Increase of Nonspecific Resistance to Infection by Protodyne, a Protein Component Derived from Bacterial Protoplasm (32739)

F. M. BERGER, G. M. FUKUI, B. J. LUDWIG, AND J. P. ROSSELET
Wallace Laboratories, Cranbury, New Jersey 08512

It has been known for more than 70 years that a temporary increase in host resistance to many bacterial infections can be produced in animals by injection of immunologically unrelated Gram-negative organisms. This enhanced resistance does not depend to any major extent on circulating antibodies since it cannot be transferred with the serum of protected animals (1). Landy (2,3) and Rowley (4) indicated that the agent principally responsible for this increase of the nonspecific resistance of the host is endotoxin, a toxic lipopolysaccharide previously isolated from bacteria by Boivin *et al.* (5,6), and others. Subsequent investigators demonstrated that endotoxin is contained only in the bacterial cell walls and that bacterial protoplasm is substantially free from this material (7).

It was observed that endotoxins prepared by various methods of extraction, while differing markedly from each other in their chemical composition and toxic properties, often retained their ability to increase the host's nonspecific resistance to infections and disease (8). Certain protein-containing bacterial extracts appeared to be much less toxic than more highly purified lipopolysaccharides (8,9). The question arose whether the protein moiety in these extracts served merely as a diluent or actively contributed to the protective activity of these preparations. To resolve this question, work was undertaken to separate the bacterial cell wall from the bacterial protoplasm and to compare endotoxin-free proteinaceous material derived from the cell protoplasm with classical, highly purified cell wall-derived lipopolysaccharide (endotoxin).

Materials and Methods. Separation of endotoxin from the protoplasmic material was carried out by a combination of physical and chemical methods. *E. coli* cells grown on a semisynthetic medium at 30°C for 20 hours were disrupted in an RF-1 Ribi refrigerated cell fractionator (I. Sorvall, Inc., Norwalk, Connecticut) (10) at 30,000 psi. After separation of the cell wall material from the protoplasm by centrifugation, both fractions were subjected to Westphal's extraction procedure (11). This method, which utilizes 50% aqueous phenol extraction at 68°C, achieves separation of proteinaceous material from lipopolysaccharides. The endotoxin and nucleic acids are contained in the aqueous phase. The protein remaining in the phenol layer was further purified by methyl alcohol precipitation, centrifugation, and dialysis.

This protein fraction of the protoplasm, which we call protodyne, and the lipopolysaccharide (endotoxin) prepared from the cell walls of the same organism by the Westphal method (11), were evaluated for their ability to protect mice from infections with various microorganisms. The toxicity, pyrogenic properties, and the ability to induce the Shwartzman reaction of the two substances were also studied.

The ability of protodyne to enhance nonspecific resistance to infections was evaluated in mice of the Swiss Webster strain, utilizing *Salmonella typhimurium, Salmonella typhosa, Pseudomonas aeruginosa, Klebsiella pneumoniae, Streptococcus mastitidis,* and *Diplococcus pneumoniae* as the infectious agents. The microorganisms, with the exception of *Pseudomonas aeruginosa,* were administered intraperitoneally. With all germs, the number of viable cells given was adjusted in preliminary titrations to produce deaths in 90% of animals within 48 hours.

Protodyne or endotoxin was usually given intraperitoneally at 5 different dose levels. Groups of 20 mice were used at each dose level and protodyne or endotoxin administered in a single injection 24 hours prior to infection. In the case of *Pseudomonas aeruginosa,* the mice were infected intravenously 48 hours after treatment with protodyne or endotoxin. When 90% of the un-

TABLE I. Increased Resistance to Infection Produced in Mice by Protodyne and Endotoxin.

Organism	Number of viable organisms given	Protodyne		Endotoxin		Relative potency[c]
		PD_{50} (mg/kg)[a]	Confidence limits[b]	PD_{50} (mg/kg)	Confidence limits	
Salmonella typhimurium (3S)	1.12×10^8	0.052	0.030–0.090	0.0028	0.0013–0.0058	19
Salmonella typhosa (Ty 2)	4.55×10^8	0.032	0.015–0.067	0.0019	0.0011–0.0032	17
Pseudomonas aeruginosa (25-A-2)	4.6×10^7	0.44	0.27 –0.70	0.025	0.014 –0.045	18
Klebsiella pneumoniae (B)	3.9×10^7	0.54	0.23 –1.24	0.0052	0.0029–0.0095	104
Streptococcus mastitidis (SP_1)	9.7×10^4	0.59	0.33 –1.06	0.0038	0.0019–0.0077	155

[a] Dose protecting 50 per cent of animals from death by infection.

[b] 95% confidence limit.

[c] Endotoxin = 1.

treated control animals were dead—usually 24 to 48 hours after infection—the median protective dose, PD_{50}, that is, the dose at which 50% of the treated animals survived, was calculated (12). Each experiment was repeated 3–5 times with similar results.

Results. Protodyne increased the ability of the animal to resist infections by all the microorganisms that were used (Table I). For at least two reasons the increase of resistance was considered to be due to a nonspecific increase of host resistance and independent of the presence of specific antibodies. First, the animals were protected against a number of different, immunologically unrelated strains of microorganisms, and second, the increase of resistance occurred soon after administration of protodyne, at a time before effective levels of antibodies could have been formed.

Although protodyne increased host resistance to infection by all microorganisms, larger amounts of protodyne than of endotoxin were needed to produce this effect. Endotoxin and protodyne also differed from each other in the relative amounts needed to produce increased resistance against infection with different microorganisms. In the case of endotoxin, doses of a similar magnitude protected animals from infections evoked by most of the microorganisms that were studied. With protodyne, on the other hand, the amount needed to obtain protection varied greatly with the microorganisms used. Thus, animals infected with *Streptococcus mastitidis, Klebsiella pneumoniae* or *Pseudomonas aeruginosa* required about 10 times as much protodyne to obtain protection than animals infected with *Salmonella typhimurium* or *Salmonella typhosa*. These results suggest that protodyne mobilizes different host mechanisms than endotoxin.

To elicit the Shwartzman reaction, protodyne and endotoxin were injected intracutaneously in the clipped ventral surface of New Zealand rabbits in doses of 100, 200, and 500 μg in a volume of 0.2 ml of saline. *E. coli* endotoxin (100 μg) or *Serratia marcescens* endotoxin (500 μg), used for provocation, were injected intravenously in a volume of 1 ml 18 hours after sensitization. The degree of erythema and necrosis was rated 5 hours after challenge. While the endotoxin produced the usual hemorrhagic and necrotic lesions, protodyne, even in very large doses, did not produce any detectable skin changes. This is of particular interest since the Shwartzman reaction is one of the most sensitive and specific tests for endotoxin.

The pyrogenicity of protodyne was also evaluated in male New Zealand albino rabbits using the usual techniques (13). Endotoxin, in doses of 0.5 μg/kg produced a maximum temperature rise of $3.5 \pm 0.07°F$, while protodyne under these conditions produced a rise of $0.8 \pm 0.1°F$. When the area of the fever graph was measured (14), protodyne had only one-one hundredth of the pyrogenicity of endotoxin. Protodyne appeared quite nontoxic. Mice tolerated a dose of 400 mg/kg intraperitoneally without any discernable changes in appear-

ance or behavior. Mice receiving endotoxin in doses as low as 10 mg/kg, on the other hand, were listless and emaciated in appearance. Their fur was ruffled and they had diarrhea. The dose of endotoxin killing 50% of animals after intraperitoneal administration was 21 mg/kg with 95% confidence limits of 15–30 mg/kg.

Discussion. The occurrence of a proteinaceous substance in the protoplasm of *E. coli* that is of low toxicity and is able to increase nonspecific resistance to infections is unexpected and of considerable theoretical and practical interest. Ribi *et al.* (7) have shown that the biological properties usually associated with endotoxin are chiefly present in the extracts prepared from the cell walls. The separated protoplasm in the hands of these investigators retained only traces of these activities. These investigators, however, studied only the biological properties of crude protoplasm, of its trichloroacetic acid extracts and of the residue of the trichloroacetic acid extracts (15). The biological properties of the phenol soluble fraction containing the proteinaceous material of the protoplasm were not investigated.

Protodyne differs from endotoxin and the protoplasmic material previously described by Ribi *et al.* (15) not only in biological properties but also in chemical composition. Protodyne is predominantly protein and contains only less than 1% of carbohydrate. Endotoxin and Ribi's protoplasmic material, on the other hand, was made up predominantly of polysaccharides. The result of a typical chemical analysis of protodyne and endotoxin prepared from our strain of *E. coli,* and bringing out the differences in chemical composition of the two extracts, is given in Table II. The nitrogenous material present in endotoxin is the protein moiety described by Homma and Suzuki (16) which occurs in the bacterial cell wall and has a tendency to form complexes with endotoxin.

Protodyne can also be differentiated from endotoxin by measuring the lipid content of the two preparations. This was done by determining the fatty acid content of endotoxin and protodyne by gas chromatographic analysis of methylated chloroform extracts obtained after acid hydrolysis of the two preparations. The endotoxin fraction contained the bulk of the total fatty acids whereas the protodyne fraction had less than 1% of that amount.

TABLE II. Chemical Composition of Extracts of *E. coli.*

	Biuret protein (%)	Kjeldahl nitrogen (%)	Carbohydrate (%)
Endotoxin[a]	28.6	9.8	45.5
Protodyne[b]	94.2	14.0	0.6

[a] Endotoxin: Water soluble phase of a Westphal extract prepared from isolated cell walls.

[b] Protodyne: Phenol soluble phase of a Westphal extract prepared from isolated bacterial protoplasm.

Summary. It now appears that *E. coli* and perhaps other organisms contain not only endotoxin, but also a nontoxic proteinaceous material that is not a lipopolysaccharide and yet has the ability to increase the nonspecific host resistance against a variety of infections. This substance, called protodyne, occurs in the protoplasm and can be extracted from the separated protoplasm with hot phenol. Protodyne differs from endotoxin, which is contained in the bacterial cell walls, in consisting predominantly of proteinaceous material and in being substantially free from lipids and polysaccharides. Biologically, protodyne appears devoid of the pyrogenic and Shwartzman inducing properties that are so characteristic of endotoxin. While protodyne increases host resistance to infection with a variety of microorganisms, it differs from endotoxin in having a different degree of protective action depending on the microorganism responsible for the infection. It is possible that protodyne plays a role in protecting animals from infections prior to the onset of specific antibody formation.

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Effect of Aflatoxin on Human Leukocytes (32740)

D. A. DOLIMPIO, C. JACOBSON AND M. LEGATOR
(Introduced by J. Reilly)
Division of Nutrition, Food and Drug Administration, U. S. Department of Health, Education, and Welfare, Washington, D. C. 20204

Aflatoxin B_1, a mycotoxin produced by *Aspergillus flavus*, is carcinogenic in rats (1–3) and trout (4). Studies in tissue culture and in cell-free systems indicate that aflatoxin primarily affects the synthesis of nucleic acid (5,6). Chromosome abnormalities have been produced by this compound in seedling roots of vetch (*Vicia faba*) (7) and in a cell line derived from the kidney of a rat kangaroo (*Potorous tridactylis apicalis*) (7,8). The experiments reported in this paper were designed to investigate the effect of aflatoxin on human leukocyte culture and to characterize the type of response.

Materials and Methods. Two preparations of aflatoxin were used: an aflatoxin mixture (495) containing 15% B_1, 9% G_1, and less than 1% B_2 and G_2; and a crystalline B_1 preparation[1] (6).

Leukocyte cultures (72 hours) were set according to a modification of the method of Moorhead *et al.* (9), using plasma inocula from three healthy females. Aflatoxin 495 was added to produce final concentrations of 1.0, 5.0, 10.0, 25.0, and 50.0 μg/ml and aflatoxin B_1 at a final concentration of 40.0 μg/ml to replicate cultures.

[1] Aflatoxin was obtained from Dr. A. D. Campbell, Division of Food Chemistry, Food and Drug Administration.

Our preliminary work had shown that aflatoxin is toxic when added initially at the time of plasma inoculation. Therefore, exposure was delayed until 22 hours after inoculation. Cells were exposed to aflatoxin for either 8 or 48 hours and were harvested after 70-hours total incubation. A dose effect in percent was determined by scoring number of metaphase cells with chromosomal aberrations per total number of analyzable metaphase cells. Photomicrographs were made of all cells with microscopically detectable abnormalities, and the abnormalities were further scored as to type (break, gap, translocation, deletion, fragment), site (long arm, short arm, centromere) and karyotypic group according to the Denver Report (10).

Results and Discussion. Mitotic rate. A definite inhibition of mitosis was found after 8-hours exposure to aflatoxin (Table I). The mitotic inhibition was greater after 48-hours

TABLE I. Mitotic Index of Aflatoxin-Treated Cells at Various Exposure Times.

Length of exposure (hours)	Aflatoxin concentrations (μg/ml)					
	0	1	5	10	25	50
8	4.2	4.8	3.6	2.6	3.8	3.0
48	4.2	3.4	2.8	2.2	1.9	1.4