THE TOXICITY OF SOME BACTERIAL FILTRATES FOR MICE PRE-INFECTED WITH *EPERYTHROZÖON COCCOIDES*

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CONCOMITANT or previous infection of mice with *Eperythrozöon coccoides*, a harmless blood parasite of mice, greatly enhances the pathogenic action of mouse hepatitis virus (MHV1) (Niven, Dick, Gledhill and Andrewes, 1952); this fact has provided a useful method of studying the action of the virus (Gledhill and Niven, 1955). We now report another peculiarity of mice recently infected with *E. coccoides*. During the passage of murine liver suspensions by intraperitoneal inoculation, undertaken as a routine measure to ensure that a newly introduced group of mice were not carriers of ectromelia virus, hepatitis appeared which was due to a virulent strain of *Salmonella typhi-murium*. It was observed that the organism killed mice infected with *E. coccoides* much more quickly than it killed normal mice. Since *E. coccoides* enhances the pathogenic action of mouse hepatitis virus (MHV1) by its effect on the liver, it seemed possible that this parasite might influence the pathogenicity of the strain of *Salm. typhi-murium* (termed S_4) by increasing susceptibility to the toxic effect of this organism rather than by increasing its rate of multiplication. Experiments described in this paper show that formalin-inactivated 20-hour cultures are lethal to mice pre-treated with *E. coccoides* and that the toxic principle is present in cultures as a filtrable substance. Furthermore, filtrates of cultures of other related Gram-negative bacteria are toxic for mice infected with *E. coccoides* while filtrates of cultures of the Gram-positive bacteria tested were not. Filtrates of cultures of the Gram-negative bacteria tested are practically non-toxic for normal mice.

METHODS

Unless otherwise stated, we used throughout weanling mice aged 20–23 days of the VS strain which is not usually infected with *E. coccoides*. The bacteria were grown in Wright's broth (pH 7·0) for 18–20 hr. at 37°. Formalin was then added to the culture to give a final concentration of 0·25 per cent and the mixture was incubated at 37° for a further 24 hr. to inactivate the bacteria. As a preliminary to filtration, the cells were deposited by centrifugation for 10 min. in an angle centrifuge at about 3000 r.p.m. For most purposes the supernatant was filtered through a gradocol membrane of 1·5 μ average pore diameter (A.P.D.) standing on a membrane of 0·54 μ A.P.D. Filtrates were usually injected into mice intraperitoneally and the condition of the mice observed after 6, 12, 24, 48 and 72 hr., when the experiments were terminated. Results were judged chiefly by the total mortality within 72 hr. and to a lesser extent by illness during the first 24 hr.

Dr. Joan Taylor has confirmed that S_4 is a strain of *Salm. typhi-murium* (antigenic structure 0 = 4, 12; H = i − 1, 2). High dilutions of culture are lethal for mice and its virulence equals that of virulent strains. Other species and strains of bacteria were used as hereunder:

Salm. typhi-murium, 3 strains; one virulent, D_2 from Dr. J. S. Paterson; two avirulent, Toronto and M206 from Dr. D. Hobson.

Salmonella limete from Dr. J. S. Paterson.

Salmonella cholerae-suis, Salmonella paratyphi, Salmonella enteritidis, Salmonella newport and *Salmonella london* from Dr. Joan Taylor.

Bacterium coli 4 strains; one of uncertain origin; D433, D21C1 and E793 from Dr. Joan Taylor.

Strains of *Shigella—Sh. shigae, Sh. flexneri* and *Sh. boydii* (2 strains) from Dr. K. Patricia Carpenter.

Staphylococcus aureus, coagulase-positive strain from Dr. J. H. Humphrey.

Streptococcus pyogenes, group A strain from Dr. H. Rogers.

Erysipelothrix rhusiopathiae, mouse virulent strain Ru.

Mice were infected with *E. coccoides* by injecting them with 10^{-7} dilution of spleen suspension taken from mice which had been themselves inoculated a week before with *E. coccoides* spleen suspension diluted 10^{-8}; the inoculation of bacteria or bacterial filtrate was given 120–128 hr. after that of *E. coccoides*. This interval between the inoculation of parasite and bacteria or filtrate was selected because it was considered likely to be the most effective by analogy with the results of experiments with mouse hepatitis virus (Gledhill, 1956) and this was confirmed in experiments to be described. The mice thus pre-treated with *E. coccoides* will be termed " pre-treated mice " and mice not so treated will be termed " normal mice ".

RESULTS

The toxicity of filtrates of S_4

In order to test whether an inactivated growth of S_4 was toxic for pre-treated mice, an 18–20 hr. growth was treated with formalin to give a concentration of 0·25 per cent and after further incubation at 37° for 24 hr. it proved to be sterile. Some of this bacterial suspension in doses of 0·3 ml. was injected intraperitoneally into normal and pre-treated mice. The rest of the suspension was centrifuged to deposit the cells, and the supernatant was injected in the same dose into normal and pre-treated mice. One fraction of the deposited cells was re-suspended in a volume of broth equal to that in which they were dispersed before centrifugation and another was similarly re-suspended in supernatant. Both these bacterial suspensions were injected into normal and pre-treated mice. The results of this experiment are presented in Table I from which it is clear that the supernatant of

TABLE I.—*Enhanced Toxicity of Supernatant from Formalin-inactivated Growth of* Salm. typhi-murium *(Strain* S_4*) for* E. coccoides *Pre-treated Mice*

	Mice pre-treated with *E. coccoides*		Mice not pre-treated with *E. coccoides*	
Material*	Condition 12 hr. after inoculation	Mortality 72 hr. after inoculation	Condition 12 hr. after inoculation	Mortality 72 hr. after inoculation
Formolized whole culture	9† 1VS**	10/10	3SS 7N	0/10
,, supernatant	6† 4S	10/10	1S 2SS 7N	0/10
,, cells + broth	1† 1VS 2SS 6N	3/10	10N	0/10
Recombined cells+supernatant	6† 4S	10/10	3SS 7N	0/10
Formolized broth	5N	0/5	5N	0/5

* Mice were inoculated (0·3 ml. intraperitoneally) with materials from 20 hr. growth *Salm. typhi-murium* strain S_4 inactivated by incubation with formalin in a final concentration of 0·25 per cent.

** Symbols for condition of mice: † = dead; VS = very ill; S = ill; SS = slightly ill; N = normal.

the inactivated growth contains a substance lethal for pre-treated mice and practically non-toxic for normal mice. The cells had some toxicity for pre-treated mice which was, however, much less than that of the supernatant. Another

TABLE II.—*Enhanced Toxicity of Filtrate and Supernatant of Growths of* Salm. typhi-murium (*Strain S_4*) *for* E. coccoides *Pre-treated Mice*

Material (from 20 hr. growth of *Salm. typhi-murium*, strain S_4)	Dose of inoculum (ml.)	Mice pre-treated with *E. coccoides*		Mice not pre-treated with *E. coccoides*	
		Condition 11 hr. after inoculation	Mortality 72 hr. after inoculation	Condition 11 hr. after inoculation	Mortality 72 hr. after inoculation
Filtrate (540 mμ A.P.D.) of unformolized growth	0·3	5† 1VS 1SS 1N**	6/8	1SS 7N	0/8
Supernatant of formolized growth*	0·3	5† 5S	5/8	8N	0/8
Supernatant of formolized growth*	0·03	2VS 2SS 4N	2/8	N.T.	N.T.
Supernatant of formolized growth*	0·1	1† 3VS 3S 1N	5/8	8N	0/8
Supernatant of formolized growth*	0·3	3† 4S 2SS	6/9	1SS 8N	0/9
Supernatant of formolized growth*	0·6	N.T.	N.T.	6SS 2N	0/8
Supernatant of formolized growth heated at 70° for 30 min.	0·3	2† 4S 2SS	5/8	1SS 7N	0/8

* Formolized growth inactivated by incubation with 0·25 per cent formalin for 24 hr.
** Symbols for condition of mice: † = dead; VS = very ill; S = ill; SS = slightly ill; N = normal. N.T. = not tested.

experiment was carried out to show that the toxic principle was filtrable through a gradocol membrane impermeable to the bacteria and that filtrates of living growth contained the principle present in formolized growth. At the same time, varying doses of formolized supernatant heated to 70° for ½ hr. were introduced into treated and normal mice. The results are shown in Table II. It will be seen that none of the inocula used produced significant symptoms in normal mice whereas 60–80 per cent of pre-treated mice of all groups (except that given the smallest dose of toxic agent, *viz.*, 0·03 ml.) were killed. Further, it appeared that the toxic principle was not heat-labile. The results of the experiment are less clear-cut than those presented in Table I in which all pre-treated mice were killed by toxin. Since the failure to kill all pre-treated mice in the experiment of Table II could not be correlated with the dose of toxin given, it was suspected that, at the time when the toxin was injected, the concentration of *E. coccoides* in the pre-treated mice of this experiment was not optimal for the production of the enhancing action. This possibility was tested in the following experiments.

The effect of filtrate injected at varying times after inoculation with E. coccoides

Spleen suspension at a dilution of 10^{-7} was injected into 9 groups of 10 mice each. Exactly 24 hr. afterwards one group was inoculated with filtrate, a second group at 48 hr. and so on at daily intervals until all the groups had been inoculated. Blood smears were taken from each mouse of every group at the time of its inoculation with toxin and, by suitable marking of the mice of each group, observations on the *E. coccoides* present in each blood smear could be related to the reaction towards filtrate of the mouse from which it had been taken. A summary of the results is shown in Table III. It will be seen that the filtrate did not affect the first three groups of mice. Mice of group 4 were the first in which *E. coccoides* was plentiful in smears and although these mice became ill following the injection of filtrate,

TABLE III.—*The Relationship of Mouse Response to Culture Filtrate to the Time of Inoculation and Abundance of* E. coccoides *in Blood Smears*

Time between *E. coccoides* inoculation and filtrate inoculation in days	Abundance of *E. coccoides* in blood smears at the time of inoculation with filtrate	Result of inoculation with filtrate	
		Number ill	Number dead
1	Absent from all smears	0/10	0/10
2	,, ,, ,,	0/10	0/10
3	Absent from 4 smears, present (very scanty) in 6	0/10	0/10
4	Present in all smears, abundant in 8	10/10	0/10
5	Present in all smears, abundant in 9	10/10	5/10
6	Present in all smears, abundant in 9	10/10	6/10
7	Present in all smears, abundant in 5	6/10	4/10
8	Absent from 4 smears, present (scanty) in 6	8/10	0/10
9	Absent from 3 smears, present (very scanty) in 7	1/10	0/10

E. coccoides was given as 0·2 ml. $\times 10^{-7}$ infected spleen suspension intraperitoneally. Filtrate was 20 hr. growth of *Salm. typhi-murium* (strain S_4) formolized (0·25 per cent) and filtered through a 540 mμ A.P.D. gradocol membrane. Dose was 0·2 ml. intraperitoneally.

none of them died. The next three groups (5, 6 and 7) showed a mortality of 5/10, 6/10 and 4/10. In group 8 the majority became ill but none died, and in the last group they remained well. Thus throughout the period of enhanced susceptibility, *E. coccoides* was plentiful in blood smears; the maximal enhancement occurred at about 1½–2 days after the parasite first appeared and mice recovered normal resistance to toxin with the disappearance of the parasite from blood smears. On the other hand, there appeared to be no consistently direct relationships between the recorded concentration of parasites in individual blood smears of mice of a group and their reactions to the toxic filtrate, and for this reason the results of individual blood smears have been omitted from Table III. From previous experiments the dose of filtrate used had been found sufficient to produce death in practically 100 per cent of mice in which *E. coccoides* had produced a state of maximum susceptibility to filtrate, yet the maximum mortality in this experiment was 6/10. This might suggest that the duration of maximal or nearly maximal susceptibility in relation to the time of injection of *E. coccoides* was less than 24 hr. and further experiments would be necessary to determine this.

In a further experiment to throw light on the action of *E. coccoides* in raising the susceptibility of mice to bacterial filtrate, 1 mg. of oxytetracycline in 5 per cent gum acacia was injected subcutaneously into groups of pre-treated mice at varying times before and after the inoculation with filtrate. None of 12 mice died which had been treated with oxytetracycline 12 hr. before they were inoculated with filtrate, whereas all of 10 mice died which had been treated 6 hr. before filtrate inoculation. Other groups of mice treated with oxytetracycline less than 6 hr. before filtrate inoculation and after filtrate inoculation and an untreated group all showed a high mortality. These results were elucidated by taking blood smears from the mice of a control group (not inoculated with filtrate) at the time of its injection with oxytetracycline and 2, 4 and 6 hr. afterwards. *E.*

coccoides was very abundant in the smears taken at the time of giving the drug and 2 hr. afterwards; at 4 hr. the number of *E. coccoides* in smears had become slightly less while at 6 hr. they had practically disappeared from the peripheral blood.*

Properties of the toxic filtrate

The toxic principle of 540 mμ A.P.D. gradocol membrane filtrate of formolized 20-hr. growth of S_4 resisted boiling for 15 min. and was non-dialysable. It was readily filtrable through a gradocol membrane of 200 mμ A.P.D. but not through one of 50 mμ A.P.D. After intravenous injection results were comparable with those obtained by the intraperitoneal route, whereas only 2 of 23 pre-treated mice died after subcutaneous inoculation with 0·2 ml. of filtrate. These results are based on two independent experiments which gave completely consistent results. In a single experiment toxic filtrate was titrated in groups of *E. coccoides* pre-treated mice aged 10–12 weeks and compared with mice of the age used in other experiments (3 weeks) with the following results :

Dose of filtrate (ml.)	Mortality ratio in mice aged	
	10–12 weeks	3 weeks
0·02 . . .	2/10	4/10
0·05 . . .	5/10	3/10
0·2 . . .	10/10	10/10

Older mice thus appear to be as susceptible as younger mice and the use of older mice for experiments of the kind described here might be preferable to the use of weanling mice.

Toxicity of filtrates of other organisms for pre-treated mice

Since the above results were obtained with a single virulent strain of *S. typhi-murium* isolated in this laboratory, experiments of the same kind were carried out with other bacteria. Culture filtrates from all strains of *S. typhi-murium* whether virulent or avirulent, were lethal to a high proportion of pre-treated mice. Filtrates from other species of *Salmonella* were also toxic and the mortality produced by all the species tested, including the strains of *S. typhi-murium* (other than S_4), was 30/53 or 57 per cent. With 4 strains of *Bact. coli* 12 of 25 pre-treated mice became ill (48 per cent) and 10 died (40 per cent). Similarly, with 4 strains of *Shigella* 16 of 32 mice became ill (50 per cent) and 5 died (16 per cent). In contrast, filtrates from growths of 3 species of Gram-positive organisms, *Str. pyogenes*, a coagulase-positive *S. aureus* and a virulent strain of *Ery. rhusiopathiae*, had no visible effect on pre-treated mice. Filtrates from both the Gram-negative and the Gram-positive organisms were non-toxic, or almost non-toxic, for normal mice.

Toxicity of lipopolysaccharides from Gram-negative organisms for pre-treated mice

It seemed possible that the toxic principle in culture filtrates of Gram-negative bacteria might be related to the endotoxins which can be extracted from these

* It should be noted that the VS strain of mice does not carry *E. coccoides*. Although the response to an injection of *E. coccoides* in mice which carry this parasite has not been fully investigated, probably it would be insufficient to render them highly susceptible to culture filtrate.

organisms. Since the endotoxins of Gram-negative bacteria are their somatic antigens and consist of lipopolysaccharides united to protein (Wilson and Miles, 1955), lipopolysaccharides derived from endotoxins might prove to be much more toxic for pre-treated mice than for normal mice. Through the courtesy of Dr. Rowley and his colleagues we obtained samples of lipopolysaccharide extracted from a strain of *Bact. coli* (strain CV) and we inoculated normal and pre-treated mice with varying doses. The results of experiments carried out on different occasions were variable in both pre-treated and normal mice; for example, in one experiment a dose of 50 μg. killed one of 8 normal mice and 100 μg. killed all of 6 mice; yet in a comparable experiment on another occasion, 80 μg. failed to kill any of 14 mice and 100 μg. failed to kill any of 6 mice. Preliminary experiments appear to suggest that such discrepancies are due to a marked variation of the toxicity of lipopolysaccharide and it was also found that environmental temperature affected the mortality rate of the mice; this possibility which might have important implications is being studied further. When doses of 10 μg. and even 2 μg. were injected intravenously into groups of 5 pre-treated mice they died within 24 hr. whereas doses of 10 μg. and 50 μg. failed to kill any mice when injected by the same route into groups of 5 normal mice. In another experiment, 10 μg. killed all of 6 pre-treated mice and 5 μg. killed two of 6, whereas 50 μg. killed only one of 8 normal mice. Similar results were obtained with lipopolysaccharide from *Pasteurella pseudotuberculosis* and with that from *Chromobacterium prodigiosum*. In order to obtain the ratio of the LD_{50} of lipopolysaccharide for normal mice to that for pre-treated mice, graduated doses of the *Bact. coli* lipopolysaccharide were injected intravenously or intraperitoneally into groups of normal and pre-treated mice. The ratios obtained in four such experiments were 11 : 1, 100 : 1, $>$ 11 : 1 and $>$ 20 : 1. The failure to obtain determinate ratios in the two latter experiments was due to failure to produce a lethal effect in normal mice even with the highest doses of lipopolysaccharide used. The results, however, indicate that the ratio probably lies between 10 : 1 and 100 : 1.

DISCUSSION

The experimental observations with lipopolysaccharides from Gram-negative bacteria support the hypothesis that the toxic principle present in young culture filtrates of such bacteria is endotoxin. By concentrating filtrates we hope to show that they are as toxic as the purified lipopolysaccharide. Preliminary experiments suggest that inoculation of normal mice with filtrate increases resistance to the combined treatment with *E. coccoides* and filtrate. Attempts to produce antiserum in rabbits which neutralizes the toxin have not yet been fully successful. Investigation along these lines is being continued.

We consider that pre-treatment of mice with *E. coccoides* might be a valuable aid to investigation of the rôle of endotoxins in the pathogenicity of the coli-typhoid group which might perhaps lead to a better understanding of some forms of food poisoning and infantile diarrhoea.

SUMMARY

Filtrates of 18–20 hour growths of the coli-typhoid and dysentery groups of bacteria were toxic for mice pre-treated with *E. coccoides* when injected intravenously or intraperitoneally but not subcutaneously. Some filtrates contained a

toxic material which was lethal, especially filtrates of *S. typhi-murium* and some other *Salmonellae*. Other filtrates produced manifest illness within a few hours with complete recovery within 24 hours. All the filtrates were almost non-toxic for normal mice. In contrast, filtrates of growths of a group A streptococcus, a strain of *Staphylococcus aureus* and a strain of *Erysipelothrix rhusiopathiae* were not toxic for pre-treated mice.

The toxic principle of the filtrate resisted boiling for at least 15 minutes and was not dialysable. It was easily filtrable through a gradocol membrane of average pore diameter 200 mμ but detectable amounts did not pass through a membrane of A.P.D. 50 mμ.

The lipopolysaccharides extracted from the endotoxins of *Bacterium coli*, *Pasteurella pseudotuberculosis* and *Chromobacterium prodigiosum* were lethal for *Eperythrozöon coccoides* pre-treated mice in much smaller doses than for normal mice.

It is suggested that the toxic principle of filtrates of growths of these Gram-negative organisms might be lipopolysaccharide.

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