Protection of Mice against Lethal Staphylococcus Infection by Escherichia coli O_{86} Fractions

Abstract. Small doses of protein-lipopolysaccharides and lipopolysaccharides from *Escherichia coli* O_{86} prevent the death of mice infected intraperitoneally with a clinically virulent *Staphylococcus aureus.*

The increasing seriousness of antibiotic-resistant staphylococcal infections in man and the established effectiveness of some polysaccharides in the prevention of experimental infections due to Gram-negative bacteria prompted the present investigation. Landy (*1*) found that lipopolysaccharides from Gram-negative bacteria were quite ineffective against infection of mice with even minimal amounts of *Staphylococcus aureus.* Dubos and Schaedler (*2*) noted that mice became either more resistant or more susceptible to subsequent intravenous infection with virulent staphylococci, depending on the amount and the time interval between treatment with either Pertussis or Klebsiella vaccine, both of which contain endotoxin. Under optimal conditions, the authors noted a marked increase of resistance to staphylococcal infections. Nevertheless, the death rate of mice was still between 40 and 100 percent 12 to 15 days after the challenge. More recently these investigators reported considerable enhancement of mortality due to staphylococcal infection in mice, if minute amounts of endotoxin were given simultaneously with a challenge dose of staphylococci (*3*).

In the present experiments, litter mates of Swiss albino mice (Huntingdon Farms, Inc.), predominantly male, 5 to 6 wk old and weighing 18 to 25 g, were used. The animals were given free access to Purina Chow and water. For protection the mice were injected intraperitoneally with a solution in 0.25 ml of 0.85 percent saline of (i) protein-lipopolysaccharides or (ii) lipopolysaccharides, the purest of which contained <1 percent nucleic acids [analytical data: (i) C, 51.6; H, 8.3; N, 4.7; P, 2.6; and (ii) C, 45.5; H, 6.9; N, 1.9; P, 1.96] from blood group B active *E. coli* O_{86} (*4*), prepared by established procedures (*5*). Since both the protein-lipopolysaccharides and lipopolysaccharides showed approximately the same biological activities, no differentiation is made.

Table 1. Protection of mice by *E. coli* O_{86} (protein-) lipopolysaccharide complexes against lethal *Staphylococcus aureus* infection 16 to 72 hr later. Route: i.p., intraperitoneal; s.c., subcutaneous.

Polysaccharide complex administration		Deaths	
Amt.* (μg)	Route	No. after 20 to 30 days	Percentage
20	i.p.	4/110	3.6
2	i.p.	9/40	23
200	s.c.	15/50	30
20	s.c.	21/30	70
None		116/130	89

* Divided into two equal lots and given 24 hr apart.

The LD_{50} of this material, given intraperitoneally, was approximately 700 μg per mouse. Control animals received intraperitoneally 0.25 ml of physiological saline.

The animals were challenged intraperitoneally with the coagulase positive *Staphylococcus aureus* phage type 44A (*6*). A dose of 1.1 to 1.3 × 10^8 cocci, causing death in 85 to 100 percent of the mice within 6 to 24 hr, was administered in 0.25 ml of physiological saline. Mice are much more resistant to intravenous inoculation with this organism than to intraperitoneal injection (*6*) where the LD_{50} was 1.5 to 2.5 × 10^7 microorganisms.

Staphylococci were grown and the concentration adjusted according to the method of Higginbotham and Dougherty (*6*), except that the concentration of bacteria was initially adjusted to a standard turbidity in the Klett-Summerson colorimeter with a red No. 66 filter.

Survival of the mice was determined 16 to 36 days after application of the challenging dose of staphylococci. The similar results of 14 experiments, in which the interval between the second lipopolysaccharide treatment and challenge was 16 to 72 hr, are summarized in Table 1. Ten micrograms injected twice intraperitoneally in an interval of 24 hr prevented death due to staphylococcal challenge in 106 out of 110 mice. A similar but weaker effect was obtained when two times 100 μg of the polysaccharide material was injected subcutaneously with the challenge still being carried out intraperitoneally.

Table 2 shows the time dependence of the effect of the *E. coli* O_{86} polysaccharides between one administration of polysaccharide and challenge by staphylococci. The polysaccharide fractions gave full protection if inoculated intraperitoneally 16 to 48 hr before and over 50 percent protection when given 6 to 12 hr prior to challenge. A single injection of *E. coli* O_{86} fractions more than 75 hr or less than 5 hr prior to or shortly after administration of staphylococci, gave little or no protection. The application of *E. coli* O_{86} polysaccharide complex subcutaneously also afforded protection if the interval between injection of protective material and challenge was more than 10 hr. Numerous other polysaccharides such as blood group active preparations from *Sassafras, Taxus,* and erythrocytes (*7*), as well as the monosaccharides 2-O-methyl-L-fucose, 2-O-methyl-D-fucose (*8*), and two crystallized brain gangliosides, showed no effect at similar concentrations. In addition, force feeding of the test animals with three times 200 mg each of *E. coli* O_{86} polysaccharide over a 3-day period with the last feeding 20 hr prior to challenge did not lower the death rate.

Table 2. Influence on protection of time interval between injection of polysaccharide complex and challenge.

Interval before challenge (hr)	Deaths	
	No. after 16 days	Percentage
10 μg intraperitoneally		
2 to 4	32/50	64
5 to 6	10/20	50
8 to 9	2/20	10
11 to 12	2/20	10
16 to 48	0/50	0
No polysaccharide complex	49/55	89

These results have been uniformly reproducible over a period exceeding 1 yr. They are noteworthy in that they point to the possibility of approaching the therapy of staphylococcal infections by other than classical methods. The protective action of the polysaccharide material and the peculiar time interval essential for its effectiveness merit further investigation. It is to be noted, however, in interpreting these findings, that protection by these preparations has been tested against only one virulent strain at concentrations which, while approximately 100 percent lethal, exceed the LD_{50} only by about one decadic logarithm. Furthermore, the induced lethal staphylococcal infections in mice used in these experiments are hardly comparable to human staphylococcal infections (*9*).

Note added in proof: Similar results have now been obtained with *E. coli* O_{128} lipopolysaccharide prepared in this laboratory.

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Axenic Cultivation of Entamoeba histolytica

Abstract. *Entamoeba histolytica* (National Institutes of Health strain 200) has been maintained in axenic culture through 50 transfers over a period of 6 months. The medium used is diphasic and contains a cell-free extract of chick embryo which is essential for growth. Inocula of 50,000 amoebas yield 150,000 to 200,000 organisms in 72 hours of incubation at 35°C. An extract prepared from boiled embryos supports growth equally well.

Cultures of *Entamoeba histolytica* are readily established in association with certain species of bacteria or trypanosomatids. In the absence of these microbial associates, cultures are difficult to initiate and thus far have been established only in the presence of intact metazoan cells, namely, chick embryo cells (*1, 2*). To my knowledge there is no published report presenting unequivocal evidence of the axenic cultivation of this amoeba—that is, the indefinite subcultivation of amoebas in an environment free of metabolizing bacteria, fungi, protozoa, or metazoan cells. Evidence that axenic cultivation of a strain of *E. histolytica* has been achieved is presented in the present report.

The medium used is diphasic. The ingredients of the nutrient broth employed in both phases are listed in Table 1. To prepare the base, 1.6 g of agar are added to 80 ml of nutrient broth and the mixture is boiled, distributed in 4-ml portions to screw-capped tubes (16 by 125 mm), autoclaved for 10 min (15 lb, 121°C), cooled, and stored in the refrigerator. One week before use the base is melted, 1 ml of horse serum (inactivated 30 min, 56°C) is added per tube, and a slant with a short butt is made. After the slant hardens it is overlaid with 3.25 ml of sterile diluted nutrient broth (1:4, with distilled water) containing 0.05 percent agar and is then replaced in the refrigerator for 1 wk. Immediately before use the medium is brought to room temperature, and to the overlay are added 1.5 ml of chick embryo extract and 0.25 ml of vitamin supplement No. 109 (*3*).

Table 1. Ingredients of nutrient broth used in preparation of the diphasic medium for axenic cultivation of *Entamoeba histolytica* [*p*H adjusted to 7.2 with 1*N* NaOH; autoclaved for 10 min (15 lb, 121°C)].

Ingredient	Amount
Tryptose (Difco)	1.00 g
Trypticase (BBL)	1.00 g
Yeast extract (BBL)	1.00 g
Glucose	0.50 g
L-Cysteine hydrochloride	.10 g
Ascorbic acid	.02 g
NaCl	.50 g
KH_2PO_4	.08 g
K_2HPO_4 (anhydrous)	.08 g
Water (distilled)	80.00 ml*
Resazurin (0.05–percent aqueous solution)	0.02 ml

* Or as much as is needed.

Extracts are prepared from 11- or 12-day chick embryos. These are harvested aseptically and, after removal of the eyes and beak, are cut into two or three pieces, weighed, and processed in one of two ways. In one process the extract is prepared by a modification of the "mild extraction" technique (*4*). Embryos are quick-frozen in a Dry Ice–alcohol bath and placed in the refrigerator, and 1 hr later 3 ml of autoclaved, chilled, buffered saline (NaCl, 5.0 g; KH_2PO_4, 1.6 g; Na_2HPO_4, anhydrous, 1.6 g; distilled water, 1000 ml or as much as needed, *p*H 6.8) are added per gram of tissue. Extraction is carried out for 96 hr at 3°C. The extract is then strained through sterile cheesecloth and centrifuged for 30 min (1000*g*, 0°C). The supernatant fluid is collected aseptically, distributed in 10-ml portions to screw-capped tubes, quick-frozen in Dry Ice and alcohol, and stored at −22°C. This material, 25-percent, mild, chick embryo extract, is referred to hereafter as $CEEM_{25}$.

In the other process, boiling buffered saline is poured over the embryos (3 ml/g). The embryos are then boiled for 2 min, cooled to room temperature, and refrigerated (3°C) for 24 hr. Further processing is similar to that described for the preparation of $CEEM_{25}$. The second extract, 25-percent, heated, chick embryo extract, is hereafter referred to as $CEEH_{25}$. Each batch of extract is checked for microbial contaminants by the procedure which is outlined below.

For detection of bacterial and fungal contaminants of extracts and cultures, the following media are used: fluid thioglycollate, brain-heart infusion with 0.1 percent agar, Sabouraud's broth, trypticase soy agar plates with 5 percent sheep blood, and PPLO (*5*) agar plates (Difco) with 20 percent human blood. The fluid media, in 50-ml quantities, are inoculated with 0.25-ml samples, the plates with 0.05-ml samples. Fluid thioglycollate and brain-heart infusion are incubated at 25°, 35°, and 55°C; Sabouraud's broth and agar plates are incubated at 25° and 35°C. All are kept under observation for 7 days. In testing cultures containing antibiotics, 1-ml samples are transferred from the first set of liquid test media to a second set and also incubated for 1 wk. To detect trypanosomatids, tubes of STB medium (*6*) are inoculated with 0.25-ml samples, incubated at room temperature, and observed for 14 days.

Amoebas (NIH strain 200) grown in association with a trypanosomatid of the genus *Crithidia* (*7*) were used to initiate axenic cultures in medium containing $CEEM_{25}$. The monoxenic source cultures were started with amoebic cysts isolated with a micromanipulator after a wash in dilute HCl. Two sets of axenic cultures (I and II) were established, the first with inocula of 500,000 amoebas, the second with 150,000. Incubation was carried out at 35°C. First transfers were made after 24 or 48 hr, second and third at intervals of 48 or 72 hr. At this point multiplication of the amoebas slowed, and it was necessary to extend the time between transfers to 5 and even 7 days. After the seventh transfer, cultures showed renewed vigor, and thereafter transfers were made alternately at 72 and 96 hr. In well-established cultures, inocula of 50,000 amoebas yielded 150,000 to 200,000 organisms in 72 hr. Subcultures were made by removing the relatively amoeba-free upper half of overlay and transferring 0.5 to 1.5 ml of the remainder to fresh medium.

Set I cultures were initiated in the presence of penicillin G and dihydrostreptomycin sulfate (250 units and 0.25 mg, respectively, per milliliter of over-