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THE STIMULATION BY ENDOTOXIN OF THE NONSPECIFIC RESISTANCE OF MICE TO BACTERIAL INFECTIONS

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Summary.—The nonspecific resistance of mice to challenge was enhanced following the administration of an *E. coli* O55 B5 endotoxin. Although the route of administration of the endotoxin and the challenge organism were varied, the nonspecific resistance of the animal was enhanced in all the experiments. The efficiency of this resistance was highest when the inducing substance and the challenge dose of bacteria were administered intraperitoneally. Poly I : C and double stranded RNA were also studied but were much less effective than endotoxin in stimulating a resistance to infection.

Stimulation of the fixed macrophages could not explain fully the enhanced resistance, since the clearance rates of colloidal carbon and radioactively labelled bacteria from the blood were not significantly enhanced after the administration of endotoxin. Furthermore, splenectomized animals, and animals injected with agents which interfere with the RES activity, trypan blue and corticosteroids, still developed a degree of nonspecific resistance to infection.

DURING the last 20 years a number of workers have described the stimulation of nonspecific resistance in laboratory animals to bacterial and viral infections following the administration of endotoxins, the lipopolysaccharides isolated from the Enterobacteriacae (Rowley, 1955; Kiser, Lindh and de Mello, 1956; Dubos and Schaelder, 1956; Condie, Zak and Good, 1955; Howard, Rowley and Wardlaw, 1958; Abernathy and Spink, 1956; Wagner *et al.*, 1959). After a lull during the early 1960s, interest was revived when endotoxin was shown to be one of the numerous substances which can stimulate the appearance of circulating interferon in *in vivo* experiments (Ho, 1964). From this work has stemmed the finding that many interferon inducers are capable of inducing resistance to bacterial and protozoan infections when administered prophylactically (Weinstein, Waitz and Came, 1970; Jahul *et al.*, 1968; Remington and Merigan, 1968; Herman and Baron, 1970).

Some of the aspects producing the greatest disagreement in the literature included the dose requirements, the time after administration when protection was optimal, the duration of the protection and the spectrum of the bacterial species resisted. It was the lack of agreement between the early workers which led to this present study to investigate induced resistance in greater detail. The present investigation was designed to study the induction of a nonspecific resistance in mice following the administration of known *in vivo* interferon inducers and to provide a basis for further detailed studies on the subject.

MATERIALS AND METHODS

Animals.—BSVS male white mice weighing 20–25 g and grey mice of the LACG strain were used in all the experiments. The animals were caged in groups of 5 from weaning and were given food and water *ad libitum*.

Bacteria.—The *Salmonella dublin* strain 3246 was isolated from a case of bovine abortion and kindly supplied by Mr M. H. Hinton, MAFF Carmarthen. The *Staphylococcus aureus* S 201 was coagulase positive, haemolytic and had a phage pattern 42D/+. The *Streptococcus agalactiae* 090 group B, type Ia was originally obtained from Dr Rebecca Lancefield. All these organisms were maintained by subculture on blood agar plates. The bacteria for animal challenge were grown in 25 ml Oxoid nutrient broth in shake cultures, harvested by centrifugation (2500 *g*, 20 min) and resuspended in physiological saline at the required concentration. The streptococcal strain lost considerable viability when suspended in saline and was therefore suspended in Todd–Hewitt broth for washing and inoculation to maintain a higher viability.

Endotoxin.—*E. coli* 055 B5 endotoxin, prepared by the Boivin method (Difco Laboratories, Detroit) was dissolved in saline at such a concentration that the required inoculum could be administered in a 0·1 ml dose.

Endotoxin multiple emulsion.—A multiple emulsion containing endotoxin was prepared by a variation of the procedure described by Herbert (1965). One ml of endotoxin solution in saline (20 times the final concentration) was added through a 26 gauge needle to 1·0 ml of a 9 parts to 1 part Drakeol (Pennsylvania Refining Co., Delaware)/Arlacel A (Atlas Chemical Industries Inc., Wilmington, Delaware) mixture. The mixture was then rapidly expelled from a syringe through a 26 gauge needle; this emulsifying procedure was repeated twice and the mixture was finally ejected into 18 ml of physiological saline containing 1% Tween 80. The resulting mixture was immersed in an ice bath, and further emulsified by sonification for two periods of 20 sec with a Soniprobe (Dawe Instruments Ltd, London). Each stage of this preparation was carried out aseptically to yield a sterile permanent emulsion. This stable emulsion was not broken by centrifugation at 10,000 *g*, and the phases which separated slightly after prolonged storage at 4° were redispersed by shaking.

Polyinosinic–polycytidylic acid.—The polynucleotides were obtained from the Boehringer Corporation, Mannheim. The polymers were mixed in equimolar concentrations in phosphate buffered saline pH 7·0 (0·006 mol/l sodium phosphate, 0·15 mol/l sodium chloride) (Field *et al.*, 1972). The complex formation was determined by the hypochromic effect, immediately after mixing (Davies and Rich, 1958).

Double stranded RNA.—This was kindly donated in a freeze dried form by Beecham Research Laboratories.

Reticuloendothelial activity.—The activity of the reticuloendothelial system was studied in 3 ways. The clearance of colloidal carbon from the blood, the removal of radioactively labelled bacteria from the blood and the uptake of radioactively labelled bacteria by certain organs of the body.

Colloidal carbon (supplied by Gunter Wagner, Hanover) uptake by the reticuloendothelial system was studied by the technique developed by Biozzi, Benacerraf and Halpern (1953).

The clearance of labelled bacteria was studied using bacteria labelled by growing in a liquid medium supplemented with tritiated leucine (specific activity 250 mCi/mmol, 20 μCi/ml of nutrient media). The labelled bacteria were harvested by centrifugation and the pellet washed 3 times in physiological saline. Approximately 10^8 organisms in 0·1 ml were injected intravenously into the lateral tail vein and blood samples were taken at timed intervals up to 20 min after the injection; these samples were solubilized in Soluene (Packard Instruments), and their radioactivity determined in a Beckman DPM 100 liquid scintillation counter. The logarithmic rate of loss of radioactivity was proportional to the clearance of the bacteria.

The uptake of labelled bacteria was studied by injecting the tritiated bacteria into the mice as described above. After 30 min the animals were killed by cervical dislocation; a blood sample was taken and the spleen and liver removed. The blood sample and the whole organs were solubilized in Soluene and the radioactivity determined in the scintillation counter.

Experimental routine.—Endotoxin (75 μg) was injected intraperitoneally or intravenously 4 days before bacterial challenge; it has been shown previously that at this interval the mice had acquired a maximum resistance. After a challenge with *Salmonella dublin* they were observed twice a day for 7 days for deaths, and after the staphylococcal and streptococcal challenges they were observed 4 times a day for 3 days.

RESULTS

Toxicity of endotoxin

The endotoxin used in these studies (*E. coli* O55 B5) was of a relatively low toxicity; nevertheless, a 75 μg dose administered intraperitoneally produced a 3–5% fatality in 24 hours. It was hoped that in the multiple emulsion the endotoxin might be released slowly, thereby maintaining a low non-toxic concentration for a longer period. However, the emulsion and dialysed emulsion were both as toxic as endotoxin dissolved in saline. The technique for preparing the emulsion does not encapsulate all the endotoxin and dialysis of the emulsion did not reduce its toxicity, suggesting that the free endotoxin molecules were too large to pass through the dialysis membrane.

Pathogenicity of the challenge organisms before and after endotoxin administration

The organisms used to challenge the mice produced a range of pathological effects (Table I). The gram negative *Salmonella dublin* gave a chronic infection,

TABLE I.—*Pathogenicity of the Challenge Organisms*

Organism	LD_{50} dose (CFU)*	Average interval between LD_{50} challenge dose and death (hours)	Experimental dose (CFU)*	Average interval between exptl dose and death (hours)
Salmonella dublin	20	240	10^6	96
Staphylococcus aureus	5×10^8	5	2×10^9	5
Streptococcus 090	25	24	10^2	24

* Colony forming units.

with an eventual death even after the injection of very low numbers of organisms, whereas the 2 gram positive strains produced very acute infections with rapid death. The administration of endotoxin considerably enhanced the resistance of the mice to bacterial challenge; this is shown in Tables II, III and IV. In these experiments white and grey mice were challenged after the injection of 75 μg of endotoxin in saline or double emulsion.

TABLE II.—*The Effect of Injecting Endotoxin in Saline and in a Multiple Emulsion on the Survival of BSVS and LACG Mice Challenged with* Salmonella dublin

	Mice injected with	No. of animals tested	% Animals surviving after challenge with 10^6 CFU at (hours) 0	54	76	81	100	108	122	130	144	150	165
BSVS	75 μg endotoxin: emulsion	34	100	100	100	100	97	97	94	94	82	82	73
BSVS	75 μg endotoxin: saline	33	100	100	97	97	76	76	73	73	36	36	12
BSVS	saline	35	100	100	91	91	46	0					
	Saline emulsion control	10	100	100	100	80	80	0					
			30	72	96	103	122	170 hours					
LACG	75 μg endotoxin: emulsion	9	100	100	66	66	55	44					
LACG	75 μg endotoxin: saline	10	100	70	20	10	0	0					
LACG	saline	10	90	40	0	0	0	0					
	Saline emulsion control	10	100	50	0	0	0	0					

TABLE III.—*The Effect of Injecting Endotoxin in Saline and in a Multiple Emulsion on the Survival of BSVS Mice Challenged with Streptococci*

Injected with	No. of animals tested	% Animals surviving after i.p. challenge with 100 CFU at (hours) 0	24	36	72	
75 μg endotoxin in emulsion	10	100	100	90	50	Total survival
75 μg endotoxin in saline	10	100	100	20	0	0
Saline	10	100	90	0	0	0
Saline emulsion control	10	100	70	0	0	0

TABLE IV.—*The Effect of Injecting Endotoxin in Saline and in a Multiple Emulsion on the Survival of BSVS Mice Challenged with Staphylococci*

Injected with	No. of animals tested	% Animals surviving after challenge with 2×10^9 CFU at (hours) 0	2	4	6	24	
75 μg endotoxin in emulsion	10	100	100	80	70	70	Total survival
75 μg endotoxin in saline	10	100	100	60	20	20	Total survival
Saline	10	100	40	0	0	0	0
Saline emulsion control	10	100	60	0	0	0	0

Effect of the challenge route

In all the above experiments the intraperitoneal route of administration was used for the endotoxin and the subsequent bacterial challenges. Therefore, the protection observed could have been due to a local cellular reaction in the peritoneal cavity. Table V clearly shows that this protection was not the result of such a reaction since intraperitoneal endotoxin injections protected the mice against an intravenous bacterial challenge. Likewise, an intravenous administration of endotoxin enhanced the resistance of the mice against an intraperitoneal challenge. Nevertheless, irrespective of the route of challenge, the intraperitoneal endotoxin administration conferred a better protection (Table V).

TABLE V.—*The Effect of the Intraperitoneal Administration of Endotoxin and Intravenous Challenge of Salmonellae on the Survival of BSVS Mice*

Administration intraperitoneally with	No. of animals tested	% of animals surviving after intravenous challenge with 10^6 CFU of *S. dublin* (days) 0	4	5	6	7	8
75 μg endotoxin in emulsion	12	100	100	100	75	50	42
75 μg endotoxin in saline	9	100	100	90	50	40	10
Control	12	100	44	11	0	0	0

The subcutaneous administration of endotoxin in saline and in the multiple emulsion was also studied: although the resistance of the treated animals to bacterial challenge was enhanced, the animals developed sterile abscesses at the site of injection. These abscesses were more severe in the emulsion treated animals, due possibly to the higher local concentration associated with a slow diffusion and the adjuvant action of the emulsion.

Resistance conferred by double stranded RNA and poly I : C

Two other interferon inducers, double stranded RNA (dRNA) and poly I : C, were studied and found to induce very little resistance to infection in BSVS mice. The poly I : C showed virtually no protective effect, while the dRNA did induce a very low level of protection. The administration of these substances in a double emulsion did not enhance their activity.

Ribonuclease (RNase) present in the peritoneal cavity may have led to a partial enzymic degradation of these double stranded ribonucleic acids with a loss of biological activity. Diazani, Gagnoni and Cantagalli (1970) showed that diethylaminoethyl dextran (DEAEd) (Pharmacia, Uppsala) not only protected poly I : C against RNase activity but mouse L-cells pretreated with DEAEd produced a higher interferon titre when later treated with poly I : C.

In the present experiments, pretreatment or simultaneous i.p. treatment of mice with DEAEd did not increase the antimicrobial activity induced by poly I : C or dRNA administered intraperitoneally. Similarly, the administration of the polycation protamine sulphate (Sigma Chemical Co.) did not significantly increase the resistance induced by the two nucleic acids. The endotoxin/protamine sulphate regimen was devised on the premise that the poly-basic substance might aid the passage of the endotoxin through the negatively charged cell membrane to a possible site of induction.

The role of macrophages in intraperitoneal/intravenous experiments

The experiment described in Table V showed that the stimulation of fixed peritoneal macrophages could not fully explain the resistance displayed by endotoxin treated mice.

The direct role of the reticuloendothelial system (RES) was investigated by attempting to blockade the system and then induce nonspecific resistance in the mice. Two types of RES disrupting agents were used; trypan blue (Beeson, 1947) and various chemical derivatives of the corticosteroids. An aqueous trypan blue solution (1%) (George T. Gurr, London) was injected intravenously (0·1 ml) on the 2 consecutive days before and on the day of endotoxin administration. A similar dose regimen was used for Voren (Abbotts Laboratories Ltd, dexamethasone-21 pyridine-4-carboxylate), Betsolan (Glaxo Laboratories Ltd, betamethasone) and cortisol (Koch Light Laboratories Ltd, 4-pregnen-11 β-21 diol 3,20 dione), such that each dose contained 200 μg of active corticosteroid. The trypan blue increased the lethal effect of the endotoxin and decreased the resulting induced resistance, but the RES blockaded animals were still protected against bacterial infection when compared with the controls. The corticosteroid injections reduced the amount of endotoxic shock, as shown by Mills (1971), and decreased the animal's resistance to bacterial challenge; this resistance, however, was not reduced to the level existing in the control animals.

Splenectomized animals.—One of the major organs of the RES was removed when groups of BSVS mice were splenectomized. Ten days after the surgery the mice were injected with 75 μg endotoxin, and 4 days later challenged with *Salmonella dublin*. The splenectomized animals appeared to be no more susceptible to the endotoxic shock, and to have acquired the same degree of nonspecific resistance as the intact control animals receiving similar doses of endotoxin and challenge bacteria.

Blood clearance and tissue uptake studies.—The activity of the RES was studied further in 2 experiments involving the clearance from the blood of colloidal carbon and tritium labelled bacteria (Table VI). Although the standard error associated

TABLE VI.—*The Rate of Removal of Colloidal Carbon and Radioactive Bacteria from the Blood of Untreated and Endotoxin Treated BSVS Mice*

	Untreated (mean ±s.e.)	Endotoxin in emulsion (mean ±s.e.)	Endotoxin in saline (mean ±s.e.)
Colloidal carbon (log optical density 620 nm min^{-1})	0·028±0·002	0·027±0·0013	0·029±0·0024
Radioactive *Salmonella dublin* (log ct/min min^{-1})	0·011±0·001	0·012±0·0012	0·013±0·0011

Observations were made 4 days after the intraperitoneal administration of 75 μg of endotoxin.

TABLE VII.—*Percentage Recovery of Radioactivity in the Spleen, Liver and Blood of Mice 30 minutes after the Intravenous Injection of Tritium Labelled Bacteria*

		Salmonella dublin % radioactivity ±s.e.			*Staphylococcus aureus* % radioactivity ±s.e.		
Animals preinjected* with		Spleen	Liver	Blood	Spleen	Liver	Blood
75 μg endotoxin	emulsion	21·7 ±0·71	40·3 ±2·86	13·08 ±1·04	2·9 ±0·18	42·5 ±2·0	0·72 ±0·058
	saline	21·2 ±1·5	41·7 ±3·22	11·71 ±1·69	2·34 ±0·12	46·2 ±1·40	0·78 ±0·067
Saline		14·2 ±2·10	47·4 ±3·2	12·9 ±0·26	3·2 ±0·38	42·7 ±2·15	0·83 ±0·17

* Intraperitoneally, 4 days before the injection of bacteria.

with both these experiments is considerable, the results indicate that the blood clearance rates are not significantly different in animals 4 days after endotoxin administration compared with the untreated controls. Table VII provides some indication of the destination of radioactively labelled bacteria after removal from the blood. Again, at 4 days after the endotoxin administration in the experimental group the 2 major organs of the RES appear to have removed similar amounts of particulate material from the blood, compared with saline controls, suggesting no marked enhancement of the macrophage activity.

DISCUSSION

The endotoxin from *E. coli* O55 B5 was selected from a number of endotoxins for its low toxicity following a series of toxicity trials. Nevertheless, some toxic symptoms were observed in the mice as the dosage was increased. Although a higher degree of nonspecific resistance to bacterial infections can be achieved by higher doses of endotoxin, the 75 μg dose used in this study constituted a compromise between an acceptable level of toxicity and a significant enhancement of resistance.

A study of the literature reveals that the doses given by different workers have varied to a considerable extent. Weinstein *et al.* (1970) used as little as 0·1 μg endotoxin to elicit a resistance against *E. coli* in mice, whereas MacGregor, Sheagren and Wolff (1969) injected 400 μg doses into mice to induce a protection

against *Plasmodium berghei*. Weinstein's dosage rate produced no enhancement of nonspecific resistance whatsoever in our strains of BSVS and LACG mice, whereas our experimental animals were unable to survive the 400 μg doses used by MacGregor. It is clear that the level of nonspecific resistance which can be induced depends on the source of the endotoxin, its dose, the strain of mice studied and the virulence of the challenge strain of the microorganism.

It was anticipated that the administration of the endotoxin in a double emulsion would lead to a reduced toxicity and a prolonged activity due to a slow release into the tissues. While this protective action of the emulsion was not observed, the double emulsion considerably enhanced the nonspecific resistance. It is possible that the double emulsion may have acted as an adjuvant, but insufficient information is available at present to be certain of the precise mechanism involved.

The apparent efficiency with which the nonspecific resistance of the mouse could be boosted depended upon the challenge microorganism used in the experiments. The type of infection produced by the challenge organisms in this present study varied considerably from a chronic infection produced by low numbers of the very pathogenic *Salmonella dublin*, to the very acute infections produced by the staphylococci and streptococci. It is unlikely that the rapid death following the staphylococcal challenge of the control mice was due to the action of injected toxin, since the staphylococcal toxin is an exotoxin and would not be present in a high concentration in the well washed suspensions injected. It is conceivable, however, that the growth conditions in the peritoneal cavity would be such that the staphylococcal generation time would be of short duration, allowing a challenge dose of $2{\cdot}0 \times 10^9$ viable organisms to produce a bacteriaemia within 5 hours, causing rapid death.

The stimulation of natural resistance was not observed in the experiments when mice were injected with poly I : C. On the other hand, Weinstein *et al.* (1970) showed that this synthetic double stranded ribonucleotide would protect mice against *E. coli* infections. One possible explanation for our lack of success may have been that while the poly I : C prepared showed a typical hypochromic effect (Davies and Rich, 1958) indicating a double stranded molecule, its movement in a caesium chloride gradient in an analytical ultracentrifuge indicated a relatively low molecular weight of 5×10^5. Morahan *et al.* (1972) observed that little interferon was produced by poly I : C of less than 10^6 molecular weight, so that a preparation of lower molecular weight may have been inadequate to induce nonspecific resistance.

From this investigation it is conceivable that increased phagocytosis may not be a major factor contributing to nonspecific resistance induced by endotoxin although other workers (Biozzi *et al.*, 1955; MacGregor *et al.*, 1969) have observed enhanced macrophage activity. Nevertheless, endotoxin may stimulate the release of antimicrobial factors into the intracellular fluids. Tew, Scott and Donaldson (1971) demonstrated the release of the cationic proteins lysozyme and β-lysin from the platelets and leucocytes into the plasma of endotoxin treated rabbits, and Brownlie (1972) showed the release of cationic proteins from cows' milk leucocytes into milk whey following endotoxin injections into the mammary gland. The nonspecific antimicrobial role of these cationic proteins and their likely importance in nonspecific resistance have been described extensively elsewhere (Hirsch, 1958; Zeya and Spitznagel, 1966; Hibbitt, Cole and Reiter, 1969;

Hibbitt, Brownlie and Cole, 1971; Gladstone and Walton, 1971; Brownlie and Hibbitt, 1972).

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