A COMPARATIVE STUDY OF ADAPTED BACTERIOPHAGE WITH SHIGELLA DYSENTERY ANTIGENS AND THE PURIFICATION OF BACTERIOPHAGE-TOXINS AND EXOTOXIN

Thesis for the Degree of Ph.D.

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By

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INTRODUCTION

The dysenteries have afflicted man for centuries. They are severe or mild inflammations of the lower intestinal tract, that are at least partially characterized by frequent painful evacuations of bloody mucopurulent stools. The organisms are present in the intestinal tract, and occasionally in the mesenteric glands, but do not invade the urinary tract or the blood stream.

There are two main types of dysentery bacilli:

1. Shigella dysenteriae which produces acid in glucose and which produces no acid in mannite, lactose, and dulcite, and does not form indole.
2. Shigella paradysenteriae (Flexner, Hiss (Y), Strong, and others) which produce acid in glucose and mannite and form indole.

The Shiga type is type-specific in the antigen content and may be differentiated from the paradysentery group by specific immune sera. It produces an exotoxin (neurotoxin) as well as an endotoxin (enterotoxin). The presence of these two toxins have been determined by a number of independent investigators during recent years.

In 1898, Shiga (1) discovered in the stools of patients, suffering from dysentery in Japan, the micro-organism which bears his name. Following Shiga's work, discoveries by Kruse in Germany and Flexner in the Philippines further confirmed Shiga's work

that bacteria cause dysentery. In recent years a number of strains of paradysentery, with some cultural and antigenic similarities to the Shiga type, have been studied but the virulence of these related types has been shown to be less than that of Shig. dysenteriae and they do not produce neurotoxin. These basic differences were the main factors for the Shiga type for research.

The general impression is that dysentery in its severest form is to be found in tropical or semitropical countries, and while it is true that most outbreaks are caused usually by the paradysentery and related groups there are occasional sporadic outbreaks of Shigella dysentery in the United States. At Owosso, Michigan, an instance of the occurrence of this type is brought to our attention. The Owosso strain of Shigella dysentery was obtained through the courtesy of the Michigan Department of Health.

The transmissible lytic substance, called bacteriophage, was discovered independently by Twort in 1915 and by d'Herelle in 1917. d'Herelle has stated that he first observed the phenomenon, in Mexico, in 1909, while working on a bacterial disease of locusts.

The nature of bacteriophage is still a subject of dispute, but the production of this substance in the absence of bacterial cells by Krueger and Baldwin (2) will lead, no doubt, to further clarification of this point. It is not the purpose of this research to delve further into the nature of bacteriophage, but to study the antigenic characters designated by Larkum (3) and later Holm, Anderson, and Leonard as "Staphylococcus Bacteriophage Toxoid. This study will emphasize research on lysed Shig. dysenteriae cells and their bacterial products.

The investigations of bacteriologists who have studied this subject indicate that bacteriophages have antigenic specificities. Animals injected with bacteriophage filtrates develop corresponding antibacterial antibodies. Larkum (3) states that bacteriophage is an antigen of toxoid character, since it does not produce hemolysis and dermonecrosis, is innocuous to rabbits, and will produce antitoxin which neutralizes the toxic properties of staphylococcus toxin. Holm, Anderson and Leonard (4) state that staphylococcus bacteriophage toxoid contains both endo-and exotoxin principles in solution which they designate "Ambo-toxid".

It is the purpose of this research to determine if these bacteriophage antigens are toxoid or toxin in character for Shig. dysenteriae and to compare bacteriophage and purified bacteriophage with Shigella bacterin, exotoxin, bacteriophage-exotoxin, endotoxin and bacteriophage-exotoxoid.

The purification of bacteriophage according to the procedure of Krueger and Tamada (11) and the subsequent discovery that Shigella bacteriophage No. 18 contained both endo- and exotoxins led the author to attempt a similar procedure for the purification of Shigella exotoxin.

EXPERIMENTAL

A review of the literature in reference to types of media required for the production of exotoxin and endotoxin indicates that several media must be used. Olitsky and Kligler (7) have carried out extensive study of the Shigella dysenteriae toxin. They differentiate quite definitely between the exotoxin and endotoxin. Their exotoxin was obtained by growing the organisms for five days in alkaline-egg broth, while their endotoxin was produced by incubating the organisms from agar growths in salt solution for two days. The filtrate from the latter medium contained the endotoxin.

Subsequent data will show that it is unnecessary to use two types of media for the production of both toxins but in the author's experiments two types of media were used in order to prepare bacteriophagic antigens in broth as well as exotoxins and endotoxins on nutrient agar.

Culture media for growth of the organisms were nutrient broth and nutrient agar. Each was adjusted to pH 7.6. Media for fermentation reactions were 1 per cent peptone water containing Andrade's indicator with 0.5 per cent each of glucose, mannite, lactose, and dulcite. The test for indole was made on 7 day old culture in 1 per cent peptone water in which one or two drops of Ehrlich's (6) reagent was added:

PREPARATION OF MEDIA

Strains of Shig. dysenteriae rapidly lose their viability if frequent transplants are not made. A semi-solid medium used once a month maintained the toxicity of the organism.

The medium was prepared as follows:

1. **Semi-solid Medium**

   Whites of 2 eggs

   Yolk of 1 egg

   200 cc distilled water

Mix the eggs thoroughly with an egg beater before adding the water. Boil 2 minutes, stirring constantly. Filter first through a wire strainer, and then through cotton. Sterilize at 15 lbs. pressure for 15 minutes.

To a sterile veal infusion agar (1.5% agar, 1% peptone, 0.5% NaCl) add sterile egg mix in the proportion of 5 cc of egg mixture to 2 cc of agar. Mix, tube under aseptic conditions, and allow to solidify. Stab cultures are made.

The medium is quite soft but will solidify somewhat on cooling.

This type of medium will not suffice to maintain the viability of organisms, except when the strains are frequently transplanted. It was found that rapid transfers of ten or more "seedings" in nutrient broth not only rendered the organisms

viable but enhanced the production of both exo- and endotoxins.

2. Nutrient Broth.

A. Formula:

Ground veal, fat free (Infusion)	500 gms.
Peptone, Proteose, Bacto	15 gms.
Sodium chloride, C.P.	5 gms.
Sterile 25% dextrose solution	10 cc.
Distilled water	1000 cc.

B. Procedure:

500 gms. of chopped, defatted veal were placed in 1 liter of distilled water and infused over night in the refrigerator. It was then boiled for an hour, passed through a press, filtered through cheese cloth and cotton, autoclaved for 20 minutes at 15 pounds pressure and cooled over night in the refrigerator. The fat was then skimmed from the surface and the volume was made up to 1 liter. The peptone and salt were added and the reaction was adjusted to pH 7.6. The media were heated to 100°C. for 10 minutes. The reaction was readjusted to pH 7.6. 100cc portions were placed in Erlenmeyer flasks and sterlized under pressure. One cc. portions of 25% sterile dextrose solution was added aseptically to each flask prior

to use. All flasks were incubated at 35-36°C. for 48 hours to check sterility.

3. Nutrient Agar.

a. Formula:

Na Cl (C.P.)	5 gms.
Neo-Peptone	15 gms.
Veal Infusion (prepared as in 2b but made double strength)	500 cc.
Distilled water	500 cc.
Agar	40 gms.

b. The medium was prepared as in 2 b.

SOURCE OF CULTURES

Shig. dysenteriae cultures were kindly supplied by a number of universities and biological laboratories. The identity of each culture was checked by fermentation tests on glucose, mannite, lactose and dulcite, by indole production in Dunham's solution and by agglutination tests with Shig. dysenteriae antisera obtained from the National Institute of Health. The cultures with their biochemical reactions are listed in Table 1.

TABLE I

THE DIFFERENTIAL CULTURAL CHARACTERISTICS OF SHIGELLA

Organism	GLUCOSE	MANNITE	LACTOSE	DULCITE	INDOLE	Remarks
#1 = #1--Northwestern University	*	-	-	-	-	
2 = #0.35 " "	*	-	-	-	-	
3 = #2 " "	*	-	-	-	-	
4 = #K7A Washington University	*	-	-	-	-	
5 = Detroit Dept. of Health	*	-	-	-	-	
6 = #1339 Parke Davis & Co.	*	-	-	-	-	
7 = #0.1675 " "	*	-	-	-	-	
8 = #202 Lederle Biological Laboratories	*	-	-	-	-	
9 = University of Wisconsin	*	-	-	-	-	
10 = " " "	*	-	-	-	-	
11 = Stanford University	*	-	-	-	-	
12 = National Type Culture (London) #4837	*	-	-	-	-	
13 = University of Cincinnati	*	-	-	-	-	
14 = National Type Culture (London) #3510	*	-	-	-	-	
15 = H^2-Shiga, Imperial University, Japan	*	-	-	-	-	
16 = I^2 " " "	*	-	-	-	-	
17 = N " " "	*	-	-	-	-	
18 = O " " "	*	-	-	-	-	
19 = OT " " "	*	-	-	-	-	
20 = -- Michigan Dept. of Health	*	-	-	-	-	

* = acid formation

PREPARATION OF EXOTOXINS

Strain No. 18 was transferred four or more times in dextrose nutrient broth. Each transplant in 10 cc of the broth included four loopfuls which perceptibly clouded the broth in four hours or less, then 0.1 cc of the last culture was transferred to a 100 cc flask containing 25 cc of nutrient broth which then was incubated for 24 hours. One cubic centimeter transplant of this 24 hour broth culture was made aseptically to Kolle culture flasks containing a layer of nutrient agar. Even distribution of organisms over the surface of the agar was obtained by making a "spreader" through a process of slightly curving about 10 cm. of a 40 cm. stiff iron wire and covering the curved area with a thin layer of absorbent cotton which was secured with coarse silk thread. This "spreader" was sterilized in a 500 cc Erlenmeyer flask at 15 lbs. pressure for 30 minutes.

The organisms were grown 20 hours on nutrient agar and the growth was loosened with a "spreader" after 5 to 10 cc of physiological salt solution had been added and allowed to macerate for two hours.

PROCEDURE FOR SEPARATION OF EXOTOXIN FROM ORGANISMS

1. Suspensions of Shigella organisms were heated at 60°c for 1 hour and checked for sterility.

2. Supernatant fluid was separated from the suspension by rapid centrifugation. The supernatant fluid which contains the exotoxin was filtered through a Pasteur-Chamberland L3.

candle and checked for sterility.

3. M. L. D. of the exotoxin was determined by intravenous injection in the marginal vein of the ear of a rabbit.

4. The precipitated cells were retained for future preparation of endotoxin.

PREPARATION OF ENDOTOXIN

Boivin and Mesrobeanu (8) (in collaboration with Calalb) have shown that by the trichloracetic acid technique, a thermostable substance can be extracted from Shig. dysenteriae bacterial bodies of S form which, when injected into susceptible animals in lethal amounts, produces diarrhea followed by death in 24 to 48 hours. This substance does not produce the characteristic paralysis of the exotoxin.

The author's technique, which was a modification of the method of Boivin and Mesrobeanu, was carried out as follows:

The cells were digested with 0.5% trypsin and 0.5% Na_2CO_3 for sixty hours and then the trypsin was replenished, but no more sodium carbonate was added, and digestion was continued for an additional sixty hours. Trichloracetic acid was added until the cells gave a pH of 3.5 and the mixture was allowed to stand three hours, then neutralized with N/1 NaOH. The mixture was now centrifuged. The supernatant liquid was dialyzed by means of a cellophane sac against distilled water for 36 hours with a change of distilled water every 12 hours. The material in the dialysis-sac contained the endotoxin. The dialysate was

filtered through a Pasteur-Chamberland L.3 candle and graduated amounts were injected intravenously into rabbits to determine the minimum lethal dose.

PREPARATION OF SHIGELLA BACTERIN

The same procedure for preparation of exotoxin was used for Shigella bacterin. The bacteral cells and bacterial exudates were left intact after heating and graduated amounts were injected intravenously into rabbits to determine the minimum lethal dose.

PREPARATION OF BACTERIOPHAGE

The preliminary work for the different antigens began with the adaptation of bacteriophage to Shigella No. 8. The final comparison was made with strain No.18 bacteriophage that had a minimum lethal dose of 4cc for a rabbit weighing 2 kilograms injected intravenously. This bacteriophage also produced paralysis and diarrhea in rabbits injected subcutaneously. Death occurred when larger doses were given.

Shigella dysenteriae was transplanted at least four successive times and additional transplants were used when four transfer loops of organisms in 10cc did not produce definite clouding in four hours or less incubation. Bacteriophage was obtained from fresh untreated sewage. The sewage was filtered through cotton, followed by filtration through filter paper coated with fuller's earth and finally by ultrafiltration to render it bacteria free. After sufficient rapid transfers the activity of the bacteriophage was determined by the methods

recommended by d'Herelle (8) as preliminary tests and by Krueger (9) for the final tests. After the bacteriophage had reached a "fixed" stage the succeeding filtrates were reserved for future animal inoculations.

Another source of bacteriophage was that obtained from Mallmann (5). The bacteriophage readily lysed Shigella strain 18 and after 10 transfers, the titre obtained was 10^{-8}. This lysing titre checked again after 50 transfers were made was 10^{-9}. Subsequent checks for 100 transfers and 150 transfers gave no increase in the titre. The large number of transfers gave an abundant supply of bacteriophage so that a check could be made as to the relative toxicity of the lysed bacterial products.

Bacteriophage with a final titre of 10^{-8}. was isolated from unaltered sewage. Bacteriophage that was six months old produced death with slightly larger doses than with the freshly prepared bacteriophage.

PREPARATION OF BACTERIOPHAGE-EXOTOXIN

Bacteriophage-exotoxin was prepared by adding bacteriophage and exotoxin in proportion to their minimum lethal dose. The M.L.D. of the bacteriophage-exotoxin was then determined.

PREPARATION OF BACTERIOPHAGE-EXOTOXOID

Bacteriophage-exotoxoid was prepared as follows:

0.4 per cent formalin (U.S.P.) was added to the flasks of bacteriophage-exotoxin. (M.L.D.-4cc)

The mixture was incubated for four weeks at 40°C. It was then checked for toxicity.

The same quantity was used for immunization as was used in bacteriophage-toxin immunizations.

PREPARATION OF PURIFIED BACTERIOPHAGE

(a) The Procedure for the Purification of Agar

The preparation of relatively pure bacteriophage has been presented by Krueger and Tamada (11). To carry out the purification process, purified agar was used as a substrate. In order to use agar the author has modified the procedure recommended by Dominikiewicz (12). Two hundred and fifty to three hundred grams of agar in a powder form was dissolved in three to four liters of hot water kept at a temperature just below the boiling point in a double boiler. The melted agar was poured in a thin stream into eight or more liters of 95 per cent alcohol, which had been slightly acidified with acetic acid while the fluid was continually stirred by means of a glass rod. The agar precipitated at once from the solution in the form of a spongy, white sediment. After the fluid had been vigorously shaken and the sediment had settled to the bottom, the alcohol was decanted, and the agar sediment was poured on a piece of fine-meshed linen, which was stretched over a battery jar of ten liters capacity. The precipitated agar was washed in 95% alcohol to remove all traces of acetic acid. It was dried in an oven for two hours at 60°C. The agar was stored in glass containers.

The removal of traces of acetic acid so that the agar was neutral was a problem that had to be solved. It was found that the particles of agar retained considerable acid even after excessive washings with neutral alcohol as recommended by Dominikiewicz (12). The precipitated agar was alternately immersed and firmly pressed out through a linen cloth four times, each, in ten 250cc quantities of 95 per cent alcohol. This process gave a neutral agar.

(b) Preparation of Relatively Pure Bacteriophage

The preparation of relatively pure bacteriophage suspension was carried out with slight modification of the procedure recommended by Krueger and Tamada (11).

It is a well-known fact that the lytic agent, bacteriophage, is commonly associated with colloidal substances of the culture medium used for its preparation. This point has been emphasized by Bronfenbrenner (13), and it appears quite obvious that these non-specific protein substances may prove quite deleterious. Andrews, Bulloch, Dreyer, Feldes, Ledingham, and Wolf (14) state:

> "Since the horse is a highly susceptible animal, a start must be made with very small doses. The actual quantity of the toxin to be injected will depend on its strength. The stronger the toxin the better, since the volume injected will be the smaller. After every injection, a careful watch must be kept on the animal. The intensity of the local reaction evidenced by infiltration at the site of injection, the temperature, appetite, and general condition of the animal are to be noted and recorded. Among the ill-effects that may follow toxin injections, the most severe are paralysis and death of the horse with fatty degeneration of the heart and degenerative changes in the kidney. A chronic malady sometimes supervenes in the animals which have undergone for some years continuous courses of antitoxin-production.

> The first sign is an enfeeblement of the antitoxin-producing power. The animal soon becomes ill and eventually dies with amyloid degeneration of the spleen and liver, and with hepatic and peritoneal hemorrhages."

Muir (15) made a similar statement but it applies more to to the results of absorption of toxins in the body, which have been elaborated following bacterial infection. The statement is as follows:

> "Toxins are absorbed from the site of bacterial growth and are carried by the blood stream throughout the body. Hence there is often seen in infections a general toxic action, the injury being more diffuse but less severe. Various retrogressive changes are thus produced in various organs -- cloudy swelling, fatty degeneration, sometimes foci of actual necrosis. These are most marked in highly specialized cells, and in organs concerned in the excretion of toxins, especially the kidneys and liver."

The apparatus and materials for purification of bacteriophage are explained in the accompanying diagram. Convenient dimensions for the tube are 15 cm. by 5 cm. with a total volume of approximately 295 cc. A pyrex glass cylinder was used with 2 one-hole stoppers. The cylinder and stoppers were sterilized with 70% alcohol which was later removed with sterile saline solution, since the pyrex glass occasionally would break with autoclaving.

The bridges were inverted and filled with sterile purified 3 per cent agar containing 2 per cent NaCl. by attaching to the distal end of each tube a piece of soft rubber tubing which was closed with a Mohr's tubing clamp. When the agar was hardened the lower bridge and stopper were inserted into the pyrex cylinder and successive layers of 3, 2, 1, and 0.5 per cent agar containing 0.2 per cent NaCl were pipetted into the glass cylinder,

but each layer was first allowed to congeal in the refrigerator before adding the succeeding layer. The layers were equally divided so that the total space occupied was equivalent to approximately one-half the length of the cylinder minus the space which was occupied by the rubber stopper inserts. After the agar was congealed a sufficient quantity of bacteriophage suspension was layered over it to allow a space of 1 to 2 cm. between its upper surface and the rubber stopper when the latter was placed in position.

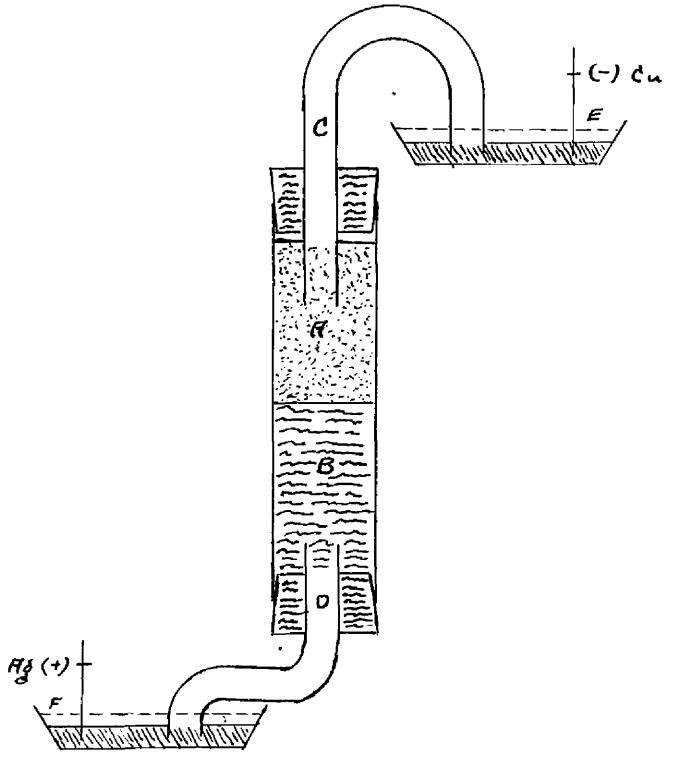
A current of 100 to 125 volts and from 5 to 10 milliamperes was supplied by connecting in series four-45 volts radio "B" batteries to which was attached direct current voltmeter and milliammeter of sufficient calibrations and a rheostat with a maximum capacity of 0.10 amps. and approximately 27,000 ohms resistance. The current was passed through the apparatus for 20 to 24 hours and the copper chloride and sodium chloride solutions in which the electrodes were immersed were changed once during that period. The electrodes of silver and copper respectively, were washed thoroughly at the same time the solutions were changed. Bacteriophage was changed once during the procedure.

The 0.5 per cent agar was sliced into sections with a sterile spatula as it was forced from the cylinder and received in a sterile Petri dish and was macerated for 12 to 15 hours in physiological saline for future purification. The remaining layers of agar were treated in the same manner and were found to give a negative reaction with Hopkins-Cole (16) reagent.

The phage concentration gave a range between 10^{-8} to 10^{-10}, which was in marked contrast to Krueger and Tamada (11), who report completely lysed susceptible bacteria at a dilution of 10^{-16}. The "layer" method has the advantages of removing a greater amount of the colloidal aggregates, and the retention of the upper layer of partially purified bacteriophage which may be used for future purification.

Fig. 1. Apparatus for preparation of relatively pure bacteriophage.

A, bacteriophage suspension. B, gels of 3 per cent 2 per cent, 1 percent and 0.5 per cent purified agar and 0.2 per cent c.p. NaCl in distilled water. C and D, glass bridges containing 3.0 per cent pure agar gel with 0.2 per cent c.p. NaCl. E, porcelain cup with crystals of $CuCl_2$ at bottom. Water to level of dotted line. Negative pole (copper) of D. C. circuit dips into crystals. F, porcelain cup with crystals of NaCl at bottom. Water to level of dotted line. Silver foil attached to positive pole of D. C. circuit dips into crystals.



Adapted from Krueger, J. Gen. Physiol., Vol. 13.

PREPARATION OF PURIFIED EXOTOXIN

The purification of Shigella bacteriophage by electrophoresis made possible the retention in part of the endo- and exotoxin. This procedure offered an opportunity for the removal of extraneous materials from toxin.

The procedure was applied to the purification of exotoxin. The exotoxin used for the purification had an M.L.D. of 0.05 cc for a 2.0 Kg. rabbit. When 10 cc of the same exotoxin purified was injected into a rabbit intravenously, no perceptible reaction occurred. When the bacteriophage filtrate of the top layer of agar (0.5 per cent), which was rejected in the purification of bacteriophage, was injected in 5 cc quantities, characteristic paralysis was followed by death. Since the 0.5 per cent layer of agar gave a weak positive reaction with the Hopkins-Cole test, this fraction was repurified by passing through another 0.5 per cent layer. This produced an exotoxin with a negative Hopkins-Cole reaction and only a slight loss in toxicity.

It is difficult to understand why bacteriophage should retain some of its toxicity when purified by electrophoresis while exotoxin under identical methods of purification should lose its toxicity. This may be due, however, to variability in the agar substrate but checks on this point failed to demonstrate this as the cause.

The major portion of the experimental work was conducted with (Shigella "O"), strain No. 18, which is one of the five cultures received from the Imperial University, Japan, in

January, 1937. This culture was demonstrated to be the most virulent.

Of the 19 strains tested, strains Nos. 8 (No. 202-Lederle, 13 (Shiga Moore), 15, 16, 17, 18 and 19 gave to rabbits pronounced reaction intradermally. Strains 15, 16, 17, 18 and 19, which were the cultures obtained from the Imperial University, were as a group the most toxic. Of these five strains, strain 18 was much more toxic than the other four. By intravenous inoculation the same relationship as indicated by intradermal tests was obtained.

Strain No. 20 gave cultural, agglutinating, and toxin characteristics like Strain 18.

Shigella culture No. 8 was used in the preliminary tests for antigenic study of bacteriophage. At first, injections with the specific bacteriophage were made daily.

The injections were made both subcutaneously and intravenously on albino rabbits.

Shigella Bacteriophage Injections:

The agglutinating titre of normal sera of all rabbits used for injections was less than 1 to 5.

The following animals received 10cc of bacteriophage, 10^{-9} as indicated, prepared from Shig. dysenteriae No. 8 and, later, followed with Shigella No. 18 bacteriophage for comparative antigenic value:

TABLE NO. II a - IMMUNIZATION OF RABBITS WITH SHIGELLA BACTERIOPHAGE

Bacteriophage-Shigella Strain No. 8

Daily Inoculation

	Rabbit #S0	Rabbit #S	Rabbit #S2	Rabbit #S5	Rabbit #S7	Rabbit #S8	Rabbit #9	Rabbit #10	Rabbit #11	Rabbit #12
Treatment*	None Control	None Control	Subc.	Subc.	Subc.	Subc.	Intv.	Intv.	Intv.	Intv.
	Wt**									
2/27/36	2.63	3.20	3.29	2.90	3.12	2.54	3.23	2.78	3.42	2.80
2/28/36	"	"	"	"	"	"	"	"	"	"
2/29/36	"	"	"	"	"	"	"	"	"	"
3/1/36	"	"	"	"	"	"	"	"	"	"
3/2/36	2.83	3.32	3.23	2.92	3.00	2.72	3.10	2.49	3.28	2.68
3/3/36	"	"	"	"	"	"	"	"	"	"
3/4/36	"	"	"	"	"	"	"	"	"	"
				Weekly Inoculation						
	Wt.									
3/10/36	2.98	3.40	3.23	3.02	2.94	2.60	3.18	2.68	3.30	2.76
3/16/36	3.08	3.48	3.29	3.00	3.08	2.72	3.18	2.72	3.18	2.74
3/21/36	3.24	3.49	3.29	Died	3.15	2.88	3.18	2.72	3.48	2.62
3/30/36	Aggl. 1-5	Aggl. 1-5	Aggl. 1-60	Litter of four	Aggl. 1-50	Aggl. 1-50	Aggl. 1-60	Aggl. 1-60	Aggl. 1-60	Aggl 1-50
	Wt.			5A						
4/9/36	3.40	3.52	3.62	Subst.	3.22	2.70	2.28	2.90	3.50	2.94
4/25/36	3.50	3.64	3.60	2.27	3.06	2.90	3.16	2.72	3.50	2.98
5/1/36	3.50	3.66	3.42	2.82	3.18	2.66	3.32	2.38	3.32	2.82

* 10 cc portions were used for injection in all cases

** Weight of all animals in Kilograms

TABLE NO. II a, continued

Date	#S0	#S	#S2	#5A	#S7	#S8	#9	#10	#11	#12
5/7/36	3.32	3.48	3.38	2.90	2.98	2.74	3.32	2.45	3.30	2.70
5/15/36	3.48	3.54	3.46	2.84	3.02	2.68	3.48	2.40	3.18	2.60
5/22/36	3.50	3.68	3.42	2.96	3.24	2.88	3.50	2.58	3.35	2.82
5/28/36	3.62	3.70	3.50	3.00	3.36	3.06	2.78	3.50	3.50	2.94
6/4/36	3.50	3.70	3.38	2.92	3.20	3.00	3.60	2.99	3.42	2.90
6/11/36	Aggl. 1-5	Aggl. 1-5	Aggl. 1-60	Aggl. 1-50	Aggl. 1-60	Aggl. 1-60	Aggl. 1-60	Aggl. 1-60	Aggl. 1-60	Aggl 1-60

TABLE NO. II b - IMMUNIZATION OF RABBITS WITH SHIGELLA BACTERIOPHAGE

Bacteriophage-Shigella Strain No. 18

4/11/37	Control Wt. 3.80	Control Wt. 3.60	1cc 3.40	2cc 3.04	5cc 3.30	10cc 3.18	10cc 3.64	5cc 3.39	2cc 3.25	1cc 3.42
4/17/37	Wt. 3.80	Wt. 3.82	2cc 3.38	2cc 3.10	5cc 3.32	* d.60h	d.24h	d.72h	1cc 3.28	1cc 3.50
4/24/37	Wt. 3.70	Wt. 3.75	2cc 3.50	2cc 3.08	5cc 3.27				1cc 3.38	1cc 3.48
4/30/37	Wt. 3.86	Wt. 3.76	2cc 3.60	2cc 3.15	5cc 3.24				1cc 3.32	1cc 3.36
5/7/37	Bled 15cc	Bled 18cc	Bled 16cc	Bled 15cc	Bled 18cc				Bled 15cc	Bled 20cc
	Aggl. 1-5	Aggl. 1-5	Aggl. 1-60	Aggl. 1-60	Aggl. 1-60				Aggl. 1-60	Aggl 1-60

* died in 60 hours

Tables III a and b show the data obtained in determining the minimum lethal dose of exotoxin. The minimum lethal dose for rabbits weighing 2.0 Kg. was found to be 0.5 cc.

Tables IV and V show the data obtained in determining the minimum lethal dose of exotoxin, when normal sera of rabbits Nos. S 0 and S respectively were added to dilutions of exotoxin and incubated at 37°C for two hours prior to injection. The minimum lethal doses were found to be the same as that obtained for the exotoxin alone.

Tables VI and VII present the data for determining the minimum lethal dose of normal pooled sera of rabbits S_2, 5_a, S_7, and S_8 and rabbits 9, 10, 11 and 12. This procedure was carried out in the same manner as for rabbits in Tables IV and V. The minimum lethal doses were found, also, to be the same as for exotoxin alone.

These demonstrate that the normal sera of the rabbits used for the preparation of immune sera were free from any interfering antibodies at the start of the experiment.

The rabbits were divided into two groups, one group receiving injections subcutaneously and the other intravenously. Rabbits S_2, 5_a, S_7 and S_8 were injected subcutaneously and rabbits 9, 10, 11 and 12 intravenously. All of the animals were injected daily for 7 days and then weekly for 10 weeks.

One week after the final injections, each rabbit was bled from the heart into clean sterile test tubes. In order to get a large proportion of clear sera, the test tubes were

examined for internal roughness and tubes were discarded that did not show an even, smooth surface. Approximately 5cc was placed in each tube, plugs were inserted and tubes slanted so that the blood came within one or two centimeters of the cotton plugs. After the blood coagulated, its surface was freed from the test tube with transfer needles, then placed in refrigerator over night, when sera was drawn off and placed in sterile tubes which were sealed and kept in refrigerator until used.

The exotoxin was previously determined to have a M.L.D. of 0.5 cc for rabbits with an average weight of 2.0 Kilograms (Table II a and b.) Table VIII presents the antibody titre for rabbits Nos. S_2, 5_a, S_7 and S_8 immunized subcutaneously against Shigella bacteriophage No. 8. Table IX presents the antibody titre for rabbits Nos. 9, 10, 11 and 12 immunized intravenously against Shigella bacteriophage No. 8. Table X presents the antibody titre for rabbits Nos. S_2, 5_a, S_7 and S_8 immunized subcutaneously against Shigella bacteriophage No. 18 and Table XI presents the antibody titre for rabbits Nos. 9, 10, 11 and 12 immunized intravenously against Shigella bacteriophage No. 18.

Table XII shows the data for intravenous injections of strain No. 18. Each animal was injected weekly for eight weeks. One week after final injection each animal was bled and the agglutination titre determined.

The normal agglutination titre for all animals used was less than 1 to 5.

The immunizing dose used for each antigen was one-half the minimum lethal dose. Rabbits LR 1, 2 and 3 were treated with exotoxin; LR 4, 5 and 6 with endotoxin, LR 7, 8 and 9 with bacterin; LR 11, 12 and 13 with exotoxin-bacteriophage; LR 14, 15 and 16 with purified bacteriophage; LR 17, 18 and 19 with bacteriophage; LR 20, 21 and 22 with bacteriophage exotoxoid. Nos. LR 10 and 23 were controls.

The agglutination titres for the respective antigens were as follows:

Exotoxin less than 1 to 5

Endotoxin ranged from 1-3000 to 1-5000

Bacterin ranged from 1-1600 to 1-2000

Exotoxin-bacteriophage from 1-20 to 1-25

Purified bacteriophage from 1-50 to 1-60

Bacteriophage 1 to 50

Bacteriophage-exotoxid 1 to 60

In order to have sufficient exo-endotoxin to carry out the tests for evaluation of antibody production of Shig. dysenteriae, various batches of the exo-endotoxin were pooled so that a constant M.L.D. could be obtained. Since the range of toxicity of the several batches was 0.05 to 0.2 cc, the different batches were mixed in such proportions that the M.L.D. of the final mixture was 0.1 cc.

The method for determination of proportion of the batches is illustrated as follows:

(1) $\frac{1}{.05} = 20$; (2) $\frac{1}{0.1} = 10$; $\frac{1}{0.2} = 5$

Solution:

0.05 = 20 _ _ _ _ _ _ 5 = 1 part

10

0.2 5 _ _ _ _ _ _ 10 = 2 parts

The ratio of 0.05 M.L.D. to .2 M.L.D. is 1 part to 2 parts, or for every cubic centimeter of 0.05 M.L.D., two cubic centimeters of 0.2 M.L.D. should be used.

For care of operation and clear comparative results, animals of approximately the same age and weight were used. This made it possible to give each animal exactly the same dosage.

DETERMINATION OF M.L.D. OF EXOTOXIN FROM STRAIN 18.

TABLE III a

Rabbit	Weight	Saline	Exotoxin	Result
1	2.08	0.4cc	0.6cc	d.29 hr.
2	2.06	0.5cc	0.5cc	d.56 hr.
3	2.08	0.6cc	0.4cc	Slight Paralysis

DETERMINATION OF M.L.D. OF EXOTOXIN FROM STRAIN 18.

TABLE III b RECHECK OF III a

Rabbit	Weight	Saline	Exotoxin	Result
4	2.00	0.4cc	0.6cc	d.39 hr.
5	2.02	0.5cc	0.5cc	d.72 hr.
6	2.01	0.6cc	0.4cc	Paralysis

DETERMINATION OF M.L.D. OF EXOTOXIN MIXED WITH NORMAL SERUM OF RABBIT NO. SO.

TABLE IV

Rabbit	Weight	Serum	Saline	Exotoxin	Results
1	2.02	1cc	0.4cc	0.6cc	d.38 hr.
2	2.00	1cc	0.5cc	0.5cc	d.60 hr.
3	2.01	1cc	0.6cc	0.4cc	Paralysis

DETERMINATION OF M.L.D. OF EXOTOXIN MIXED WITH NORMAL SERUM OF RABBIT NO. S.

TABLE V

Rabbit	Weight	Serum	Saline	Exotoxin	Results
1	2.00	1cc	0.4cc	0.6cc	d.40 hrs.
2	1.99	1cc	0.5cc	0.5cc	d.59 hrs.
3	1.98	1cc	0.6cc	0.4cc	Lived

DETERMINATION OF M.L.D. OF EXOTOXIN MIXED WITH POOLED NORMAL SERUM OF RABBITS S2, 5a, S7, and S8

TABLE VI

Rabbit	Weight	Pooled Sera	Saline	Exotoxin	Results
1	2.03	1cc	0.4cc	0.6cc	d.39 hr.
2	2.02	1cc	0.5cc	0.5cc	d.79 hr.
3	2.04	1cc	0.6cc	0.4cc	Slight Paralysis

DETERMINATION OF M.L.D. OF EXOTOXIN MIXED WITH POOLED NORMAL SERUM OF RABBITS 9, 10, 11 and 12

TABLE VII

Rabbit	Weight	Pooled Sera	Saline	Exotoxin	Results
1	2.01	1cc	0.4cc	0.6cc	d.52 hr.
2	2.01	1cc	0.5cc	0.5cc	d.92 hr.
3	2.00	1cc	0.6cc	0.4cc	Lived

DETERMINATION OF M.L.D. OF EXOTOXIN MIXED WITH SERA FROM RABBITS S2, 5a, S7 and S8 IMMUNIZED SUBCUTANEOUSLY WITH SHIGA-BACTERIOPHAGE NO.8

TABLE VIII

Rabbit	Weight	Pooled Sera	Saline	Exotoxin	Results
1	2.08	1cc	0.4cc	0.6cc	d.48 hr.
2	1.96	1cc	0.4cc	0.6cc	d.46 hr.
3	2.10	1cc	0.5cc	0.5cc	d.69 hr.
4	1.93	1cc	0.5cc	0.5cc	d.70 hr.
5	2.06	1cc	0.6cc	0.4cc	Survived
6	2.00	1cc	0.6cc	0.4cc	Survived

DETERMINATION OF M.L.D. OF EXOTOXIN MIXED WITH SERA FROM RABBITS 9, 10, 11 and 12 IMMUNIZED INTRAVENOUSLY WITH SHIGA-BACTERIOPHAGE NO.8

TABLE IX

Rabbit	Weight	Pooled Sera	Saline	Exotoxin	Results
1	2.04	1cc	0.4cc	0.6cc	d.56 hr.
2	2.08	1cc	0.4cc	0.6cc	d.50 hr.
3	2.01	1cc	0.5cc	0.5cc	d.84 hr.
4	2.00	1cc	0.5cc	0.5cc	d.74 hr.
5	2.03	1cc	0.6cc	0.4cc	Survived
6	1.99	1cc	0.6cc	0.4cc	Survived

DETERMINATION OF M.L.D. OF EXOTOXIN MIXED WITH SERA FROM RABBITS S2, 5a, S7 and S8 SUBCUTANEOUSLY WITH SHIGA-BACTERIOPHAGE NO.18

TABLE X

Rabbit	Weight	Pooled Sera	Saline	Exotoxin	Results
1	1.99	1cc	0.4cc	0.6cc	d.74 hr.
2	2.02	1cc	0.4cc	0.6cc	d.79 hr.
3	2.04	1cc	0.5cc	0.5cc	Survived
4	2.01	1cc	0.5cc	0.5cc	Survived
5	2.00	1cc	0.6cc	0.4cc	Survived
6	1.98	1cc	0.6cc	0.4cc	Survived

DETERMINATION OF M.L.D. OF EXOTOXIN MIXED WITH SERA FROM RABBITS 9, 10, 11 and 12 IMMUNIZED INTRAVENOUSLY WITH SHIGA-BACTERIOPHAGE NO.18

TABLE XI

Rabbit	Weight	Pooled Sera	Saline	Exotoxin	Results
1	2.03	1cc	0.4cc	0.6cc	d.83 hr.
2	1.98	1cc	0.4cc	0.6cc	d.83 hr.
3	2.02	1cc	0.5cc	0.5cc	Survived
4	2.00	1cc	0.5cc	0.5cc	Survived
5	2.03	1cc	0.6cc	0.4cc	Survived
6	1.99	1cc	0.6cc	0.4cc	Survived

RECORD OF INTRAVENOUS INJECTIONS WITH SHIGA-18-ANTIGENS

TABLE XII
Rabbit

LR#1	x	3/2	3/9	3/15	3/22	3/28	4/4	4/11	4/18	4/25	Aggl
EXO-	*	.05cc	.05cc	.05cc	.05cc	.05cc	.05	.05	.05	Bled	1/5
TOXIN	/	2.70	2.70	2.47	2.48	2.40	2.56	2.69	2.78	12cc	
LR#2											
EXO-		.05cc	.05cc	.05cc	.05cc	.08cc	.1cc	.1cc	.1cc	Bled	1/5
TOXIN		2.90	2.84	2.84	2.70	2.70	2.66	2.84	2.98	15cc	
LR#3											
EXO-		.05cc	.05cc	.05cc	.05cc	.1cc	.1cc	.1cc	.1cc	Bled	1/5
TOXIN		3.0	3.0	2.88	3.00	3.00	2.70	2.80	2.90	15cc	
LR#4											
ENDO		.1cc	.1cc	.1cc	.1cc	.1cc	.1cc	.1cc	.1cc	Bled	1/5000
TOXIN		2.48	2.30	2.40	2.40	2.60	2.72	2.80	2.80	15cc	
LR#5											
ENDO		.1cc	.1cc	.1cc	.1cc	.15cc	.15cc	.15cc	.15cc	Bled	1/3000
TOXIN		3.10	3.10	3.00	3.10	3.00	2.86	2.86	2.86	20cc	
LR#6											
ENDO		.08cc	.08cc	.08cc	.08cc	.08cc	.08cc	.08cc	.08cc	Bled	1/4000
TOXIN		2.00	2.20	2.12	2.32	2.30	2.36	2.36	2.48	15cc	
LR#7											
SHIGELLA		1cc	1cc	1cc	1cc	1cc	1cc	1cc	1cc	Bled	1/2000
BACTERIN		2.48	2.40	2.48	2.62	2.72	2.60	2.56	2.60	16cc	
LR#8											
SHIGELLA		1cc	1cc	1cc	1cc	1.5cc	1.5cc	1.5cc	1.5cc	Bled	1/1600
BACTERIN		2.80	2.70	2.82	2.82	2.88	2.85	2.84	2.80	20cc	
LR#9											
SHIGELLA		1cc	1cc	1cc	1cc	1.2cc	1.2cc	1.2cc	1.2cc	Bled	1/1800
BACTERIN		2.62	2.67	2.48	2.54	2.58	2.40	2.54	2.68	15cc	
LR#10											
CONTROL		2.73	2.83	2.98	3.18	3.32	3.43	3.56	3.60	Bled	1/5
										10cc	

x date

* dosage

RECORD OF INTRAVENOUS INJECTIONS WITH SHIGA-18-ANTIGENS, cont.

TABLE XII Rabbit

										Aggl
LR#11										
EXOTOXIN-	*2cc	2cc	2cc	2cc	2cc	2cc	2cc	2cc	Bled	1/25
BACTERIOPHAGE/	2.64	2.78	2.64	2.70	2.70	2.60	2.80	2.82	20cc	
LR#12										
EXOTOXIN	2cc	2cc	2cc	2cc	2cc	2cc	2cc	2cc	Bled	1/20
BACTERIOPHAGE	1.92	1.92	1.80	1.76	1.84	1.88	1.80	1.74	16cc	
LR#13										
EXOTOXIN	2cc	2cc	2cc	2cc	2cc	2cc	2cc	2cc	Bled	1/25
BACTERIOPHAGE	2.28	2.38	2.40	2.30	2.42	2.60	2.74	2.78	20cc	
LR#14										
PURIFIED	2cc	2cc	2cc	2cc	2cc	2cc	2cc	2cc	Bled	1/60
BACTERIOPHAGE	3.11	3.18	3.24	3.12	3.12	3.00	3.18	3.32	20cc	
LR#15										
PURIFIED	2cc	2cc	2cc	2cc	2cc	2cc	2cc	2cc	Bled	1/50
BACTERIOPHAGE	2.36	2.36	2.44	2.52	2.58	2.64	2.72	2.94	20cc	
LR#16										
PURIFIED	2cc	2cc	2cc	2cc	2cc	2cc	2cc	2cc	Bled	1/60
BACTERIOPHAGE	3.00	3.14	3.18	3.28	3.10	3.10	3.20	3.08	20cc	
LR#17										
	2cc	2cc	2cc	2cc	2cc	2cc	2cc	2cc	Bled	1/50
BACTERIOPHAGE	1.86	1.92	2.04	2.08	2.16	2.16	2.16	2.10	12cc	
LR#18										
	2cc	2cc	2cc	2cc	2cc	2cc	2cc	2cc	Bled	1/50
BACTERIOPHAGE	2.16	2.08	2.20	2.06	2.22	2.20	2.10	2.10	12cc	
LR#19										
	2cc	2cc	2cc	2cc	2cc	2cc	2cc	2cc	Bled	1/50
BACTERIOPHAGE	3.60	3.72	3.64	3.66	3.80	3.66	3.54	3.62	20cc	
LR#20										
BACTERIOPHAGE	2cc	2cc	2cc	2cc	2cc	2cc	2cc	2cc	Bled	1/60
EXOTOXOID	1.70	1.92	2.14	2.36	2.58	2.80	2.92	3.00	18cc	
LR#21										
BACTERIOPHAGE	2cc	2cc	2cc	2cc	2cc	2cc	2cc	2cc	Bled	1/60
EXOTOXOID	2.50	2.50	2.78	3.00	3.18	3.42	3.52	3.64	20cc	
LR#22										
BACTERIOPHAGE	2cc	2cc	2cc	2cc	2cc	2cc	2cc	2cc	Bled	1/60
EXOTOXOID	2.02	2.04	2.30	2.46	2.54	2.68	2.76	2.82	20cc	
LR#23										
CONTROL	2.18	2.18	2.36	2.50	2.66	2.85	3.06	3.11	3.15	1/5

* dosage

/ weight

To determine the comparative value of the antibodies produced neutralization toxicity tests were made:

Exo-endotoxin was added to the various antisera and incubated at 37° C. for two hours for combination before injections were made.

Table XIII gives, alternating, a comparison of exo-endotoxin with and without normal serum. The following tables give the comparative values for antibody production in albine rabbits after intravenous injections of exotoxin-endotoxin with immune bodies of (XIV) Shigella Bacterin, (XV) Exotoxin, (XVI) Bacteriophage-exotoxin, (XVI) Purified Bacteriophage, (XVIII) Endotoxin, (XIX) Bacteriophage, (XX) Bacteriophage-exotoxoid.

Table XIII Exo-Endotoxin With and Without Normal Serum

Rabbit	Weight	Pooled Normal Sera	Physical. Saline	Exo-Endo-Toxin	Results
1	1.81 Kg.	1.0 cc	0.0 cc	0.4 cc	d. 16 hrs.
2	1.78	0.0 cc	1.0 cc	0.4 cc	d. 12 hrs.
3	1.82	1.0 cc	0.2 cc	0.2 cc	d. 24 hrs.
4	1.76	0.0 cc	1.0 cc	0.2 cc	d. 22 hrs.
*5	1.83	1.0 cc	0.3 cc	0.1 cc	d. 48 hrs.
6	1.79	0.0 cc	1.0 cc	0.1 cc	d. 60 hrs.
7	1.80	1.0 cc	0.35 cc	0.05 cc	survived
8	1.81	0.0 cc	1.00 cc	0.05 cc	survived
9	1.82	1.0 cc	0.375cc	0.25cc	survived
10	1.77	0.0 cc	1.375cc	0.25cc	survived

* M.L.D. Exo-Endotoxin = 0.1 cc

Table XIV Bacterin Antisera

Rabbit	Weight in Kg.	Pooled Antisera	Physiol. Saline	Exo-Endo Toxin	Results
1	1.79	1.0 cc	0.0 cc	0.4 cc	d. 68 hrs.
2	1.82	1.0 cc	0.0 cc	0.4 cc	d. 48 hrs.
3	1.81	1.0 cc	0.2 cc	0.2 cc	survived
4	1.81	1.0 cc	0.2 cc	0.2 cc	survived
5	1.78	1.0 cc	0.3 cc	0.1 cc	survived
6	1.80	1.0 cc	0.3 cc	0.1 cc	survived
7	1.79	1.0 cc	0.35cc	0.05cc	survived
8	1.81	1.0 cc	0.35cc	0.05cc	survived
9	1.80	0.0 cc	1.35cc	0.05cc	survived
10	1.82	0.0 cc	1.35cc	0.05cc	survived

Table XV Exotoxin Antisera

Rabbit	Weight in Kg.	Pooled Antisera	Physiol. Saline	Exo-Endo Toxin	Results
1	1.79	1.0 cc	0.0 cc	0.4 cc	d. 18 hrs.
2	1.81	1.0 cc	0.0 cc	0.4 cc	d. 24 hrs.
3	1.81	1.0 cc	0.2 cc	0.2 cc	d. 72 hrs.
4	1.78	1.0 cc	0.2 cc	0.2 cc	d. 84 hrs.
5	1.79	1.0 cc	0.3 cc	0.1 cc	survived
6	1.81	1.0 cc	0.3 cc	0.1 cc	survived
7	1.81	1.0 cc	0.35cc	0.05cc	survived
8	1.79	1.0 cc	0.35cc	0.05cc	survived
9	1.78	0.0 cc	1.35cc	0.05cc	survived
10	1.81	0.0 cc	1.35cc	0.05cc	survived

Table XVI Exotoxin-Bacteriophage Antisera

Rabbit	Weight in Kg.	Pooled Antisera	Physiol. Saline	Exo-Endo Toxin	Results
1	1.84	1.0 cc	0.0 cc	0.4 cc	d.60 hrs.
2	1.81	1.0 cc	0.0 cc	0.4 cc	d.72 hrs.
3	1.81	1.0 cc	0.2 cc	0.2 cc	survived
4	1.81	1.0 cc	0.2 cc	0.2 cc	survived
5	1.78	1.0 cc	0.3 cc	0.1 cc	survived
6	1.81	1.0 cc	0.3 cc	0.1 cc	survived
7	1.79	1.0 cc	0.35cc	0.05cc	survived
8	1.80	1.0 cc	0.35cc	0.05cc	survived
9	1.81	0.0 cc	1.35cc	0.05cc	survived
10	1.80	0.0 cc	1.35cc	0.05cc	survived

Table XVII Purified Bacteriophage Antisera

Rabbit	Weight in Kg.	Pooled Antisera	Physiol. Saline	Exo-Endo Toxin	Results
1	1.81	1.0 cc	0.0 cc	0.4 cc	d. 20 hrs.
2	1.83	1.0 cc	0.0 cc	0.4 cc	d. 26 hrs.
3	1.82	1.0 cc	0.2 cc	0.2 cc	d. 72 hrs.
4	1.80	1.0 cc	0.2 cc	0.2 cc	d. 92 hrs.
5	1.84	1.0 cc	0.3 cc	0.1 cc	survived
6	1.82	1.0 cc	0.3 cc	0.1 cc	survived
7	1.79	1.0 cc	0.35cc	0.05cc	survived
8	1.83	1.0 cc	0.35cc	0.05cc	survived
9	1.80	0.0 cc	1.35cc	0.05cc	survived
10	1.78	0.0 cc	1.35cc	0.05cc	survived

Table XVIII Endotoxin Antisera

Rabbit	Weight in Kg.	Pooled Antisera	Physiol. Saline	Exo-Endo Toxin	Results
1	1.83	1.0 cc	0.0 cc	0.4 cc	d. 42 hrs.
2	1.87	1.0 cc	0.0 cc	0.4 cc	d. 24 hrs.
3	1.84	1.0 cc	0.2 cc	0.2 cc	d. 82 hrs.
4	1.86	1.0 cc	0.2 cc	0.2 cc	d. 78 hrs.
5	1.85	1.0 cc	0.3 cc	0.1 cc	survived
6	1.86	1.0 cc	0.3 cc	0.1 cc	survived
7	1.84	1.0 cc	0.35cc	0.05cc	survived
8	1.85	1.0 cc	0.35cc	0.05cc	survived
9	1.81	0.0 cc	1.35cc	0.05cc	survived
10	1.83	0.0 cc	1.35cc	0.05cc	survived

Table XIX Bacteriophage Antisera

Rabbit	Weight in Kg.	Pooled Antisera	Physiol. Saline	Exo-Endo Toxin	Results
1	1.84	1.0 cc	0.0 cc	0.4 cc	d. 48 hrs.
2	1.83	1.0 cc	0.0 cc	0.4 cc	d. 24 hrs.
3	1.81	1.0 cc	0.2 cc	0.2 cc	d. 72 hrs.
4	1.84	1.0 cc	0.2 cc	0.2 cc	d. 69 hrs.
5	1.81	1.0 cc	0.3 cc	0.1 cc	survived
6	1.80	1.0 cc	0.3 cc	0.1 cc	survived
7	1.83	1.0 cc	0.35cc	0.05cc	survived
8	1.83	1.0 cc	0.35cc	0.05cc	survived
9	1.84	0.0 cc	1.35cc	0.05cc	survived
10	1.82	0.0 cc	1.35cc	0.05cc	survived

Table XX Bacteriophage-Exotoxoid Antisera

Rabbit	Weight in Kg.	Pooled Antisera	Physiolog. Saline	Exo-Endo Toxin	Results
1	1.82	1.0 cc	0.0 cc	0.6 cc	d. 76 hrs.
2	1.84	1.0 cc	0.0 cc	0.6 cc	survived
3	1.85	1.0 cc	0.0 cc	0.4 cc	survived
4	1.84	1.0 cc	0.0 cc	0.4 cc	survived
5	1.82	1.0 cc	0.2 cc	0.2 cc	survived
6	1.84	1.0 cc	0.2 cc	0.2 cc	survived
7	1.83	1.0 cc	0.3 cc	0.1 cc	survived
8	1.85	1.0 cc	0.3 cc	0.1 cc	survived
9	1.84	1.0 cc	0.35cc	0.05cc	survived
10	1.83	1.0 cc	0.35cc	0.05cc	survived
11	1.84	0.0 cc	1.35cc	0.05cc	survived
12	1.84	0.0 cc	1.35cc	0.05cc	survived

DISCUSSION

This study was instigated primarily by the fact that Larkum had demonstrated that a staphylococcus bacteriophage, although it was not toxic, would stimulate in the rabbit the production of antitoxin. Inasmuch as the injection of Shigella bacterin into an animal causes marked toxic effect, it was hoped that Shigella bacteriophage would be non-toxic and still capable of stimulating the production of antitoxin. Such an immunizing agent would be of considerable value in the control of dysentery.

A bacteriophage was prepared for Shig. dysenteriae strain 8. This material was injected into rabbits daily for 7 days and weekly for 12 weeks (Table II). The bacteriophage was injected in 10cc doses through the entire period. The animals showed little or no toxic effect from the injections. The bacteriophage antisera were tested against a known exotoxin (Table VIII and IX), and were found to contain no antitoxin.

A second bacteriophage was prepared from Shig.dysenteriae strain 18. This bacteriophage in contrast to the first preparation showed marked toxic effect. The material had a M.L.D. of 4cc for a 2 Kg. rabbit.

determined. Accordingly bacterins, endotoxins and exotoxins were prepared. These antigens were tested on rabbits in the same manner (Table XII).

The approximate values of each antigen in protective units are presented in Tables XIV, XV, XVIII and XIX. The relative values were as follows:

Bacterin 2

Exotoxin 1

Endotoxin 1

Bacteriophage. 2

To obtain the toxicogenic effect of the endotoxin contained in the bacteriophage and the exotoxin prepared from culture filtrates, mixtures of the two were prepared. The injection dose of this mixture was limited due to the toxic nature of both substances. The preparation of the antisera is shown in Table XII.

To reduce the toxicity of the above mixture, a bacteriophage-exotoxoid mixture was prepared as previously outlined. Antisera for this material was prepared as shown in Table XII.

These antisera were then tested against exo-endotoxin to determine their relative protective values. The results are presented in Tables XVI and XX. The relative values of these mixtures were as follows:

Bacteriophage-exotoxin 2

Bacteriophage-exotoxoid . . .5

In the preparation of this bacteriophage, the Shigella culture was allowed to develop 8 hours before the addition of the bacteriophage. Later tests showed that when the culture was allowed to develop for 24 hours prior to the introduction of the bacteriophage, a very toxic filtrate was obtained. In all instances, the filtrates from strain 18 showed marked toxic effects. To eliminate the toxic effect of fresh "green" bacteriophage, the filtrates were aged in the refrigerator for periods as long as 18 months without any material diminution of toxicity. Exotoxin prepared from this same strain of Shig. dysenteriae retained its toxicity for 18 months in a similar manner.

The data indicate that the production of bacteriophage with toxic properties is dependent upon the toxic properties of the Shig. dysenteriae strain used. Shig.dysenteriae strain 8, which failed to show toxic effects, failed to show toxicogenic properties. On the other hand, a toxic strain (strain 18), when lysed, produced a toxic bacteriophage which had marked toxicogenic properties. It would appear from these data that the toxicogenic properties of Shigella bacteriophage were dependent upon the toxin content of the filtrate.

To arrive at an evaluation of the toxicogenic value of bacteriophage prepared from strain 18, toxicogenic values of bacterins, endotoxins and exotoxins of this strain were

It will be observed from these data that the bacteriophage-exotoxoid, although injected in the same amounts as the bacteriophage-exotoxin, produced considerably more protective units. Not only was the bacteriophage-exotoxoid more antigenic in the stimulation of antitoxin, but its injection was attended with considerably less toxic effect during the course of immunization. The injection of the equivalent of 2 M.L.D. doses of bacteriophage-exotoxin in the form of the bacteriophage-exotoxoid failed to show any toxic effects.

Due to the fact that bacteriophage contains considerable extranenous material from the broth and the lysed bacterial cells, it was thought advisable to purify the bacteriophage to be sure that these products and compounds might not interfere with the antigenic properties of the bacteriophage. Accordingly, purified bacteriophage was prepared. This material was injected into rabbits as indicated in Table XII. The antiserum was tested against exo-endotoxin. The results are presented in Table XVII. The relative protective value was the same as that obtained with the unpurified material indicating that the purification did not change the antigenic value.

The agglutinogenic and toxicogenic properties of the various immunizing agents did not parallel each other. For

example, in Table XII the endotoxin showed an agglutination titre of 1 to 5000 and a antitoxin titre (Table XVIII) of 1, whereas the bacteriophage-exotoxoid (Table XII) showed an agglutination titre of 1 to 60 and an antitoxin titre of 5 (Table XX).

SUMMARY

The toxicogenic value of bacteriophage appeared to be dependent upon the toxicogenic value of the Shig. dysenteriae strain used in the preparation of the bacteriophage.

There appeared to be no evidence that bacteriophage in itself had toxicogenic properties.

Bacteriophage appeared to be of value as an agent for the liberation of endotoxin and possibly exotoxin through the ability to lyse the bacterial cells.

The aging of bacteriophage filtrates did not decrease its toxic properties.

The agglutinogenic properties of the bacteriophage, exotoxins, endotoxins, and bacterins do not parallel the toxicogenic properties.

A toxoid preparation of bacteriophage-exotoxin was found to be the best means of producing antitoxin.

The toxicogenic value of purified bacteriophage was the same as the unpurified bacteriophage.

The procedure outlined in this research for the preparation of bacteriophage-exotoxoid appears to offer a practical means for immunization against Shig. dysenteriae toxin.

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