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A STUDY ON BACTERIOLOGICAL LINES OF THE ANTIGENS DERIVED FROM *BACT. DYSENTERIAE* SHIGA AND OF THEIR ANTISERA IN PROTECTIVE TESTS AGAINST THE LIVING ORGANISMS

BY DOROTHY STEABBEN, *From the Serum Department of the Lister Institute, Elstree, Herts.*

PART 1. THE PREPARATION AND EXPERIMENTAL TESTING OF ANTIGENS FROM NORMAL AND VARIANT STRAINS

Recent work on the chemical analysis of the bacillary bodies of Shiga bacilli and of the toxin produced in the course of their autolysis, particularly that carried out by Boivin and his collaborators (1937 and other papers; see list of references), Haas (1937, 1938), Istrati (1938) and by Morgan (1937, 1940), has revived an old view held by some but disputed by others that two distinct antigenic and toxic constituents are capable of demonstration, one the so-called 'endotoxin' or 'enterotoxin' and the other the neurotoxin, so called from its paralytic action in certain laboratory animals but which is in addition highly enterotropic. The former, whether it can be shown to possess specific toxic power or not, represents with certainty the 'O' antigen of the bacillus and is found only in the smooth variant. It is thermostable and toxic action has been ascribed to it from the fact that when injected into the mouse it causes, in appropriate dose, death accompanied by enteritis. As an antigen, however, it is admittedly weak though sera produced in response to its injection have been held to function as anti-endotoxins. Chemically it is, according to Boivin *et al.*, a polysaccharide phospholipin complex, whereas Morgan & Partridge (1937, 1940, 1941) believe this antigenic complex to be a polysaccharide-protein complex, the phospholipin moiety playing no part in the immunological properties exercised by the specific 'O' antigen. On the other hand, the 'neurotoxin' derived from the autolysis of the bacilli is a protein of intracellular origin diffusing readily from the bacilli especially during autolysis. It is thermolabile and a potent antigen, causing paralysis of the limbs of the mouse and rabbit, but endowed as well with enterotropic properties, producing in the rabbit even greater intestinal lesions than the so-called endotoxin. Both smooth and rough strains yield this neuro-enterotoxin.

The ill-defined implications in the terms 'endotoxin' and 'neurotoxin' have led to their replacement in the following study by the terms enterotoxin and neuro-enterotoxin which reflect perhaps more accurately the parts the toxins are believed to play in the pathogenesis of dysentery in experimental animals. In man, as is well known, the enterotropic action is dominant, neurotropic effects being only rarely observed.

The primary objective of the experimental work which follows was to assess the value, in the control of experimental infections in mice, of sera produced by immunizing the rabbit with various preparations derived from typical smooth strains and from certain variants as described below; the results on this latter head are to be found in Part 2 of this paper. In Part 1 we shall be concerned solely with the description and properties of the strains used, in so far as their study on purely bacteriological lines is calculated to throw light on the alleged dual nature of Shiga toxins and their respective specificities. The experiments in fact represent an extension in the light of present knowledge of the work carried out in this Institute by Kanai (1922) in the course of an unsuccessful attempt to confirm the findings of Olitsky & Kligler (1920).

I *The origin and general characters of the strains used*

Strain A. This is the stock smooth strain of Shiga K 624, grown always on agar; smooth character maintained by regular colony selection on agar plates: colonies smooth, domed, shining and extremely viscid except when young: it contains the smooth antigen and produces neuro-enterotoxin of high titre.

Strain B. Stock rough strain of Shiga K 624; contains no smooth antigen but produces neuro-enterotoxin of high titre.

Strain C. Obtained by passage of A through mice, grown always on haemoglobin agar; colonies smooth, shining and domed but of two kinds, one viscid and of a bluish semi-transparency by artificial light, the other (variant strain D) heavily opaque and not viscid.

Strain C contains the smooth antigen but was found in the course of mouse passage to produce only negligible amounts of neuro-enterotoxin.

Details of the mouse passages. Culture A was grown on Difco peptone agar, to each slope of which was added 0·5 ml. of a 15% solution of sheep red cells laked in sterile distilled water, filtered through a Berkefeld candle and kept at 0° C. The culture was incubated for 5 hr. or less and the growth was washed off with 2 ml. of trypsin broth, and the resulting suspension standardized by Brown's tubes to an opacity of 1000 million organisms per ml. The inoculum used was 0·45 ml. of a 5% mucin preparation to which was added 0·05 ml. of the broth suspension, that is, 50 million organisms. This dose was the least which would with certainty kill the mouse within 48 hr. Two mice were inoculated intraperitoneally, and next morning the heart blood was plated on haemoglobin agar. After overnight incubation colonies that were smooth in appearance were selected, a haemoglobin agar slope inoculated, and the mouse passage repeated. The purity of the culture was controlled by agglutination tests and the fermentation of the standard sugars. With one or two exceptions, this passage was repeated every other day for 93 times. At first the inoculum did not contain a certain lethal dose, and, on some occasions, appeared to be less rather than more virulent than the original culture. After about 50 passages the inoculum frequently killed the mice within 24 hr., but since it was advisable to recover the culture from a freshly killed mouse rather than risk contamination from post-mortem invasion, the inoculum was reduced to 25 million organisms in the same amount of mucin. This dose may or may not kill within 24 hr., but usually one of the animals survives and the culture can be recovered from it. All the mice in this series died with a severe enteritis.

The effect of incorporating the dose in mucin

In the case of strain A some direct comparisons were made with regard to the influence of the vehicle, i.e. with and without mucin (see Table 1). The difference in the mortality rates seems undoubtedly significant, but the effect lags far behind that obtained when mucin is used to enhance the lethal action of organisms such as *Bact. typhosum* and the *Meningococcus*. Although the use of mucin makes possible a lowering of the number of organisms in the lethal dose, the test dose employed in the subsequent mouse experiments was never below 50 million bacilli because serial passage through the mouse did not increase the virulence of the strain.

Table 1. *The influence of mucin as compared with broth in enhancing the lethal action of* B. dysenteriae (*Shiga*) *strain K 624 A: intraperitoneal injection in mice*

Broth				Mucin			
Dose millions	No. inoculated	No. died	Mortality rate %	Dose millions	No. inoculated	No. died	Mortality rate %
50	38	26	68	50	38	35	92
20	20	6	30	20	20	9	45
5	8	0	0	5	8	3	37

Strain D. Variant of C with properties as described above. Like C it produces negligible amounts of neurotoxin during autolysis. When injected into rabbits, it calls forth no smooth agglutinins and would seem to resemble a strain described by Haas (1937), which though smooth contained no 'endotoxin'.

Strain E. This is the fully toxic strain Lemnos no. 187 from the National Collection of Type Cultures and is unrelated to K 624. Its colonies are smooth, domed and shining but are not viscid.

The lethal dose of living bacilli from strains A, B, C and D when given to mice intraperitoneally

Lethal dose of A. The mouse is not highly susceptible to infection with the Shiga bacillus but is capable of revealing differences in virulence between strains. Within the author's experience the lethal dose of a strain unrelated to A was 300,000 organisms when suspended in mucin, whereas the dose of A required to kill a mouse within 48 hr. never fell below 25 millions (see Table 1).

Lethal dose of B. The absence of the smooth antigen in this rough strain made it appear likely that it would prove to be less 'virulent' than A. Boivin & Mesrobeanu (1938*b*) have in fact reported that the

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lethal dose of a rough culture was 100 times larger than that of a smooth culture when given to mice with mucin. In the present experiments mice died overnight when 200 million organisms of a 5 hr. culture with mucin were given. Decreasing doses were then administered, the results of which are collected in Table 2. Paralysis of the hind limbs, a characteristic effect of the neurotoxin, was not evident in mice dying within 48 hr., but it was noted in almost all those which died after the third day. The latent interval doubtless represents the time required for fixation of the toxin and for the resulting neural lesion to produce its effect. The tests as displayed in Table 2 reveal the fact that the rough form of K 624 is, unlike most pathogens, as lethal as the smooth form, an effect which would seem to be due to invasion of the blood stream when the dose is suspended in mucin and to the resulting cumulative action of the neurotoxin. The extent of the bacteriaemia in mice dying within 48 hr. is such that survival would seem to be an impossible event. This is true of both the rough and smooth forms, and the degree of bacteriaemia is in proportion to the number of organisms in the inoculum. A peritoneal film made within 24 hr. of an adequate inoculum and stained with Giemsa shows enormous numbers of leucocytes, mostly polymorphonuclear cells, packed with bacilli; the background of the film consists of innumerable free bacilli. When a mouse receives a sublethal dose of bacilli, a peritoneal film still shows large numbers of leucocytes, but these are mainly monocytes and no bacteria are to be seen. A study of Table 2 and the observations described below indicate that when a dose of the rough K 624 is not sufficiently large to cause an overwhelming bacteriaemia, the bacteria may none the less produce in the body a lethal dose of the neurotropic toxin. The lethal dose of a potent preparation of this toxin is 0·001 ml., i.e. the amount which kills mice within 7 days when injected intravenously. This dose, as will appear later, is equivalent to the amount set free by the autolysis in vitro of from 20 to 30 million organisms (see Table 4). If then a small inoculum of B multiplies sufficiently to produce one lethal dose of the toxin in the body before the organisms are eliminated, virulence as a lethal factor need not be postulated.

Table 2. *The lethal dose of the rough strain of Shiga K 624 B: intraperitoneal injection in mice with mucin*

Dose millions	No. inoculated	No. dying in (days) 1	2	3	4	5	6	7	8	11	Proportion dying	No. showing paralysis
200	4	4	.	.	.	.	.	.	.	.	4/4	0
50	15	1	5	.	1	.	3	1	1	1	13/15	6
25	7	4	.	.	1	2	.	.	.	.	7/7	5
12–16	5	1	2	.	.	.	1	.	1	.	5/5	1
5	4	.	1	.	1	.	.	.	.	.	2/4	1
1	4	.	.	.	.	.	.	.	.	.	0/4	0

The following observations were made on the passage of the rough form B from the peritoneum into the circulating blood.

Two groups of six mice each were inoculated intraperitoneally with a dose of 50 million living organisms derived from 5 hr. cultures of B. The doses given to one group were suspended in broth and to the other group in mucin. Two mice were killed in each group after 2½ hr., two mice after 5 hr., and the remaining two served as controls. When the mice were killed a drop of the heart blood was mixed with melted agar and poured into a Petri dish; the colonies were counted after overnight incubation. The plates prepared from the mice that received the broth inoculum yielded very few colonies. Of the two control mice in this group one was killed next morning and the heart blood found to be sterile; the other mouse survived. When, however, the dose was suspended in mucin the colonies on the plates made at 2½ and 5 hr. were uncountable. Of the two controls in this group, one was killed next morning, and its heart blood yielded a few colonies on the plate; the other died after 7 days. This experiment gives some evidence of an early phase of the multiplication of the invading organisms in the presence of mucin, and also indicates how the delayed toxic deaths may occur, for the examination of the mucin-control mouse which was killed overnight showed that the number of organisms was apparently declining. This was confirmed by the fact that its companion survived till the seventh day.

Lethal dose of C. As we have already stated, the smallest intraperitoneal dose with mucin which will kill a mouse within 48 hr. is 25–50 million organisms. It has remained constant for 2 years. An occasional mouse has survived, but this has been attributed to the presence of the variant D in the test culture. No record was made of late deaths due to neurotoxin, but, as might be expected from the present condition

of the culture, late deaths do not now occur, and a mouse which survives the initial infecting dose for 48 hr. survives indefinitely.

Lethal dose of D. This variant of C has not been examined in any great detail. When mice were given an intraperitoneal dose of 200 millions of a 5 hr. culture in mucin, two of four survived while all of seven mice survived a dose of 100 millions. The lethal dose is therefore a large one, and in this respect and leaving out of consideration its colonial appearance, D approximates more nearly to the criterion of a rough form than B, the more normally toxic rough form of A.

The lethal dose of heat-killed cultures of strains A, B and C when given intravenously to mice

The somatic substance—the so-called 'enterotoxin' or 'endotoxin' of the smooth variety of the Shiga bacillus—is said to be heat-stable (100° C.), whereas the neuro-enterotoxin is destroyed at 80° C. It was thought therefore that it might be possible to estimate the 'enterotoxin content' of the strains A, B and C by the intravenous injection into mice of washed bacilli killed by heat at 100° C. (10 min.). Table 3 shows that the average number of organisms constituting a lethal dose was 5000 millions when estimated by opacity or by weight. The dose was the same for all three and for a non-pathogenic *Bact. coli* used as a control. It seems probable that an intravenous inoculum of this amount in the mouse causes death

Table 3. *The lethal dose of heat-killed cultures of Shiga K* 624 (*A, B and C*): *intravenous injection in mice*

K 624 strain	Age of culture hr.	Treatment	No. of bacilli millions	Dose estimated by	Proportion of mice dying
A	5	Heated 10 min. at 100° C.	5000	Opacity	5/8
			2500		1/2
			1250		0/2
B	5	Do.	5000	Opacity	5/8
			2500		0/2
			1250		0/2
C	5	Do.	5000	Opacity	6/8
			2500		0/2
			1250		0/2
C	20	Heated 1 hr. at 58° C.; dried by evaporation	5000	Weight	2/2
			3000		0/3
			1000		1/2
			500		0/4
B. coli	5	Heated 10 min. at 100° C.	5000	Opacity	5/8

from protein shock, and Boivin (1940*a*) states in fact that the 'endotoxin' derived from the Shiga bacillus has the same pathogenic action in experimental animals as that from *Bact. coli*. The post-mortem appearances following a lethal dose were similar, namely, enteritis, pale liver, congested lungs, and sometimes blood-stained material in the caecum. Such appearances are consistent with death from protein shock, and they support the idea we have formed that the lethal action of the bacterial protein apart from the neuro-enterotoxin may be largely non-specific. It may be added that the lethal dose of C when killed by 0·5% phenol is also 5000 millions, and that this figure represents the lethal dose of the bacterial residue from its autolysate (see Table 3). When C bacilli, killed by heat at 100° C. to free them entirely from neuro-enterotoxin, are given intraperitoneally to the mouse, the lethal dose is even larger than the intravenous and contains *c.* 10,000—20,000 millions. This latter dose also represents the lethal dose by the peritoneal route of the bacterial residue from an autolysate.

The effect of heating the fully toxigenic A strain at 70° C. As Kanai (1922) observed, it is impossible to ensure complete destruction of the neuro-enterotoxin within or on the bacilli unless the organisms are heated to 80° C. or more. In the present experiments heating at 70° C. for 30 min. was employed. Thorough washing of the killed bacilli did not reduce their neuro-enterotoxic action, and toxin was still present in the washed bacterial residue from the autolysate of A. What lethal action the A bacilli had in this experiment must be attributed to their neuro-enterotoxin content, since the lethal dose is smaller than that of the same culture when heated at 100° C.

The lethal dose of autolysates (generally called toxins) of cultures of strains A, B, C and D when given intravenously to mice

Autolysates were made from the four variants A, B, C and D by the same general method. The bacillus was grown for 5 hr. or for 24 hr. on Roux bottles of Difco agar, and the growth was washed off with about 10 ml. of saline per bottle, centrifuged once, and resuspended in a known volume of saline. The concentration of organisms per ml. was estimated by means of Brown's opacity tubes. The reaction of the saline suspension was adjusted to *p*H 9·0, toluol was added and the preparation incubated at 37° C. for 7 days; the *p*H was adjusted each day if necessary, and finally the supernatant was centrifuged, filtered through a Berkefeld candle and stored at 0° C.

Toxin was prepared in this way on several occasions from the rough strain B in particular, and the resulting toxicity by the intravenous route was relatively constant, the lethal dose for the mouse being about 0·001 ml. Comparative tests of the neuro-enterotoxin production of the rough culture B and of the mouse-passaged culture C unexpectedly showed that the latter culture, in the course of passage through the mouse, had almost completely lost its toxigenic power. Many tests have been made with consistent results; a typical experiment will be found in Table 4, from which it will be noted that the autolysate from a relatively small number of organisms of the rough strain B kills the mouse, whereas

Table 4. *The toxicity of autolysates (toxins) derived from K 624 variants: in order to compare the toxicity of the autolysates used the doses are expressed as the number of bacilli that correspond to the amount of autolysate injected; intravenous injection in mice*

Strain	Age of culture used for autolysate hr.	Vol. of autolysate ml.	Dose millions	Proportion of mice dying
A	24	0·01	300	6/6
		0·001	30	1/6
	5	0·2	2500	2/2
		0·1	1250	4/4
		0·01	125	1/2
B	24	0·01	168	3/3
		0·001	16	3/3
	5	0·01	40	6/6
		0·001	4	3/6
C	24	0·2	9600	2/2
		0·1	4800	0/3
	5	0·2	2600	0/6
		0·1	1300	0/6
D	24	0·5	25000	4/4
		0·2	10000	1/7

the corresponding number of organisms of the mouse-passage strain C is 100–200 times larger. C, however, has retained some capacity to produce neuro-enterotoxin because the serum from a rabbit immunized with it was found to contain 5 units of antitoxin per ml. The smooth culture A produced toxin of distinctly lower titre than that of the rough, while culture D, the variant of C above mentioned, apparently yielded none.

The lesions produced by injection into animals (mouse, guinea-pig and rabbit of (1) *living bacilli of the feebly toxigenic strain C and* (2) *of neuro-enterotoxin prepared from the rough organism B*

Though one cannot draw conclusions as to the participation of specific toxins from the post-mortem picture after infection with the living organism or compare such picture with that following the injection of a toxin, it was considered desirable to ascertain the type of lesion produced by C in view of its feeble power to yield toxin in vitro.

Mouse intraperitoneally. The usual autopsy finding is severe enteritis with occasional petechiae in the bowel. The animals die in 24–48 hr. with no preceding paralysis. The picture does not differ materially from that following intraperitoneal injection of A or B.

Guinea-pig intraperitoneally. As guinea-pigs are notoriously more resistant than rabbits the doses used were of the order of 500–1000 millions. Some died in less than 24 hr., while others were very ill for

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24 hr. and either died late (e.g. after 8 days) or recovered completely. At autopsy there was acute congestion of intestinal vessels and of abdominal viscera, but no intestinal haemorrhages were present as in the rabbit.

Rabbit intraperitoneally. Inoculations of 500–2000 millions with mucin caused death in 16–40 hr., while smaller doses of 50 millions did not necessarily kill but produced a marasmic condition. At autopsy, in addition to general congestion, there were widespread petechiae from the upper intestine down to the caecum which was filled with blood-stained grumous material. Paralysis was not observed. The haemorrhages in the intestine were more extensive than those noted after injection of neuro-enterotoxin alone.

Injection of neuro-enterotoxin prepared from B. It was thought that the intradermal injection of neuro-enterotoxin prepared from B might give some information as to the part it plays, if any, in the production of necrosis in the intestinal mucosa. Similar observations have been made by Istrati (1938).

The reaction produced in the skin of the guinea-pig. Doses of 1/10, 1/50, 1/100, 1/200, 1/400 and 1/800 ml. of the toxin (mouse L.D. = 1/1000 ml.), each made up to a volume of 0·2 ml. in saline, were injected intracutaneously into the shaven flanks of three guinea-pigs. The reactions in all three guinea-pigs were similar; the doses of 1/10 and 1/50 ml. caused a little swelling and induration, which by the fourth day showed very slight necrosis and then disappeared. The four smaller doses caused no reaction in any of the guinea-pigs. The aggregate amount of the inocula in each guinea-pig was nearly 140 lethal doses for the mouse, but the animals showed no symptoms of illness.

The reaction produced in the skin of the rabbit. Doses of 1/50, 1/100, 1/200, 1/400 and 1/800 ml. of the toxin, each made up to a volume of 0·2 ml. with saline, were injected intracutaneously into the shaven flank of a rabbit. On the first day there was an irregular area of slight redness of about 2 cm. diameter at the site of all the injections, on the second day the redness had faded but the rabbit was very weak and unable to stand, and on the third day it was dead; many haemorrhagic patches 0·5–1·0 cm. diameter were seen along the whole length of the mucous surface of the caecum. The aggregate amount of the inocula was about 38 lethal doses for the mouse. This result illustrates the difference in susceptibility between the guinea-pig and the rabbit to the action of the Shiga neuro-enterotoxin. The experiment shows that the neuro-enterotoxin causes no marked reaction in the dermal tissues of either the rabbit or the guinea-pig, and it is in conformity with the conclusion of Kanai (1922), Istrati (1938) and of Bovin (1940) that this toxin is strongly enterotropic as well as neurotropic. As the toxin was prepared from the rough strain B the result was not complicated by any enterotropic action that might be ascribed to the somatic smooth antigen.

Brief Summary of Part 1

The washed bacilli of normal smooth strains of *Bact. dysenteriae* Shiga are known to contain the specific antigen (a polysaccharide-protein-phospholipin complex), together with elements which in the course of autolysis yield what has long been known as the Shiga toxin (chemically a protein) endowed both with neurotropic and with enterotropic affinities. Consequently the finding that after repeated passage through mice a smooth variant C appeared whose autolysates proved almost completely devoid of neuro-enterotropic power, made it possible to prepare bacterial material for test which contained the specific antigen only and thus gave an opportunity of determining whether the latter contained what might reasonably be called an endotoxin. Injected in the living state C would appear to produce enteric lesions like the fully toxigenic strain A from which it was derived, though neither C nor A proved so highly enterotropic as the rough strain B; C, however, appeared to be unable to develop a neurotropism in vivo, just as its autolysates had also failed to do in vitro.

The bacilli of C when killed by heat at 100° C. should contain the endotoxin if such were present, but experiments carried out both with the parent smooth strain A, with the rough strain B and with C showed that only massive doses of the heat-killed bacilli given intravenously were capable of causing the death of mice. Similar numbers of heat-killed bacilli of a non-pathogenic *Bact. coli* acted similarly, and the clinical and post-mortem appearances were consistent with death from protein shock. Lest it might be said that heat at 100° C. may have greatly reduced the potency of some specific toxin contained in the bacilli, experiments with C bacilli killed by phenol showed that this less drastic method of killing the bacteria also failed to reduce the massive dose found to be necessary to kill mice.

The significance of these and other findings reported in Part 1 will be discussed more fully at the end of Part 2, which deals with the properties of antisera obtained by immunization of rabbits with the various antigens investigated in Part 1.

PART 2. THE PROTECTIVE OR PROPHYLACTIC EFFECT OF EXPERIMENTAL SERA PREPARED IN RESPONSE TO ANTIGENS INVESTIGATED IN PART 1

Antibacterial sera. Seven such sera (nos. X, XII, XIV, XVI, XVIII, XIX and XXI) were obtained from rabbits after intravenous injection of graduated doses of six different antigens as follows:

(1) Formol-killed bacilli of C, the feebly toxigenic variant obtained by mouse passage of Shiga K 624 (A). Two sera, X and XII, were so prepared, and by the customary assay for antitoxin content were found to possess only 5 i.u. per ml. The agglutinin titres of the two sera were respectively 800 and 1000.

(2) *Formol-killed rough bacilli* (*B*). Serum XXI.

(3) *Heat-killed C bacilli.* Serum XIV. Agglutinin titre 300.

(4) *Heat-killed A bacilli* (smooth and fully toxic). Serum XVI. Agglutinin titre 100.

(5) *Heat-killed rough bacilli* (*B*). Serum XVIII. Agglutinin titre not satisfactorily determined.

(6) *Acetone-dried rough bacilli* (*B*). Serum XIX. Agglutinin titre not satisfactorily determined.

The high temperature employed in the case of antigens 3, 4 and 5 was designed to secure the destruction of any neuro-enterotoxic antigen contained in the bodies of the washed A and B bacilli and possibly to some negligible extent in those of C.

Antineuro-enterotoxic sera (usually designated antitoxic sera and assayed as such). Sera XXI, XXII and '257'. Autolysates of the rough variant B were used as antigen for the immunization of rabbits yielding sera nos. XX and XXII. Formalin was added to a toxin solution in a concentration of 0·5% and the solution maintained at 37° C. until the lethal dose for the mouse had increased to 0·1 ml. Graduated doses were then injected intravenously into rabbits followed by injections of unmodified toxin subcutaneously. When pooled and filtered, sera XX and XXII were found to contain respectively 500 and 350 units per ml. Serum '257' was a concentrated horse serum obtained by immunization with toxin from the rough strain B together with bacterial suspensions of the smooth A strain. The serum was consequently both antitoxic and antibacterial. The antitoxin content was 3000 units per ml., and it had an agglutinin titre for smooth A bacilli of 600.

Method of test. Mice were used for the protective experiments. In general the test dose of living bacilli whether of C, A, B or the heterologous strain E was 50 millions injected intraperitoneally with mucin. The sera to be tested for protective power were as a rule employed in descending concentration from 0·3 to 0·01 ml. and made up to a constant volume of 0·5 ml. which was injected intravenously in the tail vein. When washed heat-killed bacilli were used as test, the dose injected intravenously was 5000 millions.

Experimental results

It is not proposed to discuss in detail the eleven tables in which the experimental results are displayed, as each is provided with information regarding the conditions of the experiment and the numerical data. It will be sufficient in this brief survey to refer to Table 12, in which an attempt has been made to summarize the main findings to which the reader's attention is directed.

(A) The experiments performed with sera prepared from formol-killed A, C and B bacilli are displayed in tables or sections of Tables 5, 6, 10 and 11. They show that sera prepared from formol-killed smooth bacilli possess high protective value (50–100% protection) against the living smooth bacilli (C, A or E), but little or no protection against heat-killed bacilli whether smooth or rough or against a dose of pure toxin. Only serum XXI prepared from the formol-killed rough bacilli (Table 11) is capable of giving a moderate degree of protection (50%) against infection with the living rough bacilli.

(B) Experiments with sera prepared from heat-killed A, C, or B bacilli and from acetone-dried rough bacilli are displayed in Tables 5, 6 and 11. It will be observed that the protective capacity of these sera against infection with living smooth bacilli lags considerably behind that shown by sera prepared from formol-killed bacteria, viz. 20–30% protection only. They afford no protection whatever against an intravenous dose of heat-killed bacilli (Table 6). Against infection with the living rough bacilli only serum XIX prepared from the acetone-dried rough bacilli is capable of giving moderate protection (50%). That prepared from heat-killed rough bacilli has no such protective action.

(C) The experiments performed with sera prepared by immunization with toxin (from 'rough' autolysates) are displayed in Tables 7–9. They show that 100–250 units of a rabbit antitoxic serum containing 500 units per ml. are quite unable to give protection against the standard dose (50 million) of living smooth bacilli. Another antitoxic rabbit serum containing 350 units per ml. was tested for its protective

Table 5. *Prophylactic action of antibacterial sera given intravenously 24 hr. before test dose. Test dose: 50 million bacilli of 5 hr. cultures of A, B, C or E with mucin intraperitoneally*

Serum no.	Agg. titre	Units/ml.	Antigen	Serum dose ml.	Infecting strain	Proportion of mice dying	Mortality rate %
			(*a*) Tested against homologous strain				
X	1/800	5	Formolized 5 hr. culture C	0·5	C	0/5	0
				0·2		0/5	0
				0·1		0/5	0
				0·05		0/10	0
				0·02		0/5	0
				0·01		0/25	0
				0·005		5/40	12·5
				0·002		17/35	48
				0·001		55/65	84
						(controls)	90
			(*b*) Tested against variants of homologous strain				
XII	1/1000	5	Formolized 5 hr. culture C	0·02	A	2/10	20
				(controls)	A	5/5	100
				0·02	B	10/10	100
				(controls)	B	5/5	100
				0·02	C	2/10	20
				(controls)	C	5/5	100
				0·01	A	5/10	50
				(controls)	A	5/5	100
				0·01	B	8/10	80
				(controls)	B	2/5	40
				0·01	C	4/10	40
				(controls)	C	5/5	100
			(*c*) Tested against heterologous toxic strain E				
XVI	1/100	0	Culture A heated 10 min. at 100° C.	0·05	E	7/10	70
XIV	1/300	0	Culture C heated 10 min. at 100° C.	0·02	E	7/10	70
XII	1/1000	5	Formolized 5 hr. culture C	0·01	E	3/10	30
				(controls)	E	9/10	90
			(*d*) Lower protective value of antisera derived from heated antigens (composite results)				
XIV	1/300	0	Culture C heated 10 min. at 100° C.	0·3	C	0/4	0
				0·2	C	15/25	60
XVI	1/100	0	Culture A heated 10 min. at 100° C.	0·01	C	4/5	80
				0·005	C	5/5	100

Table 6. *Failure of antibacterial sera to protect when given prior to a test dose of vaccine (A, B, or C) killed by boiling for 10 min. the serum (0·1 ml.) was injected intravenously 24 hr. before an intravenous dose of 5000 million bacilli*

Test culture	Serum used	Proportion of mice dying
A	XII (anti-formolized C)	2/2
B		1/2
C		2/2
A	XIV (anti-boiled A)	2/2
B		2/2
C		2/2
A	Nil	1/2
B		1/2
C		2/2

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Table 7. *Failure of antitoxic serum no. XX containing* 500 *units/ml. to protect mice against an infection with culture C. Antigen used in its preparation: filtered autolysate derived from rough Shiga K* 624*B. Serum dose given intravenously* 24 *hr. before test dose* (50 *million bacilli of* 5 *hr. culture C with mucin intraperitoneally*)

Serum dose	Proportion of mice dying	Mortality rate %
0·5 ml. (250 units)	18/20	90
0·2 ml. (100 units)	112/129	86
Controls	90/100 (no serum)	90

Table 8. *The action of antitoxic serum no. XXII containing* 350 *units/ml. in mice infected with rough K* 624*B: serum dose given intravenously* 24 *hr. before test dose* (50 *million bacilli of* 5 *hr. culture B with mucin intraperitoneally*

Exp.	Serum dose	Proportion of mice dying	Mortality rate %	Time of death in controls
1	0·2 ml. (70 units)	5/5	100	24 hr.
2	0·3 ml. (100 units)	4/20	20	4–7 days
2	0·2 ml. (70 units)	6/20	30	4–7 days

Table 9. *Action of concentrated horse antitoxin* (*Lister routine batch no.* 257) (*antitoxic titre* = 3000 *units/ml. agglutination titre* 600) *on* (*a*) *C strain and* (*b*) *B 'rough' strain. Serum given intravenously* 24 *hr. before test dose*

(*a*) Infecting dose: 50 million living C strain with mucin intraperitoneally

Serum dose ml.	Units	Mice dying	Mortality rate %	Time of death days
0·025	88	2/10	20	3–4
0·01	37	4/10	40	2–7
0·0 (controls)	0	9/10	90	2

(*b*) Infecting doses: 50 and 100 million living B strain with mucin intraperitoneally. Serum dose given intravenously 24 hr. before test dose

Serum dose (ml.)	0·25	0·115	0·06	0·03	0·015	0·0
Units	800	400	200	100	50	0
Mice dying at dose 100 million	2/2	2/2	2/2	1/2	2/2	2/2
Mice dying at dose 50 million	2/2	1/2	2/2	2/2	2/2	2/2
Time of death			1–3 days			

Table 10. *Effect of antibacterial serum XII on injection of rough toxin. Serum dose given intravenously* 24 *hr. before toxin. Test dose* 25 M.L.D. *toxin intravenously*

Serum dose ml.	Toxin dose ml.	Mice dying	Mortality %	Time of death days
0·1	0·25	10/10	100	1–2
Controls	0·25	5/5	100	1–2

Table 11. *Effect of antisera to rough bacilli on infection with living rough strain B. Serum doses given intravenously* 24 *hr. before test dose. Test dose* 50 *million rough strain B with mucin intraperitoneally*

Serum	Serum dose	Antigen	Mice dying	Mortality rate %	Time of death days
XVIII	0·2 ml.	Heated rough bacilli	10/10	100	1–7
XIX	0·2 ml.	Acetone dried rough bacilli	5/10	50	1–7
XXI	0·2 ml.	Formalized rough bacilli	5/10	50	1–7
	0·2 ml. (normal serum controls)	—	10/10	100	1–7

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Table 12. *Concise summary of results detailed in Tables* 5–11

Table	Type of serum	No.	Antigen used	Tested against	Percentage protected	Index
5	AB	X	Formolized C	Living C	100	+ + +
5	AB	XII	Formolized C	Living A	50–80	+ +
5	AB	XII	Formolized C	Living rough (B)	0–20	±
5	AB	XII	Formolized C	Living C	60–80	+ +
5	AB	XII	Formolized C	Living E	70	+ +
6	AB	XII	Formolized C	Heated A, B or C	16·6	±
10	AB	XII	Formolized C	Rough toxin	0	±
5	AB	XIV	Heated C	Living C	20–40	+
5	AB	XIV	Heated C	Living E	30	+
5	AB	XVI	Heated A	Living E	30	+
6	AB	XVI	Heated A	Heated A, B or C	0	±
11	AB	XVIII	Heated B	Living B	0	±
11	AB	XIX	Acetone dried B	Living B	50	+ +
11	AB	XXI	Formolized B	Living B	50	+ +
7	AT	XX	R toxin (*a*)	Living C	10–15	±
*8	AT	XXII	R toxin (*b*)	Living B	70–80	+ +
9	AT + AB	'257'	R toxin + smooth bodies	Living C	60–80	+ +
9	AT + AB	'257'	R toxin + smooth bodies	Living B	0–10	±

Symbols: AB = antibacterial; AT = antitoxin; + + + = 80–100 % protection; + + = 50–80 %; + = 0–20 %.

* See text, below.

Table 13. *Protective action of antisera to chemically isolated Shiga antigens. Serum given intravenously* 24 *hr. before test dose. Test dose* 50 *million living bacilli of* 5 *hr. culture of C with mucin intraperitoneally*

Serum no.	Agg. titre	Antigen	Serum dose	Proportion of mice dying	Mortality rate %
54/57	400	'Primary extract' of the polysaccharide of the Shiga bacillus	0·1	2/9	22
			0·05	4/10	40
			0·02	6/10	60
			(Controls	5/5	100
73	400	Purified Shiga polysaccharide combined with typhoid polypeptide	0·05	0/5	0
			0·02	3/5	60
			(Controls)	5/5	100

effect against the living rough bacilli (Table 8), and in one case gave protection rates of 70–80 %, the control animals dying in 4–7 days with paralytic symptoms. On another occasion, however, 70 units of the same serum gave no protection whatever, the controls, like the test animals, dying in 24 hr. In an effort to explain this discrepancy a duplicate set of control mice was inoculated and one was killed each day and its heart blood plated. The blood was either sterile or contained few organisms suggesting that the death of the mice was due to the effects of toxin and not to the immediate results of invasion by the rough bacilli. In this case, therefore, the antitoxin protected most probably not against a bacterial inoculum insufficient to establish infection but against toxin liberated before the organisms were eliminated. The result, in fact, emphasized the greater importance of the toxic factor in the outcome of infections with the living rough bacilli.

An antitoxic serum prepared in the horse (Table 9) and destined for use in human dysentery was also tested and found to give good protection (60–80 %) against living smooth bacilli, a result simply explained by the fact that smooth bacterial bodies as well as toxin were employed in the course of immunization. Against the living rough bacilli, however, this horse antitoxic serum was quite impotent when given in doses ranging from 50 to 800 units.

On a review of all the evidence it would seem most probable that in the experiment (Exp. 2, Table 8) yielding a discrepant result, the test dose had been overestimated, and on the occasion in question was less than 50 millions. This dose caused death of the controls in 4–7 days from liberated toxin, while the test animals protected by the antitoxin survived.

Discussion

As I have said at the commencement of this paper, the primary objective of the work was to obtain, from experiments on mice, information regarding the prophylactic value of sera prepared by immunization with the smooth somatic Shiga antigen against subsequent infection with the living smooth bacilli. It was realized that if ordinary bacteriological methods were to be employed to secure the necessary smooth antigen, the fullest consideration had to be paid to knowledge now available regarding the properties, both biological and chemical, of smooth and rough antigens and of the toxic antigen yielded by autolysates of smooth and rough bacilli. The work already cited of Boivin and his collaborators, of Haas, of Istrati and of Morgan, on the separation by chemical means of the somatic antigen from the so-called neuro-enterotoxin obtained from autolysates, has revealed the fact that the former is a polysaccharide-protein-phospholipin complex and the latter a protein. Further, these workers have demonstrated in mouse experiment that the chemically isolated somatic antigen is toxic and indeed represents the 'endotoxin' of Shiga with properties essentially enterotropic. On the strength of these new findings accumulated during the past eight years, support would appear to be given to the view originally put forward by Olitsky & Kligler (1920) that the neurotropic and enterotropic actions observed in Shiga infections are due to separate toxins. As is well known, chemically isolated somatic antigens have also been recovered from various Gram-negative pathogens, particularly those of the Salmonella group, and such antigens have been shown to be toxic with ill-defined enterotropic properties shared by all members of the group. In the case of the Shiga bacillus the matter is complicated by the fact that the rough organism is, like the smooth, endowed both with enterotropic and with neurotropic affinities and indeed is more enterotropic than the latter, so that, as matters stand at present, smooth Shiga strains would appear to contain three toxins, viz. (*a*) thermostable enterotoxin contained in the smooth soma only, (*b*) a thermolabile enterotoxin, and (*c*) a thermolabile neurotoxin, the two latter being found combined in autolysates prepared either from smooth or from rough bacilli.

The somatic antigen used in this work for immunization purposes was obtained at first from 5 hr. cultures of a smooth strain, which at this stage of incubation would contain little or no neurotoxin. Autolysates prepared from this strain after it had been repeatedly passaged through mice were found to have completely lost their capacity to yield neurotoxin, or preferably neuro-enterotoxin, for this latter term defines more accurately the particular biological property of the autolysate and is so used in this paper.

This strain was consequently of great value as a source of somatic antigen for immunization purposes. Both formol-killed and heat-killed washed bacilli of this particular variant, as well as heat-killed organisms of other smooth and fully toxic strains, were used to secure somatic antigen for immunization purposes.

Doses of these heat-killed smooth organisms and of the heat-killed rough strains were tested for toxicity by intravenous inoculation of mice, and it was found that only massive doses of the order of 5000 millions were capable of causing the death of mice. This minimal lethal dose was the same whether heat-killed smooth or rough organisms were tested, and heat-killed bacilli from a non-pathogenic *Bact. coli* were found to be toxic in similar doses—a finding which reflects a statement by Boivin that the endotoxin properties of the chemically isolated smooth Shiga antigen are common not only to endotoxins similarly prepared from *Flexner* bacilli but also from *coli* bacilli. The impression derived from the results of these experiments with heat-killed smooth or rough bacilli as toxic agents for mice was that death was due to the sheer mass of bacterial colloid and consequently of the nature of protein intoxication or shock.

Endotoxin in the sense of that shown to be present in the chemically isolated and dried products must also have been present in the dose of the heat-killed or formol-killed bacilli, but how far it was able to exert an independent action in the crude bacillary mass administered must remain for the present undecided. The specificity of the reaction could not be demonstrated, for sera prepared from smooth bacilli failed to neutralize even one lethal dose of the heat-killed bacilli given intravenously. Even if antibacterial serum was allowed to remain in contact with the heat-killed bacilli before infection of the mixture, death ensued as quickly as in the controls; in such inocula, the bacilli were, of course, agglutinated, making a coarser emulsion. In the case of inert colloids, a coarse emulsion is, on the whole, more harmful than one in a state of very fine division (Steabben, 1925).

Boivin *et al.* (1940*b*) and Haas (1937) claimed to be able to neutralize the chemically isolated endotoxin with antibacterial sera and not with normal sera, but it is admittedly clear from their protocols that

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such sera were extremely weak, being capable in the highest concentrations of neutralizing only a very few lethal doses. It should also be kept in mind that their dry product was found to be toxic only in quantities of the order of 0·1–0·2 mg. The minimal lethal dose of the polysaccharide antigen isolated by Morgan (1937), viz. 0·05–0·1 mg., has been calculated to represent the product of some 4500 million organisms. This dose will kill only a proportion of the mice injected within 48 hr. A dose of the dry product which will kill all the mice injected within 48 hr. has been shown to be 0·25–0·5 mg., so that the equivalent number of organisms from which this amount of the polysaccharide complex was derived must be very large and of the order of 10,000–20,000 millions. The chemically derived somatic antigen (Morgan, 1937) produces in the rabbit a serum which is protective against infection with the living smooth bacilli, but it is less effective than that made from the formol-killed bacilli. Serum XXII, which, in a dose of 0·01 ml., protected completely against a lethal dose of the living Shiga bacillus (with mucin), was obtained after ten injections in the rabbit, comprising in all only 2000 millions of the formol-killed bacilli. The sera referred to in Table 13, one of which protected in a dose of 0·05 ml., were obtained after six injections of the polysaccharide extract, totalling 0·3 mg. and equivalent to at least 12,000 millions of the organism. It would therefore appear that this antigen lacks some constituent of the bacterial substance which is in part responsible for the protective action of an ordinary immune serum. Though this anti-extract serum does protect the mice, it is possible that its action is not directed primarily against any specific endotoxin contained in the smooth bacilli. As with other purely antibacterial sera its tropin content would probably be the chief agent in arresting early multiplication of the invading bacteria.

In the autolysate of the rough strain which yields the most potent neuro-enterotoxin, do the entero-tropic and neurotropic elements reside in separate toxin molecules? or are the neurotropic effects and the enterotropic effects that may be exhibited by the toxin solely dependent on differences between hosts? We have here an excellent analogy with diphtheria toxin, and at the present time the view that paralytic sequelae following diphtheria are due to the action of a special component of the diphtheria filtrate designated by Ehrlich as toxone is no longer current.

The solutions of problems such as these, however, must await, in the case of *Bact. dysenteriae* Shiga and no doubt in the case of *C. diphtheriae* as well, further chemical attack on the phenomena of diffusion and autolysis under varying conditions in vitro. Moreover, the picture cannot be reasonably complete until further information is available regarding the elaboration of toxin in the body of the infected host. To turn to the prophylactic effect of antibacterial sera and antitoxic sera against infection with the living bacilli, the former especially, if prepared from formol-killed smooth organisms, are found to possess high value, whereas the purely antitoxic sera fail to show any such action. Conversely, the antibacterial sera prepared from smooth somatic antigen fail to arrest intoxication with the neuro-enterotoxin. They are equally ineffective against infection with the living rough organisms. In fact, the only sera that seemed to be capable of giving some degree of protection against infection with the living rough organisms were one prepared from acetone-dried rough bacilli and another from formol-killed rough bacilli. Doubtless the lack of protective value against the living rough bacilli shown by the antibacterial serum made from the smooth bacilli was due to the absence from the antigen of the rough substance while that prepared from the heat-killed rough bacilli was inactive because of some denaturation by heat of the rough somatic antigen. Regarding this antigen in the case of the Shiga bacillus our information is meagre.

One inference with a very practical bearing emerges from the experimental findings, namely that for the treatment of dysentery in man antitoxic serum containing an antisomatic or 'anti-endotoxic' component may be of prime therapeutic importance as an adjunct to chemotherapy not only in effecting the neutralization of toxin but also in arresting bacterial invasion if given at the earliest possible moment in the disease before toxaemic symptoms have appeared. Such a serum possessing both antitoxic and antibacterial components should also be of value in prophylaxis of dysentery in man in the face of a prevailing epidemic.

Summary and conclusions

1. An examination by bacteriological methods has been made of the antigens present in the washed bacilli of smooth Shiga strains and certain derived variants, including the rough type.

2. The washed formol-killed smooth bacilli kill mice only in massive doses of the order of 5000 millions, suggesting death from protein shock; no protection against a fatal issue

is afforded by antibacterial sera. This finding is discussed in relation to recently acquired information obtained by chemical methods on the occurrence of a specific enterotropic toxin in the smooth bacilli, as distinct from the enterotropic toxin present together with a neurotoxin in autolysates of smooth and rough bacilli.

3. Antibacterial sera prepared by immunization with the smooth antigen have high protective value against infection with the living smooth bacilli but not against pure toxin, whereas purely antitoxic sera have no value against infection with the living smooth bacilli.

4. From experiments so far performed, protection against infection with the living rough bacilli has been afforded only by sera prepared from formol-killed rough bacilli and from acetone-killed rough bacilli.

5. From findings reported in (3) inferences are drawn with regard to improvements in the manufacture of dysentery serum for use in man, both prophylactically and therapeutically.

6. The presence of two distinct antigens in the Shiga bacillus has been clearly demonstrated, since the protective values of their corresponding antisera are sharply defined, and do not overlap. The existence of the neurotoxin as a separate entity, unrelated to the smooth 'endotoxin', has also been shown by its derivation from a completely 'rough' strain, but though the smooth somatic antigen gives rise to a highly protective antiserum, in these experiments it has not been shown, *per se*, to be a specific toxin.

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