STUDIES ON THE FLEXNER GROUP OF DYSENTERY BACILLI

III. Antibody Response in Man Following the Administration of the Specific Antigen of Type V Shigella paradysenteriae (Flexner)*

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The prophylactic immunization of man against bacillary dysentery has proved to be a difficult problem. The severe reactions evoked by the toxic microorganisms used in vaccines (1, 2), the multiplicity of bacterial types encountered in the disease, and the difficulties experienced in evaluating the efficacy of vaccines are all factors which have impeded progress in the control of the disease by immunological measures.

During the past year we have been engaged in a study of the specific antigens of the Flexner group of dysentery microorganisms. Our purpose has been to learn something of the chemical nature of these substances and something of their immunological and toxic properties as well. The specific antigens of the Flexner organisms are lipocarbohydrate-protein complexes similar to those isolated by other investigators from various Gram-negative bacilli (3–5). These substances appear to reflect the essential immunological properties of the microorganisms from which they are derived. They are toxic and give rise in experimental animals to antisera which agglutinate the homologous organism, precipitate the antigen, and confer passive protection on mice against lethal infections.

Numerous attempts have been made to modify or detoxify vaccines of dysentery bacilli for purposes of prophylactic immunization, but thus far this has not been satisfactorily achieved (2, 6–11). In an effort to avoid the toxic reactions evoked by the injection of certain Gram-negative organisms, other investigators have separated the O antigens from extraneous cellular elements and have used these substances experimentally in man (12, 13). Because organisms of the *Shigella paradysenteriae* (Flexner) group are those most frequently encountered in areas where bacillary dysentery is prevalent (14–21) and because of the difficulties experienced with the use of whole bacterial vaccines, we have studied the response in man to the injection of the specific antigen of the Type V organism. The antigen of this type cross-reacts with other organisms of the group and was, therefore, chosen in the present study.

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That this highly purified substance when administered intracutaneously in appropriate amounts gives rise to demonstrable circulating antibody in human beings without causing severe reactions is seen in the following account.

EXPERIMENTAL

Specific Antigens.—The specific antigen and the polysaccharide used in this study were isolated from an acetone-killed culture of a strain of *Shigella paradysenteriae* (Flexner) Type V obtained from the collection of the Army Medical Center. The preparation and purification of these substances have been described in Paper I of this series.

The antigen was dissolved in pyrogen-free physiological saline and sterilized by filtration through a Berkefeld candle.

Injection of the Antigen.—A group of 19 healthy adult individuals who gave no past history of bacillary dysentery was divided into 3 groups, each of which received the indicated doses of the antigen intracutaneously at weekly intervals.

	Group I: 5 subjects	Group II: 8 subjects	Group III: 6 subjects
Dose	5.0 μg.	2.5 μg.	Prolonged course
	7.5 "	7.5 "	(See text)
	10.0 "	15.0 "	
Total.....	22.5 μg.	25.0 μg.	40 to 100 μg.

The subjects were bled before and 3 weeks following the course of injections.

All the volunteers developed a local reaction following the administration of the antigen. The reaction consisted of erythema, swelling, and tenderness which appeared in about 4 hours, reached a maximum of 4 × 6 cm. in 18 hours, and completely subsided in 36 hours. The reaction in 4 of the subjects in group I and in 5 of the subjects in group II was associated with a lymphangitis and an epitrochlear and axillary adenopathy. In the entire group 10 individuals received an initial dose of 5 μg. or over, and 9 received an initial dose of 2.5 μg. Four of the former and only one of the latter in this group developed a generalized systemic reaction, consisting of malaise, occipital headache, and in some instances chills and fever. The systemic reactions followed the first injection only. In every case the local reaction became less severe on subsequent inoculations in spite of the increasing dose (22, 23). Each of the subjects in group III received a somewhat different course of injections with the total doses varying from 40 to 100 μg. No subject in group III received a single dose greater than 25 μg. since this seemed to be the greatest amount that could be tolerated without systemic reactions. After 3 or 4 injections, 4 of the subjects in group III developed an immediate allergic type of reaction (24) consisting of a wheal with pseudopods and a surrounding erythematous flare. This reaction subsided in 20 to 30 minutes and was followed by the usual type of reaction. This allergic response could be avoided by decreasing the interval between injections.

Homologous Agglutinins.—Preliminary tests indicated that the maximum response as measured in terms of homologous agglutinins occurred 2 to 3 weeks following the last injection. Accordingly, all the individuals were bled 3 weeks following the last dose.

The sera of each individual obtained before and after the course of injections were titrated in parallel. A standardized suspension of formol-killed Type V organisms was prepared. Serial dilutions of serum were mixed with the bacterial suspension and incubated at 56°C. for 3 hours, then placed in the icebox overnight.

The titration of 3 sera, an excellent, an average, and a poor serum, is shown in Table I. Here it is seen that in each instance following the course of injec-

TABLE I

Agglutinin Titration of Serum of Three Subjects before and after Inoculation with Specific Antigen of Type V Shigella paradysenteriae (Flexner)

Subject	Serum	Final dilution of serum								Increase in agglutinins
		1:12.5	1:25	1:50	1:100	1:200	1:400	1:800	1:1600	
La	Pre-treatment	2*	1	0	0	0	0	0	0	32-fold
	Post-treatment	4	4	4	4	4	3	1	0	
Go	Pre-treatment	2	½	0	0	0	0	0	0	8-fold
	Post-treatment	4	4	4	3	1	0	0	0	
Ho	Pre-treatment	3	3	2	0	0	0	0	0	2-fold
	Post-treatment	4	4	4	2	0	0	0	0	

* 4 = complete agglutination with clear supernatant liquid.
½ = agglutination detectable with hand lens.
0 = no agglutination.
These notations are used in all subsequent tables.

tions a definite increase in specific bacterial agglutinins resulted. Table II summarizes the titrations of the sera of all the subjects and indicates the average increase for each group. The subjects in group I showed an average increase of fivefold and those in groups II and III of approximately sixteenfold in bacterial agglutinins.

The prolonged course of injections given to the subjects in group III was not accompanied by a further increase in the agglutinin response. The titer of the sera of these individuals reached a plateau, and subsequent injections apparently failed to increase the amount of circulating antibody.

Heterologous Agglutinins.—The serum of each individual was tested for agglutinins against the heterologous types of Flexner organisms. The cross-agglutination reactions of one of the more potent sera are given in Table III. The results presented in Table III indicate that the antiserum evoked by the

Type V specific antigen agglutinates not only the homologous organisms but those of heterologous strains as well. The cross-reactions are similar to those exhibited by the serum of rabbits immunized with Type V organisms as indicated in Table IV.

TABLE II

Distribution of the Increase in Specific Agglutinins Following Inoculation with Specific Antigen of Type V Shigella paradysenteriae (Flexner)

Group	No. of subjects	Increase in agglutinins						Average increase in agglutinins for each group
		0	2-fold	4-fold	8-fold	16-fold	32-fold	
		No. of subjects						
I	5		1	2	2			5-fold
II	8			2	3		3	16-fold
III	6			1	2	1	2	~ 17-fold

TABLE III

Homologous and Heterologous Agglutinins in the Serum of a Subject Inoculated with the Specific Antigen of Type V Shigella paradysenteriae (Flexner)

Serum	Test suspension	Final dilution of serum						
		1:25	1:50	1:100	1:200	1:400	1:800	1:1600
	type							
Post-treatment.....	V	4	4	4	4	4	3	1
	W	2	1	1	0	0	0	0
	X	3	2	1	½	0	0	0
	Y	4	4	4	4	4	1	0
	Z	4	4	4	4	4	3	0
	VZ	4	4	4	4	4	2	0
Pre-treatment.....	V	2	1	0	0			
	W	0	0	0	0			
	X	1	1	½	0			
	Y	3	2	0	0			
	Z	0	0	0	0			
	VZ	0	0	0	0			

Mouse Protection Tests (Homologous).—For purposes of determining the average increase in mouse-protective antibodies the sera of the individuals before and after injection of the specific antigen were in each instance pooled. The tests were performed with a mouse-virulent Type V strain. Varying concentrations of serum were injected subcutaneously into mice 18 hours before the intraperitoneal injection of the challenging dose of organisms. The bacteria were obtained from a 6 hour culture and were thoroughly mixed with a sterile 5 per cent suspension of Wilson's granular mucin Type 1701-W (25). The challenging dose consisted of 5400 organisms, representing more than 1500 M.L.D. The 50 per cent survival end point was determined by the method of Reed and Muench (26).

The results of the protection tests given in Table V indicate that a tenfold increase in mouse-protective antibodies can be demonstrated in the pooled

TABLE IV

Homologous and Heterologous Agglutinins in Serum of a Rabbit Injected with Heat-Killed Type V Shigella paradysenteriae (Flexner) Organisms

Test suspension	Final dilution of serum							
	1:50	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400
type								
V	4	4	3	3	2	1	½	0
W	3	2	1	0	0	0	0	0
X	1	½	0	0	0	0	0	0
Y	3	3	2	1	½	0	0	0
Z	4	3	2	2	1	1	½	0
VZ	4	3	3	2	2	1	½	0

TABLE V

Homologous Protective Antibodies in Pooled Sera of Individuals before and after Inoculation with Type V Antigen

Serum	Amount used	No. of mice		50 per cent end point
		Survived*	Died	
	cc.			*cc.*
Pooled pre-treatment	0.05	0	4	
	0.10	0	4	
	0.20	2	2	0.20
	0.30	4	0	
	0.40	4	0	
Pooled post-treatment	0.005	0	4	
	0.010	0	4	
	0.020	2	2	0.02
	0.040	4	0	
	0.060	4	0	
None	—	0	3	—

Challenging dose of organisms: 0.05 cc. of a 10^{-4} dilution of culture + 0.95 cc. mucin = 5400 organisms = > 1500 M.L.D.

M.L.D.: 3 organisms in mucin killed all control mice injected.

* Survived more than 5 days.

sera of the immunized subjects. This increase corresponds well with the increase in agglutinin titer of the pooled sera.

Mouse Protection Tests (Heterologous).—The capacity of the pooled sera to protect mice against infection with the heterologous, cross-reacting Type Z

organism was likewise determined. Since the Type Z strain was not as virulent as the Type V, the challenging dose of organisms (40,000 bacilli) was 133 L.D. $_{50}$. The results presented in Table VI indicate that there is a twofold increase in heterologous Type Z mouse-protective antibodies in the sera of the inoculated individuals.

Injection of Specific Polysaccharide.—In addition to the specific antigen the fractionation of extracts of the Type V Flexner organisms yields a specific

TABLE VI

Heterologous Protective Antibodies in Pooled Sera of Individuals before and after Inoculation with Type V Antigen

Serum	Amount used	No. of mice		50 per cent end point
		Survived*	Died	
	cc.			*cc.*
Pooled pre-treatment	0.20	0	4	
	0.24	0	4	
	0.28	2	2	0.296
	0.32	2	2	
	0.36	4	0	
Pooled post-treatment	0.10	0	4	
	0.14	1	3	0.156
	0.18	4	0	
	0.22	4	0	
	0.26	4	0	
None	—	0	3	—

Challenging dose of organisms: 0.5 cc. of a 10^{-4} dilution of culture + 0.5 cc. mucin containing 40,000 organisms equivalent to approximately 133 L.D.$_{50}$.

L.D.$_{50}$: approximately 300 organisms.

* Survived more than 5 days.

polysaccharide. This preparation is not toxic when injected into mice in quantities as great as 20 mg. Although this material does not stimulate the production of antibodies in rabbits, it was tested in a group of human volunteers in order to determine whether, like the pneumococcal polysaccharides (27–29) it would be antigenic in man.

Ten volunteers were divided into 3 groups as indicated below. Single intracutaneous injections were given, except to the subjects in group III. These individuals received 2 injections administered 1 week apart.

	Group I: 4 subjects	Group II: 3 subjects	Group III: 3 subjects
Dose	50 μg.	100 μg.	100 μg.
			100 "
Total			200 μg.

All individuals were bled before and 3 weeks following the inoculation. In no case was there a systemic reaction. The local reactions were mild, consisting of slight swelling and erythema which reached a diameter not greater than 1.5 cm. There was no adenopathy or lymphangitis. Two of the 10 subjects developed agglutinins; one had received 50 μg. and the other 100μg. of polysaccharide. The former subject showed a sixteenfold increase and the latter a fourfold increase in homologous agglutinins. The more potent serum was tested for mouse-protective antibodies against the Type V organism. The protocol is given in Table VII.

TABLE VII

Mouse-Protective Test of Serum of a Subject Inoculated with Specific Polysaccharide of Type V Shigella paradysenteriae (Flexner)

Serum of subject Sc	Amount used	No. of mice		50 per cent end point
		Survived*	Died	
	cc.			*cc.*
Pre-treatment	0.05	0	3	0.090
	0.10	2	1	
	0.20	3	0	
	0.30	3	0	
Post-treatment	0.01	0	3	
	0.02	0	3	
	0.03	1	2	0.032
	0.04	3	0	
None	—	0	3	—

Challenging dose of organisms: 0.05 cc. of a 10^{-4} dilution of culture + 0.95 cc. mucin = 3000 organisms = > 1500 M.L.D.

M.L.D.: 3 organisms in mucin killed all mice injected.

* Survived more than 5 days.

It can be seen from Table VII that the injection of the Type V specific polysaccharide produced a threefold increase in homologous mouse-protective antibodies in the serum of one of the subjects.

DISCUSSION

The use of immunologically active and antigenic bacterial products for the experimental immunization of man has been reported by a number of investigators. The protein-free capular polysaccharides of Pneumococcus appear to function well in the production of specific antibodies when administered intradermally to human beings in minute amounts (27–29). The somatic antigens of the typhoid (12) and Shiga dysentery bacillus (13) have likewise been found to evoke specific agglutinating and mouse-protective antibodies in human

subjects. Indeed, in one well established study (30, 31), it was shown that a marked lowering in morbidity and mortality in a large group of individuals resulted from the injection of an extract containing the endotoxin of *Eberthella typhosa*. In all these studies it has been the objective of these investigators to avoid the severe and toxic reactions which accompany the use of whole vaccines. Save in the case of the pneumococcal polysaccharides it cannot be said that this objective has been reached, for toxicity is an inherent property of the somatic antigens of all the Gram-negative organisms that have been investigated.

Although it has been possible to demonstrate a correlation between agglutinins, mouse-protective antibodies, and actual immunity in certain infectious diseases, it has thus far not been demonstrated for bacillary dysentery. In the latter disease the organisms are confined to the gastrointestinal tract and only rarely cause bacteremia. It would seem, therefore, that circulating antibodies do not necessarily reflect the state of local tissue immunity in the gastrointestinal tract (32). In spite of numerous attempts to determine the efficacy of vaccination against the Flexner dysentery bacilli, considerable confusion and differences of opinion have resulted. Indeed, it has not been fully determined whether an actual attack of bacillary dysentery confers any immunity to subsequent infection (33–35). However, evidence gathered from carefully controlled bacteriological and epidemiological studies seems to support the view that an attack of dysentery does confer an active immunity against subsequent infections with bacilli of the homologous type. That Flexner dysentery vaccines may have prophylactic value is also suggested by a recent study of their use in the control of institutional dysentery (36).

In the foregoing account the experimental inoculation of human subjects with the specific somatic antigen of Type V Flexner dysentery bacillus has been described. This purified material, free from contaminating cellular constituents, gives rise to reactions in human beings which are no more severe than those described in connection with typhoid-paratyphoid vaccines. In response to the inoculations of the antigen the individuals developed specific bacterial agglutinins, and mouse-protective antibodies as well. Subjects injected with the specific antigen of the Type V bacillus developed a high titer of agglutinins which diminished moderately after a period of 6 months. At this time the titer of circulating antibodies can be increased by a small recall dose. Therefore, if agglutinins in any way reflect immunity, the duration of the effect is sufficient to be of practical value.

One of the factors which must be taken into consideration in the development of an effective immunizing agent is the multiplicity of species and types involved in the etiology of bacillary dysentery. In the present study it has been shown that immunization with the specific antigen of Type V evokes agglutinins which cross-react with other organisms of the Flexner group. In

the sera of the vaccinated individuals it has also been possible to demonstrate the presence of antibodies capable of protecting mice against infection with a cross-reacting Type Z strain. It may be pointed out that the V, Z, VZ, and Y types of *Shigella paradysenteriae* (Flexner) are intimately interrelated serologically (36, 16). With this in mind and with a knowledge of the relative frequency of the etiological agents in bacillary dysentery it would seem that a mixed antigen containing Types V and W *Shigella paradysenteriae* (Flexner), *Shigella sp. Newcastle* and *sp. Sonné* antigens would anticipate the greatest number of infections.

The specific polysaccharide of the Type V *Shigella paradysenteriae* (Flexner) when injected into a number of volunteers gave rise in one instance to mouse-protective antibodies. Thus, it appears that antibodies directed against this one portion of the antigenic complex are capable of protecting mice from lethal infection with the homologous organisms. Although the response to the injection of the polysaccharide is irregular and uncertain, the results suggest that the toxic portion of the full antigen may not be necessary for the production of prophylactic immunity. The carbohydrate used in the present study was obtained by fractionating a crude extract of the organisms. There is some evidence that this preparation is partially depolymerized. A polysaccharide subsequently obtained by chemical degradation of the antigenic complex was found to be more active immunologically and was likewise non-toxic. Future tests with this more active preparation, it is hoped, may prove it to be more consistent and reliable as a non-toxic antigen in human beings.

SUMMARY

1. Volunteers have been immunized with the purified specific antigen of Type V *Shigella paradysenteriae* (Flexner). The subjects developed a high titer of bacterial agglutinins and mouse-protective antibodies. The agglutinin titer fell moderately after a period of 6 months. The subjects responded fairly well to a small recall dose of the antigen.

2. Two individuals from a group of 10 injected with the specific polysaccharide obtained from Type V organisms responded with an increase in bacterial agglutinins. Mouse-protective antibodies were demonstrable in the one serum tested.

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