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Observations on Mode of Action of Endotoxin in Chick Embryos. (29012)

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Despite the promising observations of Smith and Thomas(1), the chick embryo has not been widely used either for bioassay of endotoxin or for experimental study of the mode of action of endotoxin. Yet the chick embryo has many attributes which make it nearly an ideal tool for these purposes. Smith and Thomas pointed out that the 10-day embryo is highly susceptible to endotoxin; that this susceptibility depends to a large measure on route of inoculation and age of the embryos used, and that it is possible to protect the embryos against the lethal effect of endotoxin with 17-hydroxycorticosteroids. In addition, chick embryos are readily available and can be handled in large numbers conveniently. They may be regarded as essentially and naturally both germ-free and immunologically virgin and thus avoid the complications of variations in microflora and immunologic experience in the animal hosts generally used. It is possible to obtain blood, fluids and tissue for biochemical and histological observations, and death ensues within a matter of hours after inoculation of endotoxin into susceptible embryos and provides an easily recognizable endpoint for experimental observation.

In previous studies with chick embryos in this laboratory(2) we encountered the phenomenon, described earlier by Smith and Thomas, of the decrease in susceptibility to endotoxin with increasing embryonic maturity. The change from exquisite sensitivity to marked refractoriness to endotoxin becomes manifest during a short span of embryonic development. Although Smith and Thomas suggested that the functional maturation of the adrenal cortex could account for the disappearance of susceptibility, this hypothesis, because of the broad spectrum of adrenal cortical effects, adds little to the understanding of the mode of action of endotoxin. The above considerations suggested that further investigation of the susceptible and resistant embryos might yield information on the problem of mode of action of endotoxin at least in this experimental system.

The present report concerns additional confirmation of the Smith and Thomas phenomenon of disappearance of susceptibility to endotoxin with increasing age and with preliminary examination of some potential mechanisms of endotoxin action in the chick embryo model.

Materials and methods. Test substances, diluted in sterile physiological saline, were inoculated in 0.1 ml volumes intravenously into chick embryos at 11 or 15 days of incubation. The embryonated eggs were obtained from a single flock and were generally delivered at the ages of 8, 10 and 12 days of incubation for use at appropriate times thereafter. They were kept in a humidified incubator at 37-38°C until inoculated and were observed daily for 3 days after inoculation, although specific deaths usually occur within 12 hours. Intravenous inoculation was performed by modification of a previously described technique(3). Three sides of a rectangular window approximately 2 × 5 mm were cut over a prominent allantoic vein (while candling) using a hand drill fitted with 2 abrasive discs separated by a collar of approximately 2 mm width. With this device, 2 parallel sides of the window could be cut simultaneously. The shell flap was then lifted off with an 18-gauge needle. To allow for fixation of the vein and prevent hemorrhage the windows were removed an hour or more before inoculating. Inoculations were performed while candling using a tuberculin syringe fitted with a 27-gauge disposable needle. A simple stand was attached to the candler for resting the egg while it was being injected. Immediately following the inocula-

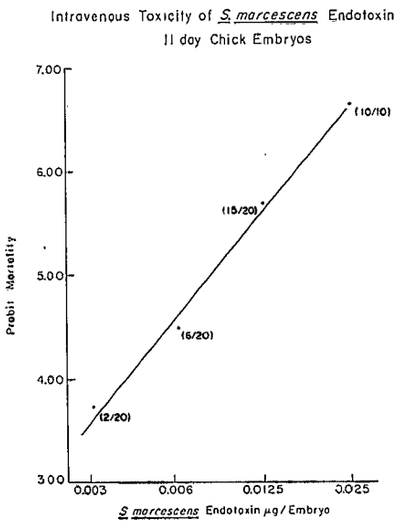

FIG. 1

tion, it was found to be of some benefit to blot the inoculation site with moderate pressure with a piece of surgical gauze. Occasional eggs which were observed to bleed under the membrane or into the allantoic cavity had a high rate of mortality and were discarded. Some external bleeding when the needle was withdrawn was not harmful, however. Mortality of saline-inoculated eggs was less than 5% and an experienced worker could inject 200 previously cut eggs in one hour. Covering of the window was found unnecessary. For most of the work, a commercial preparation (Difco) of endotoxin from *Serratia marcescens* was employed, but other endotoxins prepared in this laboratory by the aqueous ether/ethanol technique of Ribi *et al* (4) were also tested. All products for inoculation into chick embryos were sterilized by filtration through Millipore filters. Representative embryos were cultured routinely to assure freedom from contamination. LD_{50} values were approximated by the graphic method of Miller and Tainter(5).

For chemical determinations, blood was obtained from embryos by removing the shell at the air sac end and carefully nicking blood vessels with a sharp scalpel. Blood glucose determinations were made by the glucose oxidase method(6) and plasma catecholamines were determined by the method of Weil-Malherbe and Bone(7).

Results. A typical dose-response curve for *S. marcescens* endotoxin administered intravenously in 11-day embryos, presented in Fig. 1, illustrates the applicability of the chick embryo for bioassay of endotoxin. Repeated titrations on the same preparations were found to be highly reproducible with standard errors usually approximating 25% or less. Although previously Smith and Thomas(1) employed 10-day embryos, in the present study it was found that there is very little difference in susceptibility between 10- and 11-day embryos. The latter were selected for routine use since they appeared to withstand the trauma of inoculation somewhat better and their veins were more developed and more suitable for inoculation. In accord with the observations of Smith and Thomas(1), endotoxin was considerably less effective when administered on the chorioallantoic membrane and was essentially innocuous when administered at 100 µg levels *via* the allantoic route of inoculation.

Comparison of the toxicity of several endotoxin preparations for 11- and 15-day embryos (Table I) confirmed and extended the observations of Smith and Thomas(1) regarding the difference in susceptibility between the two age groups. The magnitude of the change in susceptibility to endotoxin during that time period was found to be 10,000-fold or greater with the endotoxin preparations tested. The data serve further to illustrate the exquisite susceptibility of the

TABLE I. Toxicity of Endotoxin Preparations for 11- and 15-Day Chick Embryos.

Endotoxins	LD_{50} (µg/embryo) 11-day	15-day
S. marcescens	.008	100.0
Vibrio cholerae, N-1, Ogawa	.005	>100.0
" " D344, Inaba	.01	>100.0
" " 569 B, Inaba	.14	>100.0
El Tor vibrio, 26-3, Ogawa	.008	>100.0
" " " 7738, Inaba	.033	>100.0

* Inoculated intravenously with 0.1 ml of serial 10-fold or 2-fold dilutions.

younger embryos and their usefulness as an assay system.

As a working hypothesis, it was considered that if the toxicity of endotoxin is mediated by other compounds released in the embryo following administration of endotoxin, then the responsible compounds might duplicate the endotoxin pattern of high toxicity for the younger embryo and low toxicity for the older, endotoxin-resistant, embryos.* Since the catecholamines have been widely implicated in endotoxin phenomena in other systems, it was of interest to evaluate the toxicity of these compounds for the chick embryos.

The LD_{50} of epinephrine and norepinephrine for 11-day embryos was found to be 9.2 and 16.0 μg respectively. Fifteen-day embryos were somewhat more tolerant, with LD_{50} values of 85 μg of epinephrine and 200 μg of norepinephrine. It must be considered that a portion of this observed increase in tolerance was accounted for by weight change in the embryo during the interval under study. According to Romanoff(8) the average weight of 11-day embryos is 3.49 g and that of 15-day embryos is 12.5 g. Thus, although there was some real change in susceptibility to the catecholamines with increasing age, the magnitude of the change in susceptibility did not begin to approach that observed with endotoxin. In additional contrast to the results with endotoxin, the sensitivity of the younger embryos was similar when the drugs were administered on the CAM. However, the embryos did tolerate doses by the allantoic route which were lethal when injected intravenously. Catecholamine determinations on pooled plasma from endotoxin-inoculated embryos did reveal some small increases in plasma catecholamine levels following endotoxin administration but the results were highly erratic. The normal levels of 6.5 μg of epinephrine and 3-4 μg of norepinephrine/liter of plasma occasionally showed as much as a 2-fold rise during 4 hours following a lethal dose of endotoxin. These levels were considerably below the amounts which were found to be acutely toxic. Epinephrine and endotoxin administered simultaneously in marginally lethal dosage did not act synergistically; *i.e.*, endotoxin did not enhance the sensitivity of the embryos to epinephrine and *vice versa*. It is also significant that dibenzyline (20 μg), which protected 11-day embryos against 50 μg doses of epinephrine or norepinephrine, gave no evidence of protecting embryos given LD_{15}, LD_{50} and LD_{85} doses of endotoxin.

Histamine and serotonin were not highly toxic for 11-day embryos, the LD_{50} being above 100 μg/embryo (the highest level tested). Acetylcholine also was not highly toxic; it was not lethal at 100 μg/embryo, but the majority of embryos succumbed to 1000 μg.

In contrast, insulin in small amounts was toxic for the younger embryos with an LD_{50} of approximately 0.1 unit/embryo ($\cong$ 4 μg/embryo), but doses of 20 units/embryo (the highest level tested) were tolerated by most of the older embryos. Thus, there was an increase in tolerance to insulin of greater than 200-fold during the 11- to 15-day period.

Glucose determinations on the pooled blood of inoculated embryos revealed (Table II) that both insulin and endotoxin caused a severe hypoglycemia prior to death in the younger embryos. However, the blood sugar response of the embryos to insulin was more immediate than that to endotoxin in which case there appeared to be a lag of approximately 2 hours before hypoglycemia became apparent. The effects of the two agents differed more markedly in the 15-day embryos. In the older embryos, insulin still produced a rapid and severe hypoglycemia, but the em-

* This hypothesis may not be entirely sound because (a) the acute administration of a suspected compound may not duplicate its effects upon continuous release such as might be expected following endotoxin; (b) endotoxin could enhance the sensitivity of the embryo to a compound whose release occurs naturally or in response to the endotoxin; and (c) embryos of both ages could be equally sensitive to the hypothetical suspect compound which, however, might not be formed or released in the older embryos. The latter could be manifest also if the older embryos have developed a mechanism for direct detoxification of the endotoxin.

TABLE II. Effect of Endotoxin or Insulin on Blood Glucose Levels in Chick Embryos.

Age of embryos (days)	Treatment	Exp No.	Time (hr) 0	2	4	8	20
11	Saline controls	1	100‡	98	102	—	—
		2	96	—	94	—	—
	Endotoxin*	1	104	91	21	Death	
		2		84	11	"	
	Insulin†	2	—	29	2	"	
15	Saline controls	3	112§	84	86	—	—
		4	111	112	118	115	111
	Endotoxin*	3	115	127	76	—	—
		4	—	119	101	97	84
	Insulin†	4	—	49	10	7	71

* 0.1 μg *S. marcescens* endotoxin in 0.1 ml saline, I.V.
† 0.4 μ insulin in 0.1 ml saline, I.V.
‡ Blood glucose levels, mg %: each value represents pooled blood of 20 embryos.
§ Each value represents pooled blood of 8 embryos.

bryos apparently were able to compensate and recover. Endotoxin in the older embryos caused only a slight decline in blood sugar, more gradual and less severe than that in the younger embryos, after a suggestion of a hyperglycemic phase.

Smith and Thomas(1) observed extravasation and "sludging" of blood after a short latent period in their endotoxin treated embryos followed by complete cessation of blood flow in the extra-embryonic membranes and death. Their observation that congestion and perivascular hemorrhage were the only obvious histopathological changes was confirmed in this laboratory, suggesting that intravascular events leading to clotting could account for the "toxicity" of endotoxin in this system. Similarly, the circulatory stasis could account for the hypoglycemia. However, heparin at 5.0 units per embryo failed to have any protective effect when administered simultaneously with 0.02 μg of *S. marcescens* endotoxin. (The LD_{50} of heparin was found to be 50 u/embryo and 5 u of heparin was not lethal.)

In another vein, some evidence has suggested that host reactivity to endotoxin may, in part, be dependent on the presence of antibody(9,10). Accordingly, it was of interest to determine whether antibody to endotoxin was present at any stage in the chick embryo. Antibody to cholera endotoxin was sought in the serum of chick embryos of different ages and adult chickens of the same flock by means of the highly sensitive vibriocidal antibody titration(11). No activity could be demonstrated in pools of serum from embryos of 11, 13, 15 and 19 days of age although a trace of vibriocidal antibody was detected in serum from adult chickens.

Discussion. Although epinephrine, norepinephrine, histamine and serotonin have been implicated in the vascular phenomena associated with endotoxin shock in a variety of other experimental systems(9), evidence for their participation in the lethal effect of endotoxin in the chick embryo was not established in the present study. Epinephrine and norepinephrine were found to be toxic in moderate dosage for younger embryos, but the decrease in susceptibility with increasing embryonic maturity did not approach the magnitude of the decrease in responsiveness to endotoxin during the same time interval. However, as brought out in the footnote above, this need not necessarily be the case even if these agents play a decisive role in endotoxin action in this system. Perhaps the strongest evidence against the primary participation of the catecholamines in this system is the failure to demonstrate consistent rises in the blood levels following endotoxin administration, the lack of a potentiating effect with marginally lethal doses of endotoxin and the observation that dibenzyline, which protected chick embryos against their

lethal action, had no effect on the embryos' susceptibility to endotoxin. Histamine and serotonin were less toxic even than the catecholamines by at least an order of magnitude. It remains to be determined whether endotoxin enhances the sensitivity of the embryo to these agents. Gatling(12) similarly found that endotoxin had little, if any, effect on the activity of epinephrine when both these substances were applied on the chorio-allantois. In that study, however, endotoxin, by itself, had almost no effect when given by that route in doses from 5 to 50 μg per embryo although epinephrine, norepinephrine and neosynephrine caused cephalic hematomas similar to those observed by Hook *et al*(13) and in this laboratory(2) in older embryos surviving doses of endotoxin lethal for younger embryos.

The extensive perivascular hemorrhage which was the major observed pathological manifestation preceding death could have resulted from intravascular coagulation as has been reported in other systems(14). However, heparin, which was protective against endotoxin shock in the dog(14) was not protective in the chick embryo.

The failure to detect natural antibodies against cholera endotoxin, to which the embryo is as highly susceptible as it is to other endotoxins, would seem to exclude hypersensitivity as a mechanism for endotoxin action in the chick embryo. In their study, Smith and Thomas had indicated that it was unlikely that bacterial allergy could be involved.

Perhaps the most promising avenue for further study, suggested by the work of Woods *et al*(15), is the finding of marked hypoglycemia following administration of endotoxin in the susceptible embryos coupled with absence of this response in the resistant older embryos. However, it should not be assumed that endotoxin has a direct effect on carbohydrate metabolism in the chick embryo since this could be a secondary phenomenon. That this might be the case is suggested by the delay in induction of hypoglycemia by endotoxin as compared with the rapid fall in blood sugar produced by insulin. Although the underlying mechanism of action of the two agents probably differs, it is of interest that the embryo develops the capacity to handle exogenous insulin at the same time it becomes capable of coping with endotoxin, and that the gross and microscopic pathology of embryos succumbing to insulin is similar to those given endotoxin.

The present study emphasizes the usefulness and versatility of the chick embryo for exploration of endotoxin phenomena and for bioassay of endotoxin. However, the lethal action of endotoxin in the avian embryo may not be entirely comparable to its action in mammals.

Summary. The present study confirmed the previous observation of Smith and Thomas that the chick embryo becomes refractory to intravenously administered endotoxin during the period from the 11th to the 15th day of incubation. The magnitude of the change in susceptibility is greater than 10,000-fold. Catecholamines could not be implicated in the lethal action of endotoxin in the chick embryo. Histamine, serotonin and acetylcholine were not highly toxic for the endotoxin-susceptible embryos. An anticoagulant, heparin, did not protect against endotoxin, which caused capillary stasis and perivascular hemorrhage in the embryos. Natural antibody against *Vibrio cholerae* endotoxin could not be detected in the blood during the embryonic state. A marked hypoglycemia resulted following administration of endotoxin and insulin in the younger embryos after a slight delay in the former case. Older embryos, which were markedly tolerant to insulin, developed transient hypoglycemia after insulin administration, but endotoxin caused only slight changes in the level of blood sugar in the older embryos. The versatility and usefulness of the chick embryo for further study of endotoxin phenomena and for bioassay of endotoxin was emphasized.

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