0.02	8	
R(0)4	1	0.04	8	0.4
	10	0.01	8	
" (19)4	1	0.02	8	0.03
	10	0.04	8	
S(0)2	1	0.04	6½	1.0
	10	0.04	6½	
" (17)2	1	0.04	10	0.2
	10	0.04	8	

* The pathogenicity is indicated by the smallest quantity of a 24 hour broth culture fatal to mice in 24 hours.

† Based on direct counts and controlled by dilution and plating.

‡ Animals survived doses of 5 to 10 cc. of centrifuged culture.

DISCUSSION.

From the results of these experiments it appears that both the virulent and avirulent types of each strain produce approximately the same maximum titer at some time during the growth of the cultures. Grown under the conditions previously described the strains have yielded titers in the majority of instances of 0.04 cc.

It is not to be expected that various experiments can be compared in this respect, when they are carried out on different days with different red cell suspensions. The quantities of culture fluid required to hemolyze the cell suspension in any one experiment are fairly consistent, with the exception of one in which Strain R was used in seeding. A certain degree of variation can be anticipated because of the difficulty experienced in reading the last completely hemolyzed tube in a series graded in 0.01 cc. With two of the five strains, M and R, the peaks of the hemolysin curves were simultaneous in the cultures of both the virulent and less virulent forms. With Strains J, K, and S the former lagged from 1 to $4\frac{1}{2}$ hours. This lag was apparently due to the fact that the more virulent strains grew more slowly during the first hours of incubation. This fact was evident from the amount of cloudiness in the flasks as well as from the plate counts. In the two instances in which the highest points of the hemolysin curves were reached simultaneously, the cultures of the strains which had not been passed through animals were moderately hemolytic for some time before the other flasks had any destructive action on red cells.

CONCLUSION.

Strains of streptococci whose virulence has been increased for any one species of animal do not produce greater concentrations of hemolysin than the original strain. Furthermore, there is a tendency for the original culture to grow more rapidly than the more pathogenic form, and to reach the height of hemolysin production at an earlier stage during the growth of the culture. These conclusions can probably be applied only to experiments in which the serum used in the media is from some species not employed for the animal passages.

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