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INFECTIVITY AND ANTIGENICITY OF STREPTOMYCIN-DEPENDENT *SALMONELLA TYPHOSA*

Morton Reitman

Medical Investigation Division
DIRECTOR OF MEDICAL RESEARCH

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ABSTRACT

A streptomycin-dependent mutant of Salmonella typhosa injected live in rabbits stimulated the production of O antibodies. Sera from these rabbits protected mice against challenge with virulent S. typhosa. Mutation to the dependent state did not result in loss of antigenicity. Streptomycin dependency of the mutant was demonstrated in vivo in the mouse. Antibiotic-treated mice succumbed to challenge with dependent bacilli, apparently because of presence of lethal quantities of endotoxin produced by multiplication of the mutant in the presence of streptomycin.

I. INTRODUCTION

Preliminary studies with a dihydrostreptomycin-dependent (DHSM-D) Salmonella typhosa 27 V strain showed that mutation to the dependent state resulted in loss of virulence,[1] and mice injected with this mutant in the viable state were immune when subsequently challenged with virulent organisms. Intravenous injections of viable DHSM-D cells in rabbits stimulated the production of antibody, as shown by titration of H agglutinins. This report presents further work with this strain.

II. MATERIALS AND METHODS

A. CULTURES

Salmonella typhosa 19 V is a streptomycin-sensitive, phage type E, laboratory-stock strain obtained from the University of Maryland. Salmonella typhosa 27 V is a one-step mutant derived from strain 19 V. It requires streptomycin for multiplication and grows well in media containing 10 to 600 microns per milliliter; no growth occurs in media containing more than 800 or less than five microns per milliliter. Salmonella typhosa 58 V and 0901 were obtained from Walter Reed Army Institute of Research; 58 V is a standard strain used for preparation and challenge of typhoid vaccines. Strain 0901 is a standard strain used for preparation of O antigens IX and XII.

B. EFFECT OF STREPTOMYCIN ON INFECTIVITY OF DHSM-D STRAIN

Cultures of S. typhosa 27 V were incubated on nutrient agar slants containing 20 micrograms of dihydrostreptomycin* per milliliter for 18 hours at 37°C and the growths were then washed off with saline. Viable cells were suspended in five per cent mucin** and injected in 0.5-ml amounts intraperitoneally (IP) into 16- to 18-grams mice, Swiss albino, Webster strain. Groups of 10 mice were administered 15,000 units of dihydrostreptomycin subcutaneously (SC) in three divided doses for five days; the first injection was given 30 minutes prior to challenge. Animals that died were autopsied and the survivors were killed at weekly intervals. Livers and spleens were each ground separately in a mortar and pestle containing one milliliter of saline per liver and 0.5 milli-

* Phizer.
** Wilson, Type 1701.

liter of saline per spleen. Samples of 0.1 milliliter were cultured on desoxycholate agar* with and without 200 units per milliliter of dihydrostreptomycin.

C. TITRATION OF O AGGLUTININS

White albino rabbits received a series of six injections at seven-day intervals of (a) living DHSM-dependent cells (2.67×10^9) suspended in saline, or (b) DHSM-sensitive parent strain killed with 0.3 per cent formalin.[1] Agglutinating antigen was prepared by boiling a smooth saline suspension of S. typhosa 0901 for two hours. Sera prepared from blood drawn immediately prior to injection and at designated intervals after the immunization series were kept frozen until assayed. All titers were determined at the same time by incubating antigen-serum mixtures at 52°C for 16 to 18 hours. The titer was recorded as the highest dilution of serum that caused clumping of the antigen.

D. RECIPROCAL ADSORPTION TEST

Individual sera from each group of rabbits were pooled and diluted 1:5 with saline. Each pooled serum was divided into three samples; one sample was adsorbed with the parent-sensitive strain, one was adsorbed with the dependent strain, and one was unadsorbed. The unadsorbed control serum was carried through all the steps of the procedure. Five-ml amounts of serum were added to packed cells and incubated for one hour at 50°C. The serum-antigen mixture was then centrifuged at 3000 rpm for 20 minutes and the supernatant fluid was added to fresh cells. This was repeated twice. Agglutinin titers of adsorbed and control sera were determined with antigens prepared from both parent-sensitive and mutant-dependent strains. Serum-antigen mixtures were incubated in a water bath at 50°C for one hour and then placed in the refrigerator overnight before being read.

E. PASSIVE PROTECTION TEST

Mice (14 to 16 grams) were administered intraperitoneally 0.5-ml amounts of rabbit antiserum one hour prior to a challenge dose of S. typhosa 58 suspended in mucin. Normal rabbit serum was administered as a control.

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* Baltimore Biological Laboratories, dehydrated.

III. EXPERIMENTAL RESULTS

A. EFFECT OF STREPTOMYCIN TREATMENT ON VIRULENCE OF DHSM-D STRAIN

Eight of ten mice that received streptomycin plus 10^7 viable dependent cells died within 48 hours, and four of ten treated mice challenged with 10^6 cells succumbed in the same period (Table I). Only one of ten untreated controls died. S. typhosa was recovered in large numbers from the liver and spleen of all dead animals with the exception of two carcasses that were destroyed by cannibalism before autopsies could be performed. Large numbers of typhoid bacilli were recovered from the liver and spleen of all except one of the survivors that had been treated with streptomycin. This animal had received 10^6 cells and was sacrificed on Day 14 postchallenge. Organisms recovered were only of the dependent type.

Dependent organisms were recovered in very small numbers from three untreated animals sacrificed on Day 7 and from two of the three sacrificed on Day 14. None were recovered from the untreated mice on Day 21.

It is well established that fatal infection in the mouse is due to the toxicity of the organism[2,3] and that the toxicity lies in the somatic antigens O and Vi, with the O complex being more toxic than the Vi antigen.[4] To determine if the streptomycin-dependent strain contained an endotoxin capable of causing fatal reactions in the mouse, sonic extracts of the DHSM-D strain were prepared and injected intraperitoneally. A dose of 1.9 milligrams (dry weight) killed six of ten mice within 48 hours. All mice showed symptoms of toxemia.

B. O AGGLUTININS

Figure 1 shows the O antibody titers obtained in rabbits injected intravenously with the viable dependent strain and with a formalinized dead antigen derived from the parent nondependent strain. Little difference was apparent in the titers stimulated by parenteral introduction of either strain. Peak titers of 1:2560 to 1:5120 appeared one week after the second injection and then rapidly declined to insignificant levels four weeks after the last (sixth) injection.

Titers dropped seven days after the third and fifth injections. This depression may be due to a negative phase that has been found to occur in animals containing circulating homologous antibody at the time of injection.[5] Dean and Webb[6] injected horse serum at five-day intervals in previously immunized rabbits. This was followed by an immediate fall in precipitin titer, which began to rise on the third day with maximum response on the fifth to eighth day after injection.

TABLE I. EFFECT OF DIHYDROSTREPTOMYCIN ON VIRULENCE OF DEPENDENT S. TYPHOSA IN GROUPS OF TEN MICE

Challenge Dose in 5% Mucin	Treatment	Recovery[a] of Dependent Bacilli from										Dead/ Injected
		Mice Dying on Day				Survivors Killed on Day						
		1		2		7		14		21		
		Liver	Spleen	Liver	Spleen	Liver	Spleen	Liver	Spleen	Liver	Spleen	
10^7	Streptomycin[b]	T[c]	T	T	T	2460	42680	2280	5180			8/10
		T	T	T	T							
		T	T	T	T							
		T	T									
		T	T									
10^6	Streptomycin[b]	T	T	T	T	10000	58000	11160	69600			4/10
		T	T	D[c]		840	58000	0	0			
						2760	58000	1780	34470			
10^7	Saline			D[c]		60	810	0	0	0	0	1/10
						60	920	0	230	0	0	
						40	1500	40	0	0	0	
None	Streptomycin[b]											0/10

a. Recovered on desoxycholate-streptomycin agar; no recoveries on streptomycin-free agar. Calculated from colony counts.
b. 5000 units administered subcutaneously thrice daily.
c. Abbreviations used: T = Too numerous to count; D = Destroyed by cannibalism.

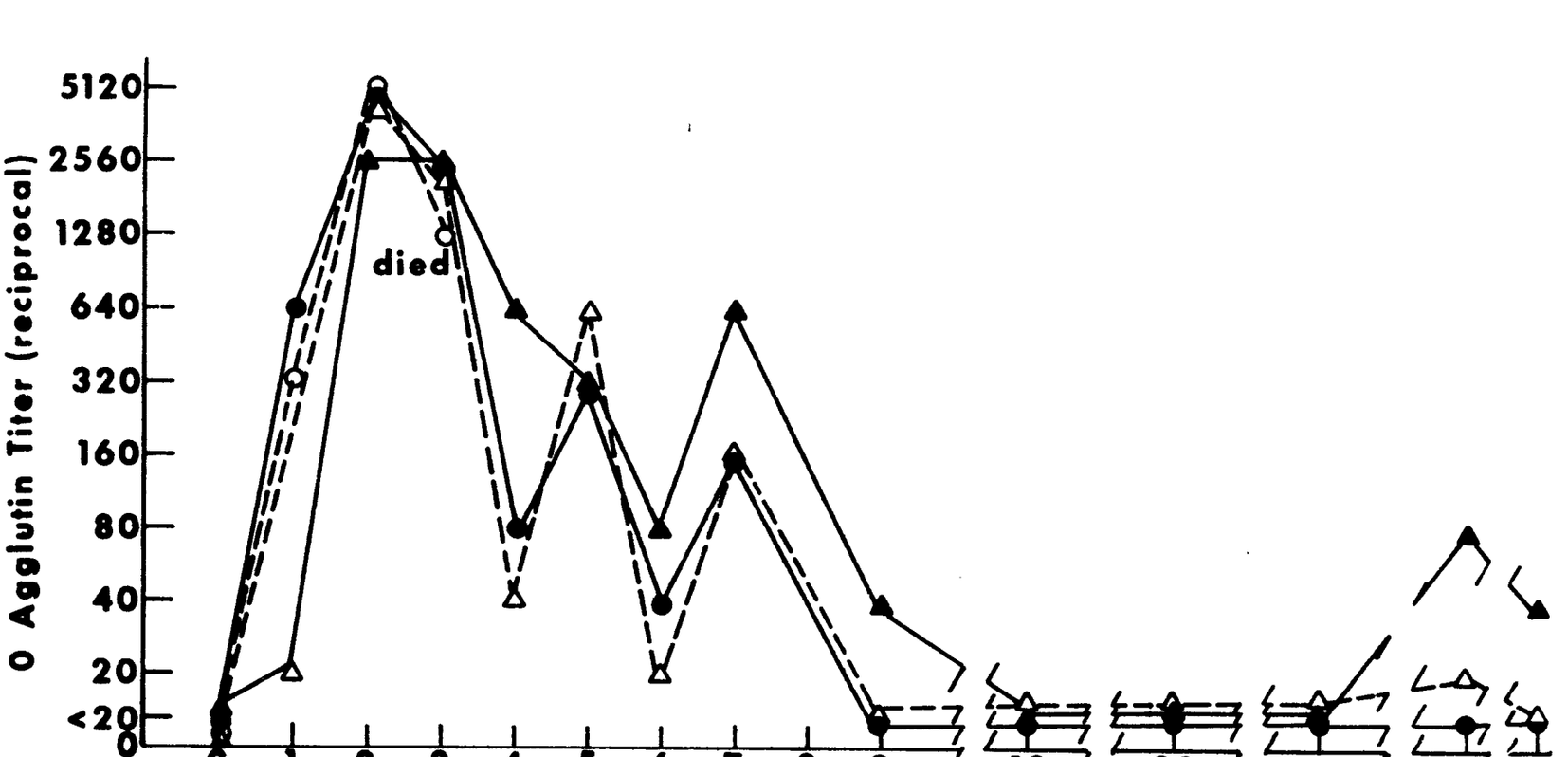

Figure 1. O Agglutinin Titers Produced in Rabbits Injected with Viable Streptomycin-Dependent and Nonviable Formalinized, Streptomycin-Sensitive Salmonella typhosa. _____ DHSM-D S. typhosa 27 V, ---- formalinized S. typhosa 19 V.

C. ADSORPTION TESTS

Reciprocal adsorption tests were made with pooled sera obtained one week after the second injection. The parent strain adsorbed all the agglutinins out of the dependent- and parent-strain-stimulated antisera. While the dependent strain adsorbed out all demonstrable agglutinins present in the dependent-stimulated antiserum, a low agglutinin level was still present in the parent-stimulated antiserum (Table II), as evidenced by the 1:40 and 1:20 titers obtained with parent and dependent antigens, respectively. The residual titers obtained were of the same order of magnitude and therefore were considered to be insignificant.

D. PASSIVE PROTECTION IN THE MOUSE

Table III shows that serum from rabbits that had received either the living dependent antigen or the killed streptomycin-sensitive antigen protected mice against approximately 40,000 LD_{50}'s of S. typhosa 58 in mucin.

E. STABILITY OF DHSM-D STRAIN

A total of 19 transfers were made on streptomycin agar during the course of these experiments. Attempts to recover streptomycin-sensitive or -resistant cells by plating large numbers of dependent cells on streptomycin-free agar were unsuccessful. In addition, no reversions to independence were noted *in vivo*.

IV. DISCUSSION

The comparable O antibody titers reported in this paper and the H antibody titers reported in a previous publication[1] obtained by injection of the viable dependent strain and formalinized sensitive cells, along with the ability of the antiserum produced to passively protect mice against large challenge doses of S. typhosa, attest to the antigenicity of both the dependent and the sensitive (parent) strain.

Research directed toward the improvement of antityphoid immunizing agents has been based mainly on attempts to prepare vaccines that will retain unaltered antigens responsible for active protection. The Vi antigen, which has been found to be an important protective antigen in mice experimentally infected with S. typhosa,[7] is considered by many workers to be important in conferring active protection in humans.[8,9]

TABLE II. RECIPROCAL ADSORPTION OF IMMUNE SERA FOR PARENT-SENSITIVE STRAIN AND MUTANT-DEPENDENT STRAIN

Immune Sera for	Adsorbed with	Agglutinin Titer when Tested Against	
		Parent Strain	Dependent Strain
Parent[a]	———	1:640	1:640
	Parent	0[b]	0
	Dependent	1:40	1:20
Dependent	———	1:1280	1:640
	Dependent	0	0
	Parent	0	0

a. Pooled.
b. Lowest dilution tested = 1:10.

TABLE III. PASSIVE PROTECTION OF MICE OBTAINED WITH ANTISERA FROM VACCINATED RABBITS. CHALLENGE WITH S. TYPHOSA 58[a]

Vaccine	Serum No.	Titer H	Titer O	Serum Amounts, ml 0.1	0.033	0.011
S. typhosa 19 V (Killed)	2	640[b]	160	0/4[c]	0/5	1/5
S. typhosa 27 V (Live)	5	640	160	0/5	0/4	0/5
S. typhosa 27 V (Live)	6	640	640	0/5	0/5	0/5
None	(Normal)	0	0	4/5		

a. Challenge dose - 3.75×10^5 in 5% mucin. All injections IP in 0.5-ml amounts.
b. Reciprocal of dilution.
c. Numerator denotes number of mice dead; denominator denotes number injected.

Because the Vi antigen is extremely labile, many workers have sought to prepare an improved typhoid vaccine by means of various killing agents without destroying the Vi immunogenic activity. It is also conceivable that the antigen responsible for active protection may be a labile-hidden antigen that has not been demonstrated. No loss in antigenicity was demonstrated in the dependent mutant, as shown by the results of the reciprocal adsorption tests. This was further borne out by tests with monospecific testing fluids.

Important safety factors to be considered in the use of dependent organisms as viable vaccines are virulence of the strain and reversion to independence. The dependent strain has been shown to be avirulent in mice even when injected in large amounts suspended in mucin.[1] Also, large quantities of viable cells caused no noticeable local or constitutional reaction in the rabbits that received intravenous injections of the viable vaccine. Vaccination of humans with a streptomycin-dependent strain of Brucella abortus produced strong post-vaccinal reactions in 14 of 80 persons.[10] Four of these individuals were known clinical cases; the rest were apparently latent brucellosis cases.

The establishment of partial or complete virulence of dependent organisms has been shown to occur *in vivo* when the substance that the organism requires is injected along with the organism.[11-15] In the experiment dealing with the effect of streptomycin on the virulence of dependent cells in mice it was found that partial virulence could be demonstrated. The failure to recover any but dependent cells from the liver and spleen of dead and surviving mice indicates that infection and lethalities were due to multiplication of dependent cells and not to reversion to streptomycin resistance.

Although the virulence experiment did not provide enough infected streptomycin-treated mice for determining the effect of treatment on prolongation of infection, it should be noted that dependent bacilli were recovered from treated or untreated mice 14 days after inoculation but no recoveries could be made at 21 days in the untreated animals. The presence of the organism in much greater numbers in streptomycin-treated mice presents further evidence of the enhancement of multiplication brought about by the presence of the antibiotic.

Although typhoid bacilli were present in fairly large numbers in the liver and spleen of streptomycin-treated animals, the failure of continued treatment to cause any deaths beyond the second day after inoculation may be due to the unavailability of the antibiotic to the organism. Rous and Jones[16] observed that living phagocytes are capable of protecting ingested typhoid bacilli from the action of potassium cyanide contained in the surrounding fluid. Protection of intracellular *Brucella* from the lethal action of streptomycin has been demonstrated with phagocytized *Brucella* and in infected mouse spleens by Magoffin and Spink.[17] Kornegay *et al*[18] reported that the liver and spleen of the mouse do not remove streptomycin from the blood or store the antibiotic. They found that intramuscular injections of 5,000 or 10,000 units per kilogram of mouse weight produced a maximum blood level of 8.5 units per milliliter, which dropped to zero at two hours. It has been reported that streptomycin that penetrates mammalian tissue cells retains its biologic properties.[19] This antibiotic has been shown to penetrate into mouse fibroblast cells and inhibit multiplication of *S. typhosa*. Thus, it is quite conceivable that only the first few injections of streptomycin might be available for the organisms in the peritoneal cavity. As long as the antibiotic is accessible to the organism in sufficient concentration, multiplication ensues, endotoxin increases and, when a toxic level is reached, the animal dies.

V. SUMMARY

Streptomycin-dependent typhoid bacilli were found to be dependent on streptomycin *in vivo* in the mouse. Avirulent dependent cells were virulent for streptomycin-treated mice.

The dependent cell was shown to contain an endotoxin lethal for mice.

Dependent *S. typhosa* stimulated the production of high-titered O agglutinins in rabbits.

Agglutinin-adsorption tests demonstrated that the dependent strain is antigenically similar to the streptomycin-sensitive parent strain.

Antiserum produced in rabbits by injection of living dependent cells protected mice against challenge with many lethal doses of virulent typhoid bacilli suspended in mucin.

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