STUDY ON TOXINS AND ANTIGENS OF SHIGELLA DYSENTERIAE

II. Active Protection of Rabbits with Whole Organisms and Fractions of Shigella dysenteriae

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Bacteria contain a variety of components that may give rise to antibodies devoid of protective power. In prophylactic immunization the presence of such useless antigens ideally should be avoided. Not only is the production of non-protective antibodies an unnecessary load, but, in some cases, particularly that of streptococci, it may be unfavorable and even dangerous. Although there is evidence of protection induced by the administration of whole-organism vaccines, the untoward toxic effects of killed bacteria and the sensitivities that may be induced by some of the components of the organisms entirely unrelated to protection make the search for purified protective antigens necessary (Dubos, 1946).

In the case of *Shigella dysenteriae*, the problem is further complicated by the claims of various investigators that different fractions of the organisms induce different protective antibodies. In the case of *S. dysenteriae*, the proponents of the two-toxin theory maintain that antibodies produced in response to the injection of the neurotoxin protect the animals against the toxin alone, and that antibacterial immunity is afforded solely by immunization with the whole smooth variant of *S. dysenteriae* or with its somatic antigen (Boivin and Mesrobeanu, 1937; Morgan and Partridge, 1941; Steabben, 1943). In a previous publication (Boroff, 1949) we have contended that there exists only one dominant antigen in *S. dysenteriae* and that the single toxin elaborated by this organism is associated with it. If this contention is true, it should also hold true that protective antibodies against one fraction of *S. dysenteriae* should protect the immunized animals against all fractions as well as against whole organisms. The following report demonstrates the active protection of rabbits by means of whole organisms and variously derived fractions of *S. dysenteriae*.

EXPERIMENTAL PROCEDURES AND RESULTS

Immunization of rabbits with S and R variants of S. dysenteriae. Smooth and rough variants of *S. dysenteriae*, strain 2308, obtained from Dr. R. Dubos, were used throughout this study. The smooth variant agglutinated with sera against all strains of *S. dysenteriae* on hand. The rough variant agglutinated neither with undiluted antisera to the smooth variant, nor with sera of rabbits immunized with the rough strains.

The organisms were grown in Roux bottles on a 2 per cent tryptone agar con-

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taining 0.1 per cent glucose and 0.1 per cent sodium chloride. The cultures were incubated at 37 C for 18 hours, and the growth was washed off with saline solution. After centrifugation, the sediment was washed twice, resuspended in saline solution, and dried by the lyophil process. All antigens described in this report were prepared from lyophilized organisms.

Since the rough variant of *S. dysenteriae* lacked somatic antigen and was nevertheless toxic according to the above-mentioned authors, the protection afforded by immunization with this variant should be directed only against the "neurotoxin," whereas injections with the smooth variant should afford antibacterial immunity. However, on the basis of our hypothesis that there is only one dominant toxin in *S. dysenteriae*, it should follow that the injection of any antigenic fraction of this organism, capable of inducing protective antibodies, will afford protection against the toxic effects of the whole organism as well as the purified toxin. These alternatives were tested by the immunization of two groups of six rabbits each with smooth and rough heat-killed variants of *S. dysenteriae*.

The organisms were suspended in sterile saline solution and heated in a water bath for 30 minutes at 56 C. If no viable organisms could be demonstrated upon subsequent tests, the suspensions were used for injections. The rabbits were given 6 intravenous injections on alternate days. The total amount injected was 57 micrograms (dry weight) of the organisms. Five days after the last injection the rabbits were bled and their sera tested for the presence of agglutinins and precipitins. Two days after the bleeding the rabbits were challenged with 1 mg of the homologous antigen. Most rabbits survived the initial challenge, but all of the normal controls died within 48 hours.

After being observed for 5 days the surviving rabbits were injected with partially purified toxin obtained from the smooth variant by Dubos' method (1946) described below. Table 1 summarizes the results. All animals injected with the toxin survived, showing that they were protected not only against whole organisms but purified toxin as well. A point of great interest was the observation that although the rabbits immunized with the rough variant showed no demonstrable antibodies, they were protected against lethal doses of smooth whole organisms and purified toxin.

Immunization of rabbits with sonic lysates of smooth and rough variants of S. dysenteriae. One gram each of lyophilized organisms of the smooth and rough variants of *S. dysenteriae* was resuspended in 40 ml of distilled water and treated in the magnetostriction oscillator for 1½ hours at 9,000 cycles per second. The treated material was centrifuged to remove the remaining organisms and debris, and the supernatant fluid filtered through a Berkefeld candle. The filtrate was a clear greenish fluid which upon lyophilization formed a white feathery substance that was readily soluble in saline solution. This substance was as toxic for mice on intraperitoneal injection as were intact organisms.

Six rabbits were injected with a saline solution of each of the sonic lysates. Each rabbit received 0.057 mg of the substance intravenously in 6 injections, over a period of 2 weeks. Unless otherwise stated, the injection schedules for all

antigens were as follows: The first dose was 0.002 mg of antigen per rabbit, the second dose 0.005 mg, the third and the fourth 0.01 mg each, and the fifth and sixth 0.02 mg each. Five days after the last injection the sera of the rabbits were tested for agglutinins and precipitins. All rabbits immunized with the smooth lysate showed the presence of these antibodies, whereas rabbits immunized with rough lysate did not. Survival of these rabbits after the injection with 20 LD_{50} of the homologous antigen proved that they were protected against this dose of toxin. A second challenge with a lethal dose of intact organisms showed that all rabbits were as resistant to whole organisms as they were to the lysates. The results of this experiment are also shown in table 1.

TABLE 1

Serological reactions and degree of active protection exhibited by rabbits immunized with whole organisms and various fractions of S. dysenteriae

RABBITS IMMUNIZED WITH	HIGHEST SERUM DILUTION GIVING COMPLETE AGGLUTINATION WITH S2308 WO	DEGREE OF PRECIPITATION REACTION WITH ANTIGEN DILUTIONS OF		SURVIVAL RATIO OF RABBITS CHALLENGED WITH 20 LD_{50} OF HOMOLOGOUS ANTIGEN	SURVIVAL RATIO OF RABBITS CHALLENGED WITH HETEROLOGOUS ANTIGEN
		1:1,000	1:10,000		
S2308 WO	1:1,024	4	3	4/6	4/4
R2308 WO	0	0	0	6/6	6/6
S2308-b	1:1,024	4	3	6/6	6/6
R2308-b	0	0	0	6/6	6/6
S2308 PT	1:1,024	4	3	5/6	5/5
R2308 PT	0	0	0	4/6	4/4
S2308 SA	1:1,024	4	0	5/6	0/5
R2308 SA	0	0	0	6/6	0/6

S, smooth variant. WO, whole heat-killed organisms.
R, rough variant. PT, partially purified toxin.
b, sonic lysate. SA, somatic antigen.

Partially purified toxin, hydrochloric acid precipitate from sonic lysate of smooth and rough cultures of *S. dysenteriae*.

Heterologous antigen challenge:
S2308 WO and R2308 WO—challenged with S2308 partially purified toxin.
S2308-b, R2308-b, S2308 PT, and R2308 PT—challenged with S2308 WO.
S2308 SA—challenged with S2308 WO.
R2308 SA—challenged with S2308 partially purified toxin.

Immunization of rabbits with partially purified toxin from smooth and rough variants of S. dysenteriae. The preceding experiment established that rabbits immunized with smooth and rough variants of *S. dysenteriae* will withstand lethal doses of the partially purified toxin. This experiment attempted to determine the immunizing potency of the purified toxin from these variants.

The toxin was obtained by the disintegration of 1 gram of dry weight of organisms suspended in distilled water in the magnetostriction oscillator at 9,000 cycles per second. The solution of the bacterial substance was centrifuged at 10,000 rpm to remove the remaining intact organisms and debris, and the clear supernatant fluid was filtered through a Berkefeld candle. The toxin was separated from the solution, according to the method of Dubos and Geiger (1946), by

precipitation with N/1 hydrochloric acid at pH 4.0 at 4 C. The precipitate was washed in acidified water, redissolved in distilled water with the aid of N/1 NaOH, and dialyzed against cold distilled water overnight. The solution was dried *in vacuo* and the dried material tested for toxicity in mice.

Fifty-seven micrograms each of toxin obtained from the smooth or rough variants of *S. dysenteriae* were injected intravenously into rabbits. Six rabbits were immunized with each preparation. Each rabbit received 6 injections given on alternate days. Five days after the last injection the rabbits were tested for the presence of circulating antibodies. Rabbits injected with toxin from the rough variant showed neither agglutinins nor precipitins. The rabbits injected with toxin from the smooth variant possessed these antibodies. The animals in both groups were then challenged with 1 mg of the homologous toxin and all survived. Five days after the first challenge each rabbit was injected with 1 mg of whole dried organisms. All rabbits survived the second challenge as well, showing that the partially purified toxin from either rough or smooth variant afforded protection against the smooth whole organism possessing presumably the toxic somatic antigen. The results are summarized in table 1.

Immunization of rabbits with diethylene glycol extracts of smooth and rough variants of S. dysenteriae. Morgan (1937) extracted a smooth strain of *S. dysenteriae* with anhydrous diethylene glycol and obtained a substance that upon chemical analysis proved to be a polypeptide-carbohydrate-lipoid complex. Rabbits injected with this substance were shown to possess in their sera agglutinins and precipitins against the homologous strain or its products. Morgan termed this substance the somatic antigen of *S. dysenteriae*. This substance was toxic for mice in 0.25-mg amounts.

Using Morgan's method, diethylene glycol extracts were obtained both from the smooth variant and the rough variant of strain 2308. After 5 days of extraction in the cold with frequent shaking, the diethylene glycol was removed by dialysis and the resultant colloidal suspension was precipitated with 66 per cent cold acetone. The precipitate was washed with acetone and alcohol and dried *in vacuo*. This substance readily resuspended in water and in this state was used for the immunization of rabbits. The method of injection and the dosages were similar to those used in the immunization with whole organisms and purified toxins.

Tests for circulating antibodies revealed that animals injected with extracts from smooth organisms possessed in their sera agglutinins against whole smooth organisms and precipitins against purified toxin from these organisms as well as the homologous antigen. Rabbits injected with the diethylene glycol extract of rough organisms possessed no such antibodies. However, neither the former nor the latter group of animals was protected against the challenge with 1 mg of smooth *S. dysenteriae*. The results of this experiment are also summarized in table 1.

Immunization of rabbits with a nontoxic variant of S. dysenteriae 2308. A nontoxic variant of the smooth strain 2308 of *S. dysenteriae* was obtained by growing a 7-hour seed culture of the toxic strain in 15 liters of veal infusion broth at pH

7.0 in a steel tank. The culture was grown for 18 hours with vigorous shaking. A growth of 10 billion organisms per ml was obtained. The organisms were separated from the medium by centrifugation, washed three times with saline solution, and lyophilized. Mice survived the intraperitoneal injection of 1 mg (dry weight) of this variant. A suspension of these organisms agglutinated, however, with all the anti-whole-organism sera on hand. Therefore, it was decided to test the protection afforded by immunization of rabbits with this nontoxic strain.

Three rabbits were given 3 intravenous injections, each consisting of 1 mg of heat-killed organisms. Five days after the last injection, the sera of these rabbits were tested for circulating antibodies. All sera showed agglutinins and precipitins. Table 2 shows the results obtained. Three days after the test bleeding, each rabbit received 2 mg of toxic organisms of the homologous strain. All rabbits survived.

Active protection afforded by immunization of rabbits with detoxified whole organisms and sonic lysates of S. dysenteriae. An earlier article (Boroff, 1949) described the detoxification of various preparations from *S. dysenteriae* by treat-

TABLE 2

Serological reactions and active protection exhibited by rabbits immunized with nontoxic strain of S2308 S. dysenteriae

RABBIT NO.	HIGHEST SERUM DILUTION GIVING COMPLETE AGGLUTINATION*	DEGREE OF PRECIPITATION REACTION WITH ANTIGEN DILUTIONS† OF		RESULT OF CHALLENGE WITH 40 LD_{50} OF TOXIC STRAIN 2308
		1:1,000	1:10,000	
1	1:1,024	4	3	Survived
2	1:1,024	4	3	Survived
3	1:1,024	4	1	Survived

* Test antigen in agglutination test, S2308 heat-killed organisms.

† Test antigen in precipitation test, S2308 partially purified toxin.

ment with ketene gas. It was shown that none of the antigenic power of these preparations was lost by this treatment and that rabbits could withstand the injection of as much as 1 mg of detoxified preparations.

To test whether rabbits could be protected against untreated preparations by injection of the detoxified antigens, six rabbits were injected intravenously with detoxified whole organisms and six with detoxified sonic lysate. Each rabbit received 57 micrograms of the respective preparation over a period of 2 weeks. Five days after the last injection the rabbits were bled and their sera titrated for agglutinins and precipitins. The results are recorded in table 3.

All rabbits showed the presence of agglutinins and precipitins. However, when challenged 2 days after the bleeding with untreated homologous preparations of whole organisms and sonic lysates, none of the rabbits survived. Apparently ketene treatment not only destroyed the toxicity of the whole organism and sonic lysate of *S. dysenteriae*, but also affected the antigen responsible for the formation of protective antibodies.

Duration of immunity in rabbits immunized with whole organisms and various

fractions of S. dysenteriae. It has been observed during the immunization of rabbits for the purpose of antiserum production that the animals could withstand, at the end of the immunization schedule, injections of the toxic material in excess of 20 LD_{50} of the antigen. In order to determine whether this active protection would last, the rabbits were tested with 20 LD_{50} of the homologous antigen 6 months after the last immunizing injection. The number of rabbits and the antigens used as well as the outcome of the challenge are shown in table 4.

TABLE 3

Serological reactions and active protection exhibited by rabbits immunized with whole organisms and sonic lysate detoxified by ketene

RABBITS IMMUNIZED WITH ACETYLATED	HIGHEST SERUM DILUTION GIVING COMPLETE AGGLUTINATION*	DEGREE OF PRECIPITATION REACTION WITH ANTIGEN DILUTIONS† OF		SURVIVAL RATIO OF RABBITS CHALLENGED WITH 20 LD_{50} OF HOMOLOGOUS UNTREATED ANTIGEN
		1:1,000	1:10,000	
Smooth whole organisms 2308	1:1,024	4	3	0/6
Sonic lysate of smooth whole organisms 2308	1:1,024	4	3	0/6

* Test antigen in agglutination test—S2308 WO.

† Test antigen in precipitation test—S2308 partially purified toxin.

TABLE 4

Active protection shown by rabbits immunized with whole organisms and various fractions of smooth and rough variants of S. dysenteriae six months after immunization

NUMBER OF RABBITS IMMUNIZED	RABBITS IMMUNIZED WITH	RABBITS CHALLENGED WITH	SURVIVAL RATIO OF RABBITS CHALLENGED WITH 20 LD_{50} OF ANTIGEN
10	S2308 WO	S2308 WO	0/10
8	S2308-b	S2308-b	3/8
3	S2308 PT	S2308 PT	0/3
3	R2308 PT	R2308 PT	0/3
3	S2308 SA	S2308 WO	0/3
5	R2308 SA	R2308 WO	0/5

S, smooth variant.

R, rough variant.

Purified toxin, trichloracetic-acid-precipitated autolyzate of S and R variants of *S. dysenteriae.*

Somatic antigen, diethylene glycol extract from S and R variants of *S. dysenteriae.*

It is apparent that only a few rabbits retained their immunity at the end of 6 months.

DISCUSSION

The data obtained in active protection experiments indicate that it is possible to immunize rabbits with either smooth or rough whole organisms or with their respective toxins and thus induce active protection against all of these substances.

Not only whole organisms or solutions of whole organisms afforded protection against each other, but chemically purified toxins protected the animals and induced the formation of identical and reciprocally absorbable antibodies.

This phenomenon of cross protection is not in accord with the concept that two toxins and two distinct antigens exist in *S. dysenteriae*. It is, however, explainable on the basis of the presence of one dominant antigen in the organism. Futhermore, the protection afforded rabbits against the whole organism of the smooth variant by immunization with a sonic lysate of the rough variant lends additional support to the latter hypothesis. The absence of agglutinins and precipitins obviously does not denote lack of protection against *S. dysenteriae*, for it has been observed that rabbits immunized with the rough variant of *S. dysenteriae* or toxic substances derived from this variant, although showing no agglutinins or precipitins in their sera, were, nevertheless, protected against lethal doses of either smooth whole organisms or the purified toxins of this variant.

The protection afforded by immunization of rabbits with rough organisms or their fractions need not, however, be ascribed to the presence of antitoxin. No toxin-antitoxin flocculation could be observed, although both antirough serum and antigen concentrations were tested over a wide range. Neither did the addition of rough toxin to smooth antitoxin inhibit subsequent flocculation with smooth variant toxin. The phenomenon of eliciting protection in the absence of demonstrable circulating antibody has been observed with organisms devoid of exotoxin. Dingle, Fothergill, and Chandler (1938) stated that guinea pigs immunized with *Hemophilus influenzae* showed no circulating antibodies although they were fully protected. Futhermore, this protective action cannot be ascribed to the antitoxin because the rabbits immunized with rough organisms or their fractions were equally protected against smooth organisms presumably possessing the toxic somatic antigen. On the other hand, the presence of agglutinins and precipitins are not necessarily a proof that animals are protected. Immunization of rabbits with ketene-detoxified smooth organisms and their sonic lysates afforded no protection to the injected animals although their sera possessed these antibodies. The lack of protective antibodies in rabbits immunized with ketene-detoxified antigen suggests that ketene destroys not only the toxicity but also the unknown factor or factors indispensable for inducing protection.

A significant observation, which may have a bearing upon the nature of vaccine to be employed for human immunization, is that a nontoxic variant of *S. dysenteriae* afforded just as good qualitative and quantitative protection to the injected rabbits as did the toxic parent strain. It must, therefore, be concluded that toxicity of the antigen is not a determining factor in inducing immunity. However, the immunity afforded by heat-killed organisms and toxic products of *S. dysenteriae* seems not to be a lasting one.

SUMMARY

Immunization of rabbits with heat-killed organisms of smooth and rough variants of *Shigella dysenteriae* or some of its fractions affords protection against both of the variants and their toxic products.

The presence of agglutinins and precipitins in the sera of injected rabbits is not an index of protection.

The toxicity of the *S. dysenteriae* organisms is not a necessary adjunct of a protective vaccine.

The immunity afforded by the injection of a heat-killed vaccine of *S. dysenteriae* is of short duration.

The results of active protection experiments support the theory of one dominant antigen in *S. dysenteriae*.

REFERENCES

BOIVIN, A., AND MESROBEANU, L. 1937 Recherches sur les toxines bacilles dysentériques. Sur la nature et sur les propriétés biologiques des principes toxiques susceptibles de se rencontrer dans les filtrats des cultures sur bouillon du bacille de Shiga. Compt. rend. soc. biol., **124**, 442–444.

BOIVIN, A., AND MESROBEANU, L. 1937 Recherches sur les toxines bacilles dysentériques. Sur le pouvoir protecteur antitoxique des sérums obtenus en injectant à l'animal l'endotoxine-antigène O du bacille de Shiga et du bacille de Flexner. Compt. rend. soc. biol., **125**, 796–798.

BOROFF, D. A. 1949 Studies on toxins and antigens of *Shigella dysenteriae*. I. Toxicity and antigenicity of whole organisms and various fractions of *S. dysenteriae*. J. Bact., **57**, 617–632.

DINGLE, J. H., FOTHERGILL, L. D., AND CHANDLER, O. A. 1938 Studies on *H. influenzae*. Part III. Failure of complement of some animal species, notably the guinea pig, to activate the bactericidal function of sera of certain other species. J. Immunol., **34**, 357.

DUBOS, R. J. 1946 The bacterial cell. Harvard University Press, Cambridge, Mass. *Refer to* p. 270.

DUBOS, R. J., AND GEIGER, J. W. 1946 Preparation and properties of Shiga toxin and toxoid. J. Exptl. Med., **84**, 143–157.

MORGAN, W. T. J. 1937 Studies in immunochemistry. II. Isolation and properties of a specific antigenic substance from *S. dysenteriae* (Shiga). Biochem. J., **31**, 2003–2021.

MORGAN, W. T. J., AND PARTRIDGE, S. M. 1941 Studies in immunochemistry. VI. The use of phenol and alkali in the degradation of antigenic material isolated from *Bact. dysenteriae* (Shiga). Biochem. J., **35**, 1140–1163.

STEABBEN, D. 1943 A study on bacteriological lines of antigens derived from *Bact. dysenteriae* (Shiga). J. Hyg., **43**, 83–85.