

Endotoxin Studies in Chicks: Effect of Lead Acetate

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SUMMARY

Lead acetate administered intravenously in a mixture with endotoxin preparations potentiates the toxic effect of endotoxin for 14-21 day old meat strain chicks. A dose of 2.8 mg of lead acetate/100 g body weight is more effective with endotoxin than a dose of 2.5 mg; however, a dose of 3.0 mg or greater is toxic alone. The degree of potentiation appears to be at least 1000 fold and permits toxicity determinations using endotoxin levels ranging from 0.125 μg to 250 μg /chick. Endotoxin preparations made using NaCl, TCA or phenol-water extraction procedures, possessed toxic activity for chicks when tested by this method.

RÉSUMÉ

En ajoutant de l'acétate de plomb à une préparation d'endotoxine injectée par voie intraveineuse, on augmente les effets toxiques de cette endotoxine pour des poussins âgés de 14 à 21 jours provenant d'une souche de poulets de grill. Une dose de 2.8 mg d'acétate de plomb par 100 gms de poids vif est plus efficace qu'une dose de 2.5 mg; cependant, une dose égale ou supérieure à 3.0 mg est toxique à elle seule. Le degré d'augmentation de la toxicité est d'au moins 1000 et permet de déterminer la toxicité de taux d'endotoxine, variant entre 0.125 mg et 250 mg par poussin. Des préparations d'endotoxine effectuées selon les procédures d'extraction au NaCl, au TCA ou à l'eau phénolée s'avéraient toxiques pour les poussins lorsqu'on utilisait cette méthode.

INTRODUCTION

Common laboratory animals such as rabbits, guinea pigs, mice or rats have been shown to be more susceptible to endotoxin than have chickens (1). Jordan and Hinshaw (4) reported that chickens survived intravenous inoculation of endotoxin of up to 40 mg/kg, and Ccle and Boyd (2) found no visible reaction to 2 mg endotoxin in two-week-old chicks. It has been mainly for these reasons that the chicken has not been used extensively in endotoxin studies. However, two-week-old chicks have been shown to be susceptible to endotoxin preparations (9) and investigations have been continued to determine the effect of different methods of extraction and purification on this activity. During this study it was evident that the variable response of the chick as measured by mortality over a wide range of test levels of endotoxin would make comparisons between procedures difficult if not impossible. The report of Seyle *et al* (7) that lead acetate increased the sensitivity of rats to endotoxin about 100,000 times above normal, suggested that this substance be tested to determine whether it would have a similar effect on chicks.

MATERIALS AND METHODS

Crude endotoxins were prepared from a strain of *Escherichia coli* (serogroup O45) isolated from chickens. Cells were grown in the synthetic medium of Davis and Mingioli (3) prepared without citrate. Ten litre amounts of medium were inoculated with 1 litre of a 12 hr culture and grown with high aeration and agitation at $37 \pm 2^\circ\text{C}$. The pH was adjusted to 7.0 ± 0.05

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using 1N NaOH every 20 minutes, and maximum growth was usually obtained in five hours. The culture was cooled and immediately centrifuged in a Servall RC 2 centrifuge with a continuous flow attachment. Crude endotoxin was recovered from the supernatant by filtering it through an 0.45 μ Millipore filter, removing the media constituents by passage through Sephadex G-25 medium in a K 100/100 column and lyophilization. The cells were suspended in distilled water and lyophilized. A sodium chloride extract was prepared as follows. Forty grams of lyophilized cells were suspended in 1100 ml of 9% NaCl and stirred under refrigeration for 16 hours. Following centrifugation the sediment was resuspended in 900 ml of 9% NaCl, heated to 68°C for 30 minutes, cooled and centrifuged. The cell residue was then suspended in 900 ml of distilled water and centrifuged. The supernatants were pooled, filtered through an 0.45 μ Millipore filter and desalted using Sephadex G-25 medium grade, in a Sephadex K 100/100 column. The eluate was lyophilized and refrigerated until used. Extracts were also made with 45% phenol-water or 0.5 N cold trichloroacetic acid using established procedures (5); these were dialyzed in cold running water for 48 hours and for 36 hours in distilled water with three changes of water. They were then lyophilized and refrigerated. Weighed amounts of the lyophilized extracts were suspended in 0.85% NaCl passed through a K 50/150 column of Sephadex G-200 and collected in 20 ml fractions. Optical density of the fractions was measured at 280 and 260 m μ using a Coleman-Hitachi 124 spectrophotometer. Selected fractions were pooled, dialyzed against running tap water for 36 hours, against distilled water for 36 hours with three changes of water, and lyophilized.

The toxoided preparation was made using the NaOH technique reported by Tripodi and Nowotny (8). These authors working with *Serratia marcescens* endotoxins found that this procedure resulted in a complete loss of toxicity. An equal volume of 0.2N NaOH was added to 4 mg/ml endotoxin and stirred at room temperature for 24 hours, then neutralized to pH 7.6 with 0.1N HCl.

The endotoxin preparations used for chick inoculations were filtered through a sterile 0.45 μ Millipore filter and 0.5 ml of the filtrate was inoculated into 5 ml of tryptose phosphate broth which was in-

cubated for 72 hours as a sterility check.

Lead acetate (Pb (C₂H₃O₂)₂ · 3H₂O; Fisher Scientific Company) was prepared as a 1 or 2% solution in distilled water and autoclaved on the day of use. Appropriate concentrations were prepared with sterile distilled water.

Columbian rock chicks 14 to 21 days of age were weighed and divided into groups of similar weights. The average weight of each group was then used to calculate the dose of lead acetate per 100 g body weight. Following a series of experiments to determine the amount of lead acetate that would be tolerated in chicks, double concentrations of lead acetate were mixed with endotoxin solutions to provide a 1 ml dose for intravenous inoculation. Mortality was measured for 24 hours post-inoculation when the experiment was terminated. The author had previously determined that negligible mortality occurred after this time.

RESULTS

Several preliminary experiments were performed to establish the level of lead acetate/100 g body weight which would be tolerated in two to three week old chicks. The results as shown in Table I indicate that 3 mg caused some mortality and that this was more severe at levels of 4 and 5 mg.

TABLE I. Effect of Lead Acetate on Chicks^a

Age (days)	Lead Acetate mg/100 g	Mortality	Weight Range(g)
20	1	0/10	142-155
20	2	0/10	155-165
20	3	0/10	166-171
20	4	2/10	176-186
20	5	9/10	185-211
20	2	0/10	139-159
20	3	1/10	159-180
20	4	9/10	178-191
19	3	0/10	120-135
19	4	1/10	134-147
19	5	2/10	148-159
18	2.5	0/10	102-109
22	2.5	0/20	212-224
19	3	0/10	171-190
14	2.5	0/20	88-92
14	2.8	0/20	97-100
14	3.0	1/20	106-114

^a1 ml injected intravenously

TABLE II. Effect of Lead Acetate and Endotoxin Combined^a

Preparation	Lead Acetate	Endotoxin ($\mu\text{g/ml}$)					Percent Mortality	
		0	1	10	50	100		
NaClF1	2.5	0/20 ^b	0/6	1/6	4/6	1/6	4/6	33
	2.8	0/20	0/6	2/6	1/6	5/6	3/6	37
	3.0	1/20	0/6	1/6	1/6	2/6	4/6	27
	0		0/6	0/6	0/6	0/6	0/6	0

^aone ml injection intravenously in 18 day chicks.

^bnumber dead/number inoculated.

An experiment with three levels of lead acetate and five concentrations of endotoxin is presented in Table II. It may be noted that there was little difference between 2.5, 2.8 and 3.0 mg with respect to total mortality. While data is not presented, the administration of lead acetate and endotoxin by IV injection in opposite wing veins produced results essentially identical to those obtained by a single combined IV dose.

Groups of birds given lead acetate intraperitoneally and endotoxin intravenously, were compared to a group given both substances intravenously. Peritonitis was produced as a result of IP administration of lead acetate at 3 mg and above and the results as presented in Table III indicate that administration by this route was not satisfactory. Approximately 80-90% of the crude endotoxin applied, was recovered following passage through Sephadex G-200. Two peaks were evident spectrophotometrically and these were designated fraction 1 and 2. Fraction 1 amounted to 75-80% of the total recovered.

Fractions 1 and 2 obtained by gel filtration of an endotoxin prepared from the

supernatant of the growth medium were compared with fraction 1 obtained by NaCl extraction from cells grown in the same medium. Results (Table IV) indicate that the activities of the two endotoxins were similar and that fraction 1 was more active than fraction 2.

TABLE III. Effect of Lead Acetate and Endotoxin Administered by Two Routes^a

Lead Acetate (mg/100 g)	Route	Endotoxin ($\mu\text{g/ml}$)		
		0	10	100
8	IP	1/10 ^b	1/10	1/10
5	IP	2/10	2/10	1/10
4	IP	1/10	1/10	1/10
3	IP	0/10	0/10	1/10
2.5	IV	0/10	4/20	9/20

^aLead acetate injected intraperitoneally, endotoxin intravenously, or both as intravenous 1 ml injections to 19 day chicks.

^bNumber dead/number inoculated.

A comparison of sephadex fractions obtained from TCA, phenol-water and sodium chloride extracts along with a toxoided

TABLE IV. Effect of Lead Acetate and Endotoxin Preparations Combined^a

Preparation	Lead Acetate ($\mu\text{g/100 g}$)	Endotoxin ($\mu\text{g/ml}$)				Percent Mortality
		0	1	10	50	
NaClF1	2.5	0/10 ^b	0/6	1/6	3/6	37
	3.0	0/10	2/6	3/6	2/6	57
Supernatant F1	2.5		1/6	2/6	2/6	40
	3.0		1/6	0/6	4/6	47
Supernatant F2	2.5		0/6	0/6	1/6	7
	3.0		1/6	1/6	2/6	23

^aOne ml injection intravenously in 18 day chicks.

^bNumber dead/number inoculated.

TABLE V. Effect of Lead Acetate^a and Endotoxin Preparations

Preparation	Endotoxin (µg/ml)											Percent Mortality				
	0.125	0.25	0.5	1	2	4	8	16	31.2	62.5	125		250	500	1000	
TCAF1	0/6 ^b	0/6	1/6	0/6	1/6	1/6	4/6	3/6	3/6	4/6	5/6	6/6			39	
PWF1	0/6	0/6	0/6	1/6	0/6	0/6	0/6	2/6	3/6	3/6	3/6	4/6			22	
NaClF1		3/6	1/6	2/6	3/6	1/6	5/6	2/6	4/6	3/6					44	
NaClF1 toxoid						0/6	2/6	1/6	0/6	1/6	0/6	0/6	3/6	3/6	19	
NaClF1 IP		1/6	2/6	0/6	0/6	1/6	1/6	2/6	1/6	2/6					19	
NaClF1 IV		without lead acetate										0/6	0/6	1/6	0/6	4

^a2.8 mg/100 g B.W. with endotoxin in 1 ml to 18 day chicks.

^bNumber dead/number inoculated

NaCl preparation is presented in Table V. The results indicate comparable activity from the TCA and NaCl extractions with the phenol-water extract being somewhat less active. Toxoiding results in a considerable reduction in toxicity and there is an indication that intraperitoneal administration of endotoxin with IV administration of lead acetate does not result in as satisfactory a potentiation as when both are administered intravenously.

DISCUSSION

Fourteen to 21 day old chicks are less tolerant of lead acetate than are rats. Seyle *et al* (7) found that 5 mg of lead acetate/100 g body weight was well tolerated in rats; however, the findings presented here indicate that while chicks can tolerate a level of 2.8 mg/100 g body weight, 3.0 mg and above results in mortality.

It has not been possible to establish the degree of enhancement because of the difficulty of obtaining sterile preparations in concentrations above 1000 µg/ml. The results suggest, however, that the toxicity of endotoxin is increased in the order of 1000 fold when given with lead acetate at a level of 2.8 mg/100 g body weight.

In spite of the enhancement of the toxicity of endotoxin by lead acetate in the chick, the chick is not as susceptible to endotoxin as is the rat under similar conditions. Nonetheless, when lead acetate is administered with endotoxins, chicks appear to be of a similar order of susceptibility as mice without potentiation (6, 10).

To be effective both endotoxin and lead acetate must be given by the IV route. Although the author has found that the endotoxin itself is essentially as active in chicks when administered by either the IP or IV routes, this was not the case when IV lead acetate was used for potentiation. The converse situation, IP lead acetate and IV endotoxin, is essentially ineffective.

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