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Effect of Egg Yolk and Phosphatides on Anthrax Infection of Rats and Guinea Pigs. (29769)

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The treatment of spores of *Bacillus anthracis* with whole chicken egg yolk has been shown to reduce the number of spores required to induce lethal infection in mice(1) rats(2,3) and guinea pigs(3). Neither the component of egg yolk nor the mechanism responsible for this effect on anthrax infection have been described. Among the constituents of egg yolk are lecithin and phosphatidyl ethanolamine, phosphatides which are quite active in diverse biological systems. Their effect on anthrax infection of rats and guinea pigs has been compared with that of whole chicken egg yolk and the results indicate that the activity of phosphatidyl ethanolamine present in yolk could account for the effect of yolk on anthrax infection.

Materials and methods. B. anthracis employed in these studies was the highly virulent Vollum V1b strain. All inocula were 1 ml. Aerosolization of spores was accomplished with a modified Henderson apparatus (4). The spore suspension, containing 4×10^{10} spores/ml, was prepared in 1957 and stored as a phenolated suspension at 4°C (Fort Detrick Lot No. 189). Although the guinea pig subcutaneous lethal dose 50% (LD_{50}) was < 10 spores, the intraperitoneal LD_{50} ($IPLD_{50}$) was 190 spores. Spores were "heat-shocked" (60°C for 30 minutes) 48 hours prior to use. Vegetative cells were harvested as chains from nutrient agar plates incubated at 37°C for 18 to 20 hours following seeding with spores. Spore and chain concentrations were determined by the conventional colony count method.

Egg yolk was freshly prepared for each use and unless otherwise noted was employed in a final concentration of 80%. Phosphatides, lecithin, phosphatidyl ethanolamine (PE), phosphatidyl serine (PS) and phosphatidyl inositol (PI), were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Aqueous suspensions were utilized. Guinea pigs of the Hartley strain weighing 350 to 450 g were obtained from the Animal Farm, Fort Detrick, Md. Rats, 28-day-old Sprague-Dawley strain weanling female, were obtained from Charles River Breeding Laboratories, Brookline, Mass.

Results. As shown in Table I i.p. inoculation of guinea pigs with *B. anthracis* spores suspended in 80% fresh chicken yolk resulted in 19-fold greater infectivity than spores suspended in water. LD_{50}'s were 10 and 190 respectively, a difference significant at the

TABLE I. Effect of Chicken Egg Yolk and PE as Suspending Fluid upon the $IPLD_{50}$ of Anthrax Spores for Guinea Pigs and Rats.

Spore suspending fluid	Guinea pig $IPLD_{50}$ spores	Rat $IPLD_{50}$ spores
Water	190	1.4×10^6
80% Yolk	10	1.4×10^6
PE (15 mg/ml)	26	1.3×10^6

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0.05 level. Reconstituted dried yolk produced comparable results. In addition to enhanced infectivity, spores suspended in 80% yolk resulted in a median time to death approximately 24 hours less than that resulting from a like number of spores in water. Intraperitoneal inoculation with spores suspended in PE, 15 mg/ml, resulted in an $IPLD_{50}$ of 26 spores, a value similar to that resulting with 80% yolk as suspending fluid.

With rats, a species far more resistant to anthrax infection, striking effects upon infectivity resulted with suspension of spores in 80% yolk and PE (15 mg/ml) as compared to water (Table I). As with guinea pigs median time to death from spores in yolk or PE was approximately 24 hours less than with spores in water.

Neither yolk nor PE *per se* caused death in rats and guinea pigs; serial sacrifice of rats following intraperitoneal injection of 15 mg PE revealed no lesions on gross and microscopic examinations. Utilizing the estimate of 2% PE in yolk(5), 1 ml of 80% yolk would contain about 15 mg PE, the quantity employed. Hence, the quantity of PE present in yolk could account for the observed effect.

With both yolk and PE, the effect on infection by anthrax spores was dose dependent. In guinea pigs, the $IPLD_{50}$ for spores in 20% yolk was 46 spores compared with 10 spores when suspended in 80% yolk; proportional effects were observed with intermediate concentrations. The dose effect was more strikingly demonstrated in rats. $IPLD_{50}$ values were 1.6×10^5, 4.1×10^3 and 1.3×10^3 for spores in 1, 8 and 15 mg PE respectively.

Lecithin, the other major phosphatide of yolk, had no effect on the rat $IPLD_{50}$ when used as suspending fluid for spores (Table II); both egg and soy bean lecithin were tested.

Studies with yolk fractions provided further confirmation that PE and lecithin of yolk differed in the effect on anthrax infection. The greater alcohol solubility of lecithin permitted convenient separation of the two(6). The alcohol soluble fraction was inactive; the alcohol insoluble fraction contained the active portion of yolk.

TABLE II. Effect of Phosphatides as Suspending Fluid upon $IPLD_{50}$ of Anthrax Spores for Rats.

Spore suspending fluid*	Rat $IPLD_{50}$ spores
Water	1.4×10^3
Egg lecithin	$>1.0 \times 10^6$
PE	1.3×10^3
PS	4.7×10^2
PI	1.3×10^4

* Phosphatide concentrations were 15 mg/ml.

The failure of lecithin (phosphatidyl choline) to duplicate the effect of PE suggested that the specific group of the alpha prime carbon, ethanolamine and choline, might be determinant in the effect. PS and PI as spore suspending fluids yielded $IPLD_{50}$ values intermediate to those of PE and lecithin, PS being more effective than PI (Table II). Components of the phosphatide molecules (l-serine, d-serine and inositol were tested) had little or no effect on the rat $IPLD_{50}$ of spores.

Since it was the acidic phosphatides which were effective in reducing the infectious dose, the effects of pH were studied. Phosphate buffer, pH 4.0, the same as PE 15 mg/ml, employed as spore suspending medium gave results comparable to water. Further, PE, 15 mg/ml, adjusted to pH 7.0 resulted in reductions of LD_{50} similar to those achieved with PE unadjusted. Thus, the effect of the phosphatides was not merely one of pH.

Though, in the foregoing studies, spores were suspended in the compound under test, such was not essential. Separate but simultaneous IP injection of rats with spores and PE resulted in identical LD_{50} values. Injection of PE 24 hours before or after spore inoculation, however, completely negated the effect.

Respiratory exposure of guinea pigs to spores aerosolized from suspensions in water or in 8 mg ml PE resulted in similar mortality (Table III). With rats, the inhaled doses achieved, 7.8×10^4 spores, were insufficient to cause death regardless of whether or not PE was included in the spray suspension

PE did not react irreversibly with spores during 1 hour incubation *in vitro* at 37°C, for washing once with distilled water after such incubation yielded spores with a rat

TABLE III. Exposure of Guinea Pigs to Anthrax Spores Aerosolized from Suspension in PE, 8 mg/ml, and in Water.

	Spores suspended in: Water	PE
Spores inhaled	Dead/Exposed	Dead/Exposed
500	0/6	1/6
5000	2/6	1/6
50,000	4/6	5/6

$IPLD_{50}$ identical to that of untreated spores.

The effect of PE on anthrax infection was not limited to spores. Suspension of chains of vegetative cells in PE (15 mg/ml) resulted in $IPLD_{50}$ for rats approximately 10,000-fold less than for chains in distilled water, 3×10^4 *vs* 3×10^8 colony forming units respectively.

PE neither supported nor enhanced growth of *B. anthracis in vitro* upon addition to broth cultures. Growth *in vivo* as determined by quantitative culture of peritoneal fluid, blood and spleen from serially sacrificed, lethally infected rats was affected in that the onset of progressive terminal bacteremia began earlier in rats inoculated IP with spores in PE than with spores in water; rates of increase and ultimate bacterial populations, however, did not differ. These results are in agreement with those obtained by Klein *et al*(3) with yolk-treated spores. Stained smears made from PE-broth cultures and from the peritoneal cavity of rats inoculated with spores in PE did not, however, reveal the massive encapsulation described by others with yolk (2,7).

Discussion. Striking reductions of $IPLD_{50}$ of anthrax spores or cells for both guinea pigs and rats resulting from suspension of the inoculum in chicken egg yolk have been demonstrated confirming earlier work. Studies with phosphatide components of yolk indicate that the PE component can account for the yolk effect but that lecithin is inactive in this regard. Phosphatides related to PE, PS, and PI were, also, active.

The effects of PE on anthrax infection following IP inoculation, though not completely defined, appear to be on the incipiency of infection. They do not seem to be due to pH, irreversible reaction with spore surface, or with rates of germination *per se*.

Though the subject of intensive study over many years, the precise pathogenesis of anthrax infection is not completely clear. The demonstration that incorporation of egg yolk into the inoculum of anthrax spores or vegetative cells markedly alters the host-microbe interaction in favor of the bacillus provided a useful investigative tool, particularly in species highly resistant, *e.g.*, rats. Klein and co-workers have employed such a model to define quantitative aspects of infection(3,7). Their studies, duplicated in part herein, indicate that the yolk effect is upon the incipiency of infection with earlier appearance of progressive bacteremia in animals infected with yolk-treated spores. Study of the details of the yolk effect is complicated, however, by the chemical complexity of the material, *i.e.*, whole chicken egg yolk. The present observations wherein a single chemical constituent of yolk (PE) and certain closely related compounds (PS and PI) have been shown to account for the activity of yolk offer a potentially superior model for detailed study of mechanisms of host resistance to anthrax.

Summary. Suspension of anthrax spores or vegetative cells in phosphatidyl ethanolamine or the related phosphatides, phosphatidyl serine and phosphatidyl inositol, markedly reduced the intraperitoneal median lethal dose for guinea pigs and Sprague-Dawley rats, thus duplicating the effect of whole chicken egg yolk. Quantitative considerations indicated that the amount of phosphatidyl ethanolamine present in yolk could account for the effect of yolk. Lecithin, the other major phosphatide of yolk, was ineffective. Phosphatidyl ethanolamine appeared to influence the initial phases of host-bacterium interaction.

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