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TECHNICAL MANUSCRIPT 455

TOXICITY OF PASTEURELLA TULARENSIS KILLED BY IONIZING RADIATION

Marshall E. Landay

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APRIL 1968

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DEPARTMENT OF THE ARMY
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TECHNICAL MANUSCRIPT 455

TOXICITY OF *PASTEURELLA TULARENSIS* KILLED BY IONIZING RADIATION

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Project 1B014501B71A

April 1968

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

ABSTRACT

Suspensions of approximately 2×10^{11} viable *Pasteurella tularensis* per ml were killed by exposure to 1×10^{6} r of gamma radiation. The irradiated suspensions, initially containing about 10 LD_{50} per ml for mice by intraperitoneal injection, immunized mice against challenge with fully virulent strains of *P. tularensis*. Toxicity and immunizing activity of the suspensions decreased significantly within a few days at 5 C. Mice were protected against the toxin by immune serum or by prior injection of endotoxin of *Escherichia coli*. Cortisone did not protect against the newly prepared suspension, but was effective against aged suspension. Lethal doses of newly prepared suspension for guinea pigs and rabbits were approximately 0.5 ml and 2 ml, respectively; cortisone protected rabbits but not guinea pigs against lethal challenge. Pyrogenic effects resembling those shown by endotoxin-containing suspensions were demonstrated in rabbits. An interpretation of the results is proposed, postulating two toxins, one labile and associated with the immunizing activity of the suspension, the other more stable and resembling classical endotoxin.

I. INTRODUCTION

Pasteurella tularensis vaccines rendered nonviable by exposure to ionizing radiation provoked immunity in white mice such that significant numbers of the animals survived challenge with the fully virulent SCHU strain of P. tularensis.[1] The significant degree of immunity produced was in marked contrast to the negligible protection against virulent strains afforded these animals by vaccines killed by other methods.[1] In these and subsequent studies it was noted that injection of the irradiated organisms into mice in somewhat greater numbers than were required for immunization frequently killed the animals within 24 hours. The toxicity appeared to resemble that associated with viable suspensions of P. tularensis[2] and with living rickettsial and viral suspensions,[3] although in these cases, the toxicity disappeared when the organisms were killed. Toxicity for white mice decreased rapidly when irradiated suspensions were held at 4 C, and attempts to obtain the toxin in soluble form and separate it from the cells were unsuccessful.* Further observations on the toxin and the responses to it in animals are presented in this report.

II. MATERIALS AND METHODS

A. BACTERIAL CULTURES

The LVS strain of P. tularensis, an attenuated strain employed as a living vaccine,[4] was used for preparation of irradiated suspensions. The culture was held in the lyophilized state, and new working cultures were prepared at monthly intervals. In one experiment, cultures of LVS that had undergone repeated transfer were also used. These cultures, designated LVS-O and LVS-ND, contained nonimmunogenic mutants that yielded gray colonies on solid medium.[4] The virulent SCHU S4 strain of P. tularensis was used for challenge of immunized mice.

B. BACTERIAL SUSPENSIONS

Cultures were grown with shaking in 500 ml volumes of peptone-glucose-cysteine broth.[5] A 5% volume of stock culture was used as inoculum. After incubation for 16 to 18 hours at 37 C the cells were harvested by centrifugation and resuspended in 1/30th the culture volume of a solution containing 0.1% gelatin and 0.9% sodium chloride, hereafter referred to as gelatin saline. Viable counts performed on modified SB agar[6] indicated approximately 2×10^{11} organisms per ml.

* Gordon, M., unpublished data.

C. IRRADIATION

The suspensions were exposed to 1×10^6 r of gamma radiation from a Co^{60} source* at a dose rate of approximately 1×10^5 r per minute. Sterility of the irradiated suspensions was established by the absence of colonies when 1-ml portions were inoculated onto SB agar medium and incubated 4 days at 37 C. The irradiated suspensions were stored at 5 C and used within 4 days, except as otherwise indicated.

D. ANIMALS

These were from random-bred colonies maintained at Fort Detrick. Female mice weighing 22 to 25 g of the Bagg strain of Swiss-Webster were used. Female guinea pigs of the Hartley strain that weighed approximately 300 g were used. Rabbits were New Zealand White adults of either sex weighing approximately 2.5 kg.

E. TOXICITY TITRATIONS

Serial twofold dilutions of the irradiated suspensions were prepared in gelatin saline, and 1 ml doses were injected intraperitoneally into mice, ten in each group. Deaths were recorded for 3 days, and the LD_{50} was calculated by the method of Reed and Muench.[7] Tests of significance were carried out by the two-sample normal approximation test.[8]

F. ENDOTOXIN

Lipopolysaccharide B from Escherichia coli was obtained from Difco Laboratories.

G. ANTISERA

Immune serum was obtained from rabbits immunized by repeated subcutaneous injections of irradiated suspension. When hemagglutination titers with polysaccharide-treated erythrocytes[9] reached 1:1280 or greater, serum was obtained, pooled, and stored at -20 C. Convalescent serum was obtained from a rabbit that had survived infection with a partially attenuated culture of the SCHU strain of P. tularensis. Normal serum for controls was pooled and stored in the same manner. Portions of immune and convalescent sera were absorbed by treatment with 1/10th volume or with 1/20th volume, respectively, of packed P. tularensis obtained by centrifugation of irradiated suspensions. The mixtures were shaken overnight at 4 C, and the absorbed sera were recovered by centrifugation.

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* We are indebted to Mr. Fred Shorten of the National Bureau of Standards for making available the radiation source.

III. RESULTS

A. TOXICITY OF IRRADIATED SUSPENSIONS

Irradiated suspensions were titrated for toxicity in mice soon after irradiation and after they were held for various intervals at approximately 5 C. Three of the suspensions were prepared from two stock cultures in which serial transfer had allowed the establishment of nonimmunogenic mutants. Results of titrations of four preparations over a 22-day period are presented in Figure 1. All of the suspensions contained approximately 10 LD_{50}/ml initially, and all decreased significantly in toxicity when held at 5 C. Although mice used in these titrations received doses as great as 10 LD_{50}, no appreciable number died in less than 8 hours. In a typical titration, 72% of the deaths occurred on the 1st day, 26% on the 2nd, and 2% on the 3rd.

Mice injected intraperitoneally with 1 ml of a 4-day-old suspension were sacrificed after 4, 8, 12, and 16 hours and their tissues were examined. No gross lesions were found. Microscopic examination of tissues taken 4 hours after injection revealed congestion and focal nuclear fragmentation in the spleen and focal necrosis in the liver. Necrosis had increased in the spleen and liver after 8 hours and had become extensive after 12 and 16 hours. Congestion of the kidney was noted after 8, 12, and 16 hours. Similar changes were found in unsacrificed animals that died after similar periods.

Lethal doses for guinea pigs and rabbits were not determined with the same precision as those for mice. Experiments with smaller groups of animals indicated, however, that the LD_{50} for guinea pigs was approximately 0.5 ml of 1-day-old suspension injected intraperitoneally, and the LD_{50} for rabbits was approximately 2 ml injected intravenously. There were no striking symptoms of intoxication in any of the species. As death approached, the animals became increasingly lethargic. Diarrhea and hematuria were noted frequently in rabbits that had received a lethal dose.

B. IMMUNOGENICITY

The effects of immunizing dose and of aging of the suspension on the protective activity in mice were investigated to study the relationship between toxicity and antigenicity. In a typical experiment, twofold serial dilutions were prepared from a suspension after it had been held at 4 C for 1, 4, and 7 days. Groups of ten mice were immunized with 1.0-ml quantities of each dilution. Early deaths caused by toxin occurred in groups receiving higher doses of suspension, groups in which half or more of the animals survived were challenged after 2 weeks with approximately

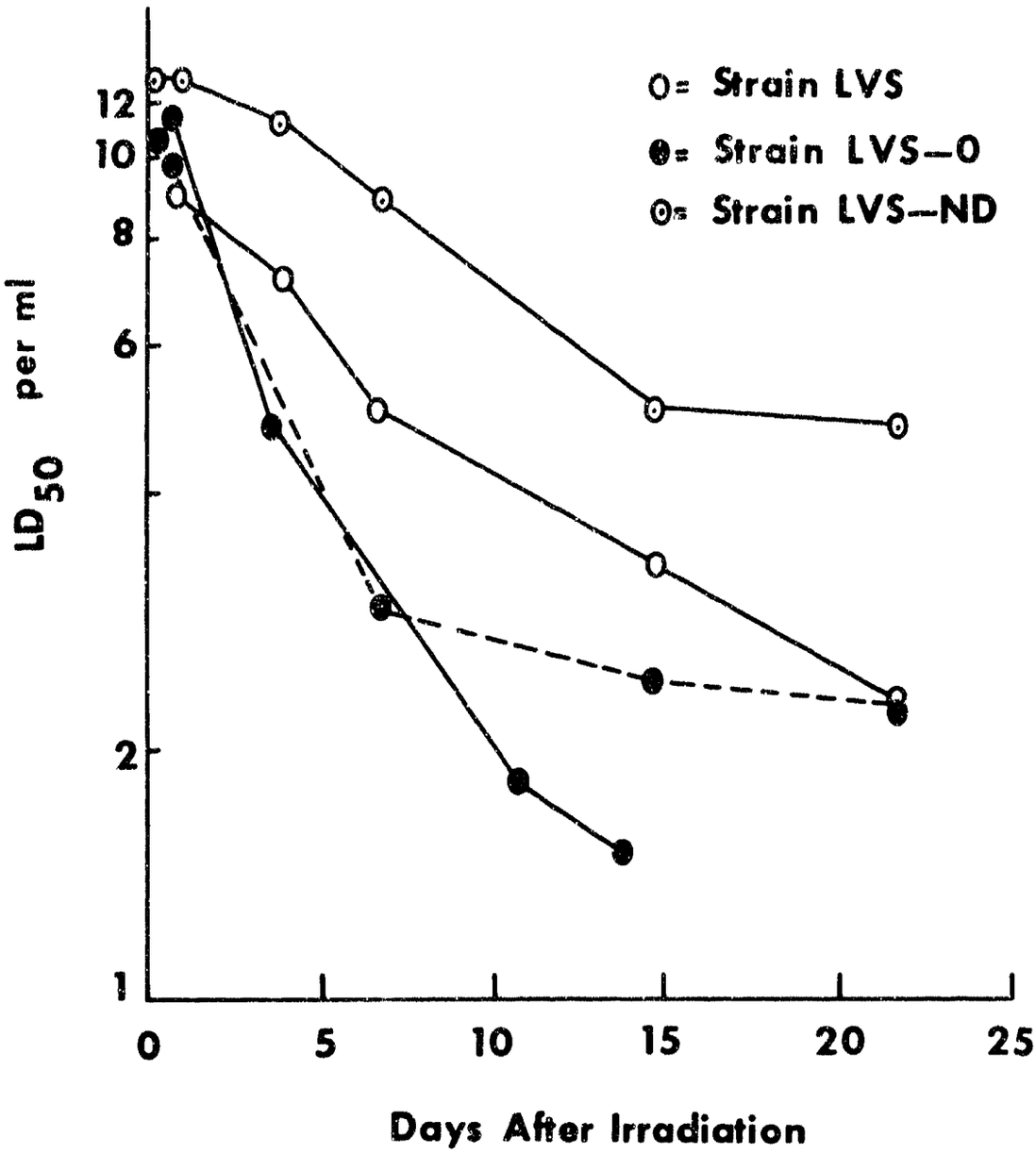

Figure 1. Deterioration at 5 C of the Toxicity for Mice of Irradiated Suspensions. The four suspensions were derived from three cultures of the LVS strain of P. tularensis.

100 cells of the virulent SCHU strain of P. tularensis. Deaths were recorded for 21 days; survival percentages are recorded in Figure 2. Unimmunized control animals died within 5 days. It is evident that the immunizing activity of the suspensions approached their maximum values only in a limited range of dilutions, and that the immunizing activity, like the toxicity, declined rapidly at 4 C. Moreover, the decline in antigenicity that occurred on standing was not overcome by an increase in dose. Additional observation on immunogenicity will be presented in a subsequent paper.

C. PYROGENIC EFFECTS

Normal rabbits were injected intravenously with graded doses of irradiated suspension, two animals per dose. Controls received gelatin saline. Rectal temperatures were measured at intervals up to 71 hours, and the mean temperatures of the experimental groups relative to the controls are recorded in Figure 3. Injection of the suspension produced a biphasic fever curve, with the first peak between 2 and 6 hours, and the second peak approximately 12 hours after the injection. The height of the first peak reached a maximum with the 0.5-ml dose, then declined with further increase in dose. The height and duration of the second peak increased with dose throughout the range studied.

D. HEMATOLOGY

Mice were injected intraperitoneally with 1 ml of a 4-day-old suspension, and groups of five animals were bled from the heart after 0, 4, 8, and 12 hours. None of the mice were alive after 16 hours. Bloods from each group of animals were pooled, and white cell counts, differential counts, and microhematocrit measurements were performed (Table 1). The white cell count decreased markedly within 4 hours and remained at a low level. This change resulted primarily from a precipitous decrease in neutrophils. There was a relative lymphocytosis, although the number of circulating lymphocytes decreased moderately. The hematocrit evidently increased at 12 hours, indicating terminal hemoconcentration.

E. EFFECT OF CORTISONE

The preceding observations indicated that the effects of the irradiated suspension resembled those of classical endotoxin in some respects. Accordingly, the effect of cortisone on susceptibility to the suspension was investigated. In a representative experiment, four groups of mice were injected intramuscularly with 5 mg of cortisone acetate, a dose effective against challenge with small amounts of classical endotoxin.[10] Within a few minutes the four groups were injected intraperitoneally with 1 ml of

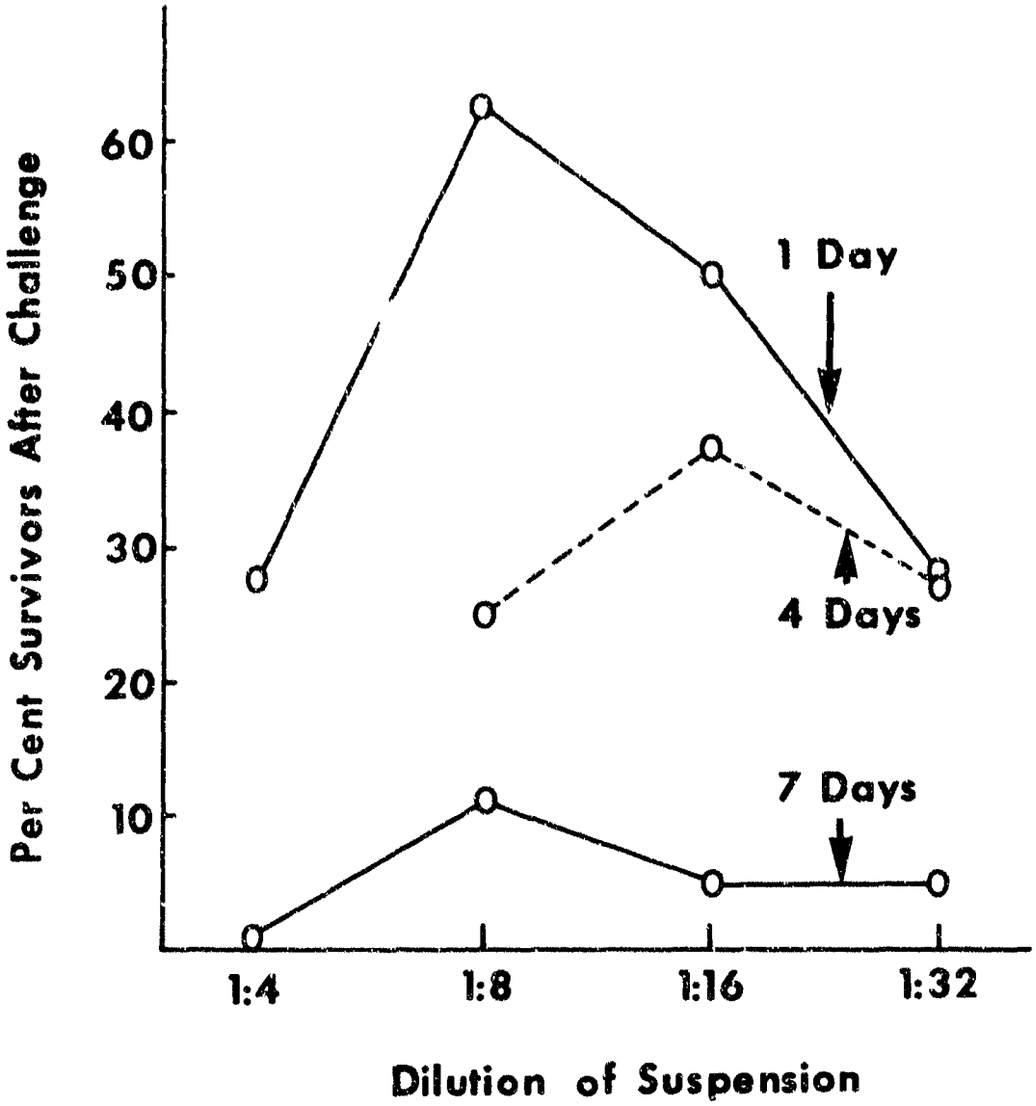

Figure 2. Immunizing Activity of an Irradiated Suspension in Mice as a Function of Dose. The suspension was tested after it had been held at 5 C for the periods indicated.

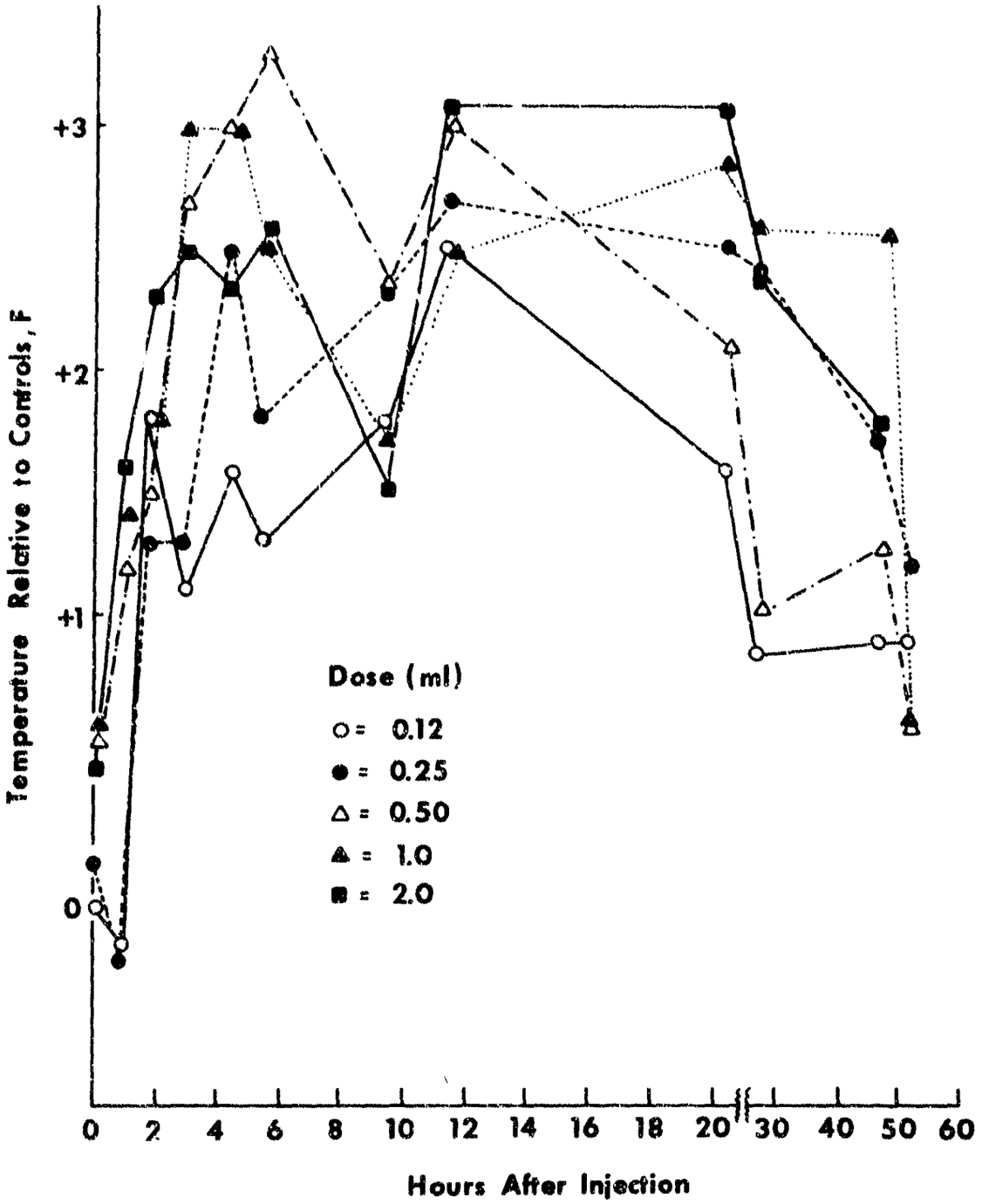

Figure 3. Mean Temperatures of Rabbits Relative to Controls after Intravenous Injection of Irradiated Suspension in the Doses Indicated.

irradiated suspension dilution 1:2, 1:4, 1:8, or 1:16, respectively. Four groups were injected intramuscularly with 0.2 ml of sterile gelatin saline and then injected intraperitoneally with 1 ml of the same dilutions of suspension. Deaths were recorded for 3 days; results are summarized in Table 2. Cortisone exerted no protective effect against the fresh irradiated suspension at any period of observation.

TABLE 1. HEMATOLOGICAL CHANGES IN MICE INJECTED WITH IRRADIATED P. TULARENSIS

Hours after Injection	WBC/mm^3	Neutrophils/mm^3	Lymphocytes/mm^3	Hematocrit, %
0	11,100	4,880	6,220	35
4	6,300	1,580	4,720	37
8	4,900	740	4,160	36
12	5,600	670	4,870	42

Experiments using irradiated suspension that had been held at 5 C for 41 days (Table 2) were carried out in the same way. Treatment with cortisone protected slightly against the aged suspension. Differences between treated and control groups were significant at the 5% level at four points of comparison and approached significance at two.

Three groups of six guinea pigs were injected intramuscularly with 12.5 mg of cortisone acetate, and three groups with gelatin saline. Within a few minutes the groups of animals were injected with graded doses of irradiated suspension. Deaths were recorded for 3 days; final results of two experiments are summarized in Table 3. The drug evidently had no effect on susceptibility of guinea pigs to the suspension.

TABLE 2. EFFECT OF CORTISONE ON SUSCEPTIBILITY OF MICE

Age of Suspension, days	Dilution of Suspension Injected (1-ml dose)	Gelatin-Saline-Treated (Controls) Survivors/Total 19 hr	24 hr	48 hr	72 hr	Survival, %	Cortisone-Treated Survivors/Total 19 hr	24 hr	48 hr	72 hr	Survival, %
<1	1:2		0/9	0/9	0/9	0		1/9	1/9	1/9	11
	1:4		1/9	0/9	0/9	0		1/9	1/9	1/9	11
	1:8		2/8	1/8	1/8	13		5/9	0/9	0/9	0
	1:16		8/9	4/9	4/9	44		8/9	2/9	0/9	0
41	Undil.	2/15	0/15	0/15	0/15	0	8/15	1/15	1/15	1/15	7
	1:2	4/15	1/15	1/15	1/15	7	11/15[a]	2/15	1/15	1/15	7
	1:4	10/15	3/15	0/15	0/15	0	14/15	14/15[a]	5/15[a]	5/15[a]	33

a. Protection significant; $P < 0.05$.

TABLE 3. EFFECT OF CORTISONE ON SUSCEPTIBILITY OF GUINEA PIGS

Dose of Suspension, ml	Survivors/Total Injected	
	Gelatin-Saline-Treated	Cortisone-Treated[a]
2	2/6	1/6
1	5/12	7/12
0.5	6/12	8/12

a. 12.5 mg cortisone acetate per animal injected intramuscularly before the irradiated suspension.

Ten rabbits were injected subcutaneously with 12.5 mg of cortisone acetate, and seven control animals with gelatin saline. Immediately all animals were injected intravenously with 3 ml of irradiated suspension. Six control rabbits died within 18 hours; the seventh was moribund at 12 hours and was sacrificed. The cortisone-treated animals remained well and appeared normal when sacrificed in pairs 1, 2, 3, 4, and 5 days after injection. Pathological changes in the control and treated animals are described elsewhere.[11]

F. PROTECTIVE EFFECT OF ANTISERUM

Immune and convalescent rabbit sera, the same sera after absorption with irradiated P. tularensis, and normal serum were tested for protective activity against the suspension in mice. Sera were injected intraperitoneally in 0.5-ml amounts, followed by 0.4 ml of irradiated suspension by the same route. Deaths were recorded for 3 days (Table 4). It is evident that both immune and convalescent sera protected mice against the toxin, and that the protective factor was removed by absorption with irradiated P. tularensis. Normal serum had no protective effect.

G. CROSS TOLERANCE WITH ENDOTOXIN

The effect of repeated injection of Escherichia coli endotoxin on susceptibility of mice to the irradiated suspension was investigated. On alternate days, mice were given six 1-ml intraperitoneal injections of graded concentrations of E. coli endotoxin. The concentrations of toxin were 0.005 mg/ml for the first dose, 0.01 mg/ml for the second dose, and 0.02 mg/ml for the remaining doses. Control mice received intraperitoneal

injections of 1 ml of gelatin saline on the same schedule. Two days after the last injection, endotoxin-treated and control groups were each divided into four subgroups that were challenged respectively with dilutions of irradiated suspension ranging from 1:2 to 1:16. Results are presented in Table 5. Prior treatment of mice with endotoxin greatly increased their resistance to irradiated suspensions.

TABLE 4. PASSIVE PROTECTION OF MICE BY RABBIT ANTISERA

Serum	Dilution[a]	Deaths/Total	Survival, %
Normal	Undil.	9/10	10
	1:4	10/10	0
Immune	Undil.	3/20	85
	1:2	2/10	80
	1:4	2/6	66
	1:8	5/6	17
	1:16	5/6	17
	1:32	6/6	0
Absorbed immune	Undil.	10/10	0
Convalescent	Undil.	0/10	100
Absorbed convalescent	Undil.	10/10	0

a. 0.5 ml of the dilution of serum was injected intraperitoneally, followed immediately by 0.4 ml of irradiated suspension.

TABLE 5. EFFECT OF PRIOR INJECTION OF ENDOTOXIN ON SUSCEPTIBILITY OF MICE

Dilution of Irradiated *P. tularensis*	Survivors/Total Injected	
	Control	Endotoxin-Treated
1:2	0/10	8/10
1:4	0/9	9/10
1:8	2/10	8/9
1:16	0/10	10/10

IV. DISCUSSION

Toxicity of killed *P. tularensis* was noted during early studies on vaccines. Suspensions killed by heat or formaldehyde produced local and generalized reactions in man[12] and edematous lesions in the skin of guinea pigs and rabbits.[13] The toxicity was evidently of a low order; lethal toxicity was not described. Significant participation of factors resembling endotoxin in the pathogenesis of tularemia was inferred on the basis of studies of the disease in man.[14] Viable suspensions were rapidly lethal for mice in doses of 1×10^9 organisms and the reaction was judged to be primarily a toxemia.[8] However, the toxicity of viable suspensions was destroyed when the organisms were killed by any of a variety of methods, and lipopolysaccharides extracted from the organisms were neither toxic nor pyrogenic.[8,15] Evidently the present irradiated suspensions retained a larger proportion of the toxicity of viable organisms than suspensions killed by other methods, a conclusion consistent with the stability of classical endotoxin in the presence of ionizing radiation.[16] Suspensions of other organisms killed by minimum irradiation may also contain endotoxin-like activities too labile to withstand classical extraction procedures.

The present studies support the concept that two kinds of toxin are responsible for the toxicity of irradiated suspensions of *P. tularensis*: a stable, endotoxin-like activity, and a relatively labile component that deteriorates within a few days at 5 C. Rabbits, more sensitive to classical endotoxin than mice, evidently respond primarily to the stable component of the suspension. The febrile response of these animals to injection of the suspension is suggestive of the presence of endotoxin. The

slower development of the fever compared with the response of rabbits to endotoxic extracts is typical of bacterial suspensions;[17] the long duration of the fever, however, resembles the response to influenza virus.[18] The protection afforded by cortisone also suggests participation of endotoxin in the lethal toxicity, because the drug evidently does not protect significantly against bacterial exotoxins.[19]

In mice, endotoxin-like activity was revealed by deterioration of the labile toxin at 5 C. Mice were protected against this residual toxicity by cortisone, although this drug was ineffective against the freshly prepared suspension. The mean toxicity for mice of 15-day-old suspensions was 3 LD_{50}/ml (Fig. 1). Thus, 1 LD_{50} represented approximately 7×10^{10} organisms on the basis of viable counts before irradiation. This toxicity is similar to the mean value of 9.4×10^{10} reported for heat or acetone-killed suspensions of Brucella abortus[20] and approaches the value of 2×10^{9} reported for heat-killed suspensions of Salmonella typhimurium.[21] Correction for the smaller size of the P. tularensis cells brings the toxicity on a dry weight basis close to that reported for S. typhimurium. Thus the endotoxin-like component of the irradiated P. tularensis resembles classical endotoxin-containing suspensions with respect to toxicity, pyrogenicity, susceptibility to cortisone, and tissue changes produced in rabbits.[11]

The labile component appears to be primarily responsible for toxicity of the fresh suspensions for mice. The observations made thus far reveal no specific symptom or morphological change sufficient to account for the lethality of the toxin. Indeed, the labile toxin has been studied thus far only in the presence of sublethal amounts of the stable, endotoxin-like component; probably the hematological and pathological changes produced by injection of the suspensions into mice are complicated by this circumstance. The lability of the toxin and its association with cells suggested a relationship to the toxins of living rickettsial and viral agents. The present exploratory studies reveal other similarities of the P. tularensis toxin to these agents: the tendency to produce terminal hemoconcentration, the frequent appearance of focal necrosis of the liver, the prolonged febrile response, and the neutralization by immune sera.[3] More detailed study of the metabolic and morphological changes produced by the labile toxin would be desirable to investigate this apparent relationship.

Neutralization of the toxicity for mice of fresh suspensions by immune or convalescent rabbit sera and absorption of the neutralizing activity by irradiated suspension may provide an experimental approach to characterization of the labile toxin. It is probable that more detailed study of the specificity of the neutralizing antibodies would permit identification of the antigens associated with the labile toxin. The tolerance to labile toxin produced by prior treatment with endotoxin of E. coli appears less likely to provide significant information regarding the nature of the toxin, because the diversity of the biological changes induced by endotoxin makes interpretation of the observed protection difficult.[22]

A most significant property of the labile toxin is its apparent association with the ability of the suspensions to immunize mice against challenge with fully virulent P. tularensis. Thus far, this association is based only on concomitant appearance and deterioration under a limited number of conditions, and must be regarded as tentative. The results suggest that the stable, endotoxin-like component does not have a similar effect on antigenicity, and indeed its toxicity may limit the level of immunity attainable with the suspension. Development of methods for isolation or independent control of the two toxicities will facilitate elucidation of their role in infection and immunization.

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13. ABSTRACT
Suspensions of approximately 2 x 10^{11} viable Pasteurella tularensis per ml were killed by exposure to 1 x 10^6 r of gamma radiation. The irradiated suspensions, initially containing about 10 LD_{50} per ml for mice by intraperitoneal injection, immunized mice against challenge with fully virulent strains of P. tularensis. Toxicity and immunizing activity of the suspensions decreased significantly within a few days at 5 C. Mice were protected against the toxin by immune serum or by prior injection of endotoxin of Escherichia coli. Cortisone did not protect against the newly prepared suspension, but was effective against aged suspension. Lethal doses of newly prepared suspension for guinea pigs and rabbits were approximately 0.5 ml and 2 ml, respectively; cortisone protected rabbits but not guinea pigs against lethal challenge. Pyrogenic effects resembling those shown by endotoxin-containing suspensions were demonstrated in rabbits. An interpretation of the results is proposed, postulating two toxins, one labile and associated with the immunizing activity of the suspension, the other more stable and resembling classical endotoxin.

14. Key Words

*Pasteurella tularensis
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Ionizing radiations
Endotoxins
Cortisone
Immunogenicity

DD FORM 1 NOV 65 1473 REPLACES DD FORM 1473, 1 JAN 64, WHICH IS OBSOLETE FOR ARMY USE.

Unclassified
Security Classification