NRDL-TR-68-146
13 December 1968

CELLULAR RESPONSE OF MICE TO INFECTION WITH PASTEURELLA TULARENSIS (LVS) FOLLOWING CONTINUOUS EXPOSURE TO LOW DOSE RATE γ RADIATION

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ADMINISTRATIVE INFORMATION

This work was done under the sponsorship of The Commission on Radiation and Infection of the Armed Forces Epidemiological Board, and was supported by the U. S. Army Research and Development Command, Department of the Army, under Research Contract No. 3A061102-B71-P-09, Basic Research in Support of Military Medicine-Radiobiology, and in conjunction with the Bureau of Medicine and Surgery, U. S. Navy Department under Work Unit MF12. 524.010-0005 as described in the Naval Radiological Defense Laboratory Annual Report to the Bureau of Medicine and Surgery (DD Form 1498) of 31 December 1967.

Research was conducted according to the principles enunciated in the "Guide for Laboratory Animal Facilities and Care", prepared by the National Academy of Sciences-National Research Council.

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ABSTRACT

Previous studies have shown that LAF_1 mice continuously exposed to radiation delivered from a Co^{60} source at 1.0-1.5 R/hr were more susceptible to subcutaneous and respiratory infections with the vaccine strain of Pasteurella tularensis, LVS, than were non-irradiated mice. Bacteriological studies indicated no significant difference in the growth or spread of the organisms in the two groups. Therefore, a histological study of lungs, liver, spleen and lymph nodes was undertaken to determine whether cellular injury in the irradiated animals might account for the increased susceptibility.

Lung washings suggested that a decrease in the number of phagocytic cells, both polymorphonuclear cells and macrophages, mobilized in the irradiated animals after infection might be one reason for the greater susceptibility. The phagocytes that were present, however, appeared to be more active as indicated by the higher percent of cells phagocytizing the organism and the greater number of bacteria ingested per cell. An alternative reason for the apparent increased activity could be that a decreased bactericidal capability of the cells permitted an increased intracellular bacterial proliferation.

Examination of sections of lung, spleen, liver and lymph nodes revealed only a more rapid appearance of lesions in the irradiated group compared to the non-irradiated mice. There was no obvious

difference, however, in the nature of the lesions.

In contrast to studies on the histology of tularemia in monkeys, in which histiocytes were the predominant phagocytic cell, the polymorphonuclear leukocyte appeared to be more in infected mice.

Immune irradiated mice responded with an increased histiocytic and plasma cell mobilization. Although some pathologic lesions were seen in these mice, they occurred to a lesser degree than in either irradiated or non-irradiated non-immune mice.

NON-TECHNICAL SUMMARY

The Problem

The growing tendency to use living vaccines for immunization of both military and civilian populations has posed the question of whether low dose rate radiation will enhance the susceptibility of individuals to infection following such immunization procedures. Previous studies have indicated that it does. The mechanism of the increased susceptibility, however, remains unclear. Therefore, the cellular response of irradiated mice to respiratory infection with the vaccine strain of Pasteurella tularensis, LVS, was compared with that of non-irradiated animals.

The Findings

A decrease in the number of phagocytic cells was noted in the lungs of irradiated mice after infection. However, more of the cells appeared to have ingested the bacteria. In addition, the average number of organisms ingested per cell appeared to be greater. This can be interpreted as evidence of greater activity of the surviving cells. Or, it might indicate increased intracellular growth of the bacteria resulting from the loss of bactericidal activity of the phagocytes because of irradiation injury. These alternative explanations are being investigated.

Examination of sections of lung, liver, spleen and lymph nodes for

differences in the cellular response showed only a more rapid appearance of lesions in the irradiated animal than in the non-irradiated mouse. No obvious difference in the type of lesions was observed.

The most predominant phagocytic cell appearing in lesions in the mouse were the polymorphonuclear cells, in contrast to the histiocytic response described in the lesions of infected monkeys. The difference in cellular response may be responsible for the greater susceptibility of mice to pulmonary tularemia following inhalation of the vaccine strain.

Immune irradiated mice responded with an increased histiocytic and plasma cell mobilization. Irradiated immune mice did develope some pathologic lesions, but to a lesser extent than did either irradiated or non-irradiated, non-immune animals.

ACKNOWLEDGEMENTS

The excellent preparation of the tissue sections by Miss Patricia Rowan, Pathology Service, is deeply appreciated. The centrifuge holders for the coverslip preparations were designed and made by Dr. P. H. Chin, Microbiology and Immunology Branch.

We are indebted to Col. Dan Crozier, M.C., US Army, Walter Reed Medical Unit and Dr. Henry T. Eigelsbach, Ft. Detrick Biological Laboratories, Frederick, Md., for generous gifts of lyophilized Pasteurella tularensis, LVS.

INTRODUCTION

A live tularemia vaccine, Pasteurella tularensis, LVS, has been found to be an effective immunizing agent in animals, including man, when administered by intradermal or subcutaneous injections, orally or as aerosol (Eigelsbach and Downs, 1961; Eigelsbach, et al, 1961; Hornick and Eigelsbach, 1966; Hornick, et al, 1966). However, recent studies have indicated that continuous exposure to Co^{60} γ radiation delivered at 1.0-1.5 R/hr increases the susceptibility of mice to infection with this strain of the organism, providing that the total accumulated dose is sufficiently high, 2000 R or greater (Hodge, et al, 1968). In addition, the immune response of mice was impaired when tested by respiratory challenge with the virulent SCHU S-4 strain, but not when challenged with LVS (Hodge, et al, 1968).

Bacteriological studies have indicated no significant difference in the growth and spread of the organism in the irradiated or non-irradiated animals infected by the respiratory route (Hodge, et al, 1968). P. tularensis was found in increasing numbers in the lungs, spleens, mediastinal and axillary lymph nodes of both groups. Only a small abortive proliferation of LVS occurred in the lungs of irradiated and non-irradiated mice immunized by subcutaneous injections of LVS.

The pathogenesis of the infection and histological changes in monkeys exposed to aerosols of P. tularensis, LVS, have been described

(White, et al, 1962; Eigelsbach, et al, 1962). However, since this strain of the organism has been found to be more virulent for mice than for guinea pigs, monkeys or man when administered as an aerosol, and since no apparent reasons for the increased susceptibility of irradiated mice were obtained by bacteriological studies, a comparison of the cellular changes in the lungs and the histology of the lungs, liver, spleen and lymph nodes was undertaken.

METHODS

MICE*

Male LAF_1, (C57L ♀ x A ♂)F_1 mice from our Laboratory colony were used in these experiments. The mice were 10-12 weeks old and the average weight was 25 grams at the time they were exposed to the Co^{60} source.

IRRADIATION

The animals were exposed continuously to γ radiation from a Co^{60} source at a dose rate of 1.1 R/hr until they had received a total dose of 2060 R. Plastic cages, housing 10 mice each, were placed on curved wooden racks so that the center of each cage was equidistant from the Co^{60} source. Doses were measured with thermoluminescent dosimeters (LiF), a Victoreen R-chamber, and Dupont films (Nos. 555 and 1290).

*In conducting the research described in this report, the investigators adhered to the "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

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The Co^{60} source was in continuous operation except for 30 minutes each week when the cages were changed. Fresh food pellets and water were also supplied at this time. No deaths occurred among the mice during the radiation exposure or among animals held as long as 60 days after removal from the Co^{60} source.

P. TULARENSIS CULTURES

A lyophilized live vaccine strain of P. tularensis, LVS, obtained from Fort Detrick Biological Laboratories, Frederick, Maryland, was suspended in a 0.1% gelatin-0.85% NaCl solution and cultured on Difco-Civil Defense Agar enriched with Difco-hemaglobin. After incubation at 37°C for 72-96 hrs, the organisms were washed from the agar plates, concentrated by centrifugation at 5000 RPM for 20 minutes and resuspended in gelatin-saline.

AEROSOL INFECTION

Within 1 hr after removal from the Co^{60} source, mice were infected by exposure to an aerosol of the organism generated by a modified Henderson apparatus described by Pribnow and Silverman (1963). P. tularensis was aerosolized in 0.1% gelatin-saline containing 0.1% antifoam (Dow Corning Antifoam B). The aerosol was sampled with AGI-30 glass impingers and the inhaled dose was calculated by Guyton's formula (Guyton, 1947). Animals were periodically sacrificed immediately after aerosol exposure to determine the number of inhaled organisms. The lungs were removed, homogenized and bacterial counts were made on Difco-

Civil Defense Agar enriched with Difco Hemaglobin. The inhaled dose of organisms determined by plate count and the dose calculated by Guyton's formula never varied by more than ± 10%. In these experiments the mice received 1.8-2.1 x 10^4 inhaled organisms.

IMMUNIZATION AND CHALLENGE

Mice were injected subcutaneously within 1 hr after removal from the source with 0.2 ml suspensions of the organism containing 1.1 x 10^2 P. tularensis, LVS. Immunity was tested by challenge 30 days after immunization with a respiratory infection of LVS administered as described above. The challenge dose was 2.1 x 10^4 inhaled organisms.

HISTOLOGICAL EXAMINATION

The cellular response in the lungs was determined by obtaining free alveolar cells using a modification of the technique described by Myrvik, et al (1961). Groups of 5 mice were sacrificed by an injection of nembutal at various times after irradiation and infection with 2.3 x 10^3 inhaled organisms, a lethal respiratory dose. The thoracic cavity was exposed and the upper portion of the trachea was clamped shut with a hemostat. One ml of TC 199 containing 0.5 units of heparin/ml was injected slowly into the lungs through the lower portion of the trachea, and allowed to remain in the lungs for approximately one minute, during which the needle and syringe were held in place. The TC 199 was then withdrawn by gently pulling on the syringe plunger. Usually 0.7-0.8 ml of the TC 199 was recovered. The cell suspension obtained was concentrated on a cover slip held in a specially designed

teflon centrifuge cup by centrifugation at 400 RPM/3 min.

After fixing the concentrated cells with 95% ethanol, the cells were stained either with Giemsa's stain for cytological identification, or with anti-P. tularensis serum conjugated with fluorescein isothiocyanate (Difco). The antiserum was diluted 1:40 in 0.85% saline. The cell preparations were incubated for 30 minutes and then washed. In order to eliminate non-specific staining, normal bovine serum conjugated with lissamine rhodamine (Difco) was used as a counterstain. This was found to have the added advantage of permitting differentiation of macrophages, polymorphonuclear cells and lymphocytes, since these cells showed a characteristic cytoplasmic staining reaction and the nuclear morphology was distinguishable.

After staining and washing, the coverslips were mounted on microscope slides for examination. The preparations stained with fluorescent antibody were examined with a Zeiss microscope using an ultraviolet light source and the appropriate filters in order to determine the presence and number of phagocytized P. tularensis.

In those instances when sufficient cell suspensions were obtained, total and differential cell counts were made using phase microscopy.

Additional groups of animals were used for histological studies of lungs, liver, spleen and lymph nodes. The tissues were fixed overnight in acetic acid-formaldehyde and were then transferred to 70% ethanol. Sections were stained with hemotoxylin and eosin, Giemsa's

and Grams's stains. Because of the small size of P. tularensis, Grams's stain usually was found to be unsatisfactory for identification and localization of the intracellular organisms.

RESULTS

A comparison of the lung washings from mice exposed to 2060 R with those from non-irradiated mice following respiratory infection with 2.1×10^3 P. tularensis, LVS, showed a slight decrease in the total number of cells obtained from the lungs of the irradiated group. The ratio of polymorphonuclear cells to macrophages, however, was similar. Polymorphonuclear cells were the predominant phagocytic cells present throughout most of the course of the infection. The data obtained in a typical experiment are presented in Table I. The percent of polymorphonuclear cells and macrophages that had phagocytized P. tularensis, LVS, and the average number of intracellular organisms detected by fluorescent microscopy in the 2 cell types are given in Table II.

Although the polymorphonuclear cells were more numerous, the macrophages were the more actively phagocytic cells. A greater percentage of these cells ingested the organisms, and the average number of organisms ingested was larger. Both types of cells appeared to be more active in the irradiated groups than in the non-irradiated as indicated by the somewhat larger percent of phagocytizing cells and the larger number of organisms ingested per cell. This was particularly true of the macrophages.

TABLE I

TOTAL CELL COUNT AND PERCENT PHAGOCYTIC CELLS IN LUNG WASHING OF IRRADIATED AND NON-IRRADIATED MICE WITH A RESPIRATORY INFECTION OF P. TULARENSIS, LVS.

	2060 R(1)			Non-Irradiated		
Day Post-Infection(2)	Total Cells x 10^4	% Neutrophils	% Macrophage	Total Cells x 10^4	% Neutrophils	% Macrophage
1	0.75	36.4	14.0	2.52	---	---
2	1.4	54.9	45.1	1.30	64.8	35.2
3	17.4	89.0	11.0	19.8	93.2	6.8
6	6.3	88.2	11.8	14.4	86.6	13.4
7	6.6	82.2	17.8	14.6	90.0	10.0
9	31.8	89.1	10.9	42.6	92.5	7.5

(1) Radiation dose rate: 1.1 R/hour.
(2) Infecting dose: 2.1 x 10^3 P. tularensis (LVS) inhaled.

TABLE II

PERCENTAGE OF PHAGOCYTIZING CELLS IN LUNG WASHINGS OF IRRADIATED AND NON-IRRADIATED MICE AND THE AVERAGE NUMBER OF ORGANISMS PER CELL FOLLOWING RESPIRATORY INFECTION WITH P. TULARENSIS.

Day Post Infection	2060 R				Non-Irradiated			
	% Phagocytizing Neutrophils	No. of Organisms Per Cell	% Phagocytizing Macrophages	No. of Organisms Per Cell	% Phagocytizing Neutrophils	No. of Organisms Per Cell	% Phagocytizing Macrophages	No. of Organisms Per Cell
1	0	0	0	0	---	---	---	---
2	3.6	2.0	34.1	6.8	0.6	0.8	21.7	4.2
3	18.3	1.6	61.2	3.8	13.8	1.3	28.5	5.0
6	34.6	2.8	73.6	6.4	12.9	1.8	40.0	3.2
7	17.1	1.8	46.0	6.9	6.5	1.4	20.5	4.2
9	5.6	1.6	72.7	2.7	5.5	1.9	7.5	0.7

The lung washings from the irradiated group contained more cellular debris, indicating a greater degree of necrosis. In addition, the number of extracellular organisms in this group appeared to be larger. Although no attempt was made to determine the number of extracellular P. tularensis, the impression was obtained that a greater proliferation of the organisms had occurred in the irradiated group than in non-irradiated mice.

Histological examination of sections of the lungs, liver, spleen, mediastinal and axillary lymph nodes obtained at 2, 4, and 8 days after respiratory infection were made to compare the pathological changes and the pathogenesis of infection in irradiated and non-irradiated, immune and non-immune animals.

The lungs of the non-irradiated, non-immune mice infected with 2.1×10^4 inhaled P. tularensis, LVS, were congested and edematous on the 2nd post-infection day. There were some foci of necrosis infiltrated by polymorphonuclear cells. A moderate perivascular and peribronchial infiltration consisting primarily of lymphocytes and histiocytes with a few plasma cells was also observed. The lungs of the irradiated group of non-immune animals at this time showed primarily a decrease in alveolar macrophages. No lesions were noted in the liver, spleen or lymph nodes of either group. However, a marked reticuloendothelial hyperplasia was observed in the spleens and lymph nodes.

On the 4th post-infection day, the congestion and edema of the lungs of the non-irradiated group had subsided. No foci of necrosis were

observed, and the interstitial infiltrate consisting of lymphocytes, histiocytes and a few neutrophils had decreased. The lungs of the irradiated group, in contrast, showed foci of reaction with consolidation characterized by necrosis and infiltrating polymorphonuclear cells and plasma cells. Some alveolar septa were thickened by infiltration of histocytes and polymorphonuclear cells. Gram's stain showed the presence of Gram negative organisms in the histiocytes. The livers of the non-irradiated group showed only a few areas having an accumulation of inflammatory cells. Those from the irradiated mice, however, had small, randomly located foci of necrosis containing neutrophils and histiocytes. The Kupffer cells appeared to be hypertrophied. The spleens of the non-irradiated mice showed no abnormalities, whereas those of the irradiated group had small necrotic foci in the red pulp extending into the follicles. Polymorphonuclear cells and histiocytes in approximately equal numbers were observed. Many of the former type of cells were necrotic. In addition, there was some pyknotic nuclear debris. The lymph nodes of the non-irradiated mice showed only a marked reticuloendothelial hyperplasia. There was an increase in the number of lymphocytes in the nodes of the non-irradiated infected animals compared with those of the irradiated mice. The nodes from the latter group appeared to be edematous. In addition, the number of intravascular polymorphonuclear cells was increased and some necrosis was observed. The necrotic areas contained large numbers of polymorphonuclear cells, fibroblasts and histiocytes. The lymph channels also were lined with polymorpho-

nuclear cells. Germinal centers were not well formed.

By the 8th day, the lungs of both groups were congested, edematous and showed necrotic foci. The cellular infiltrate of the non-irradiated animals consisted primarily of polymorphonuclear cells, whereas in the irradiated group it appeared to be mainly histiocytes with a less-marked polymorphonuclear response. Hemorrhage was pronounced in this group. A dense interstitial infiltrate characterized by lymphocytes, histiocytes and plasma cells could be seen in the lungs of the non-irradiated group. The livers of both groups had numerous foci of necrosis. The cellular response in the non-irradiated animals consisted of lymphocytes, histiocytes, reticuloendothelial cells and polymorphonuclear cells. In the irradiated mice the response was predominantly histiocytic. The Kupffer cells appeared to be swollen. Focal granulomatous lesions were present in the splenic red pulp of the non-irradiated animals. These contained histiocytes together with a few lymphocytes. The architecture of the spleens of the irradiated mice was almost completely disrupted. The follicles were sparse and small. The red pulp contained numerous degenerating cells. Mononuclear cells were present which were either reticuloendothelial cells, extramedullary hematopoietic cells or histiocytes. The lymph nodes again were characterized by reticuloendothelial hyperplasia. The normal architecture of the nodes of the irradiated animals was markedly altered due to degeneration.

Both the immunized groups of irradiated and non-irradiated mice showed less extensive histological changes after respiratory challenge

with P. tularensis, LVS. On the second post-infection day the lungs of animals in both groups were slightly edematous. A moderate perivascular infiltrate was present consisting of histiocytes, lymphocytes and plasma cells. Only a few polymorphonuclear cells were seen. A similar infiltration was noted in the alveolar septa. The major difference between the irradiated and non-irradiated immunized animals was the presence of a few areas of consolidation and necrosis in the lungs of the former group. The livers of both groups appeared to be essentially normal, although the irradiated immune animals had some small areas of focal necrosis and some extramedullary hematopoiesis. The spleens were moderately congested. The follicular structure of the spleens from irradiated animals was disrupted due to the loss of lymphocytes. Extramedullary hematopoiesis was prominent. The outer cortex of the lymph nodes of the non-irradiated immune animals were well populated with small lymphocytes in contrast to that of the irradiated immunized mice, which had only a thin layer. The former had distinct germinal centers with a few plasma cells. No true germinal centers could be found in the latter. The mid-cortex of the lymph nodes from non-irradiated animals had occasional aggregates of histiocytes in association with karyorrhectic cells and a few mast cells, while those of the irradiated immune group contained large and small lymphocytes as well as histiocytes. Mature polymorphonuclear cells were present in the medulla of this group extending along the sinuses into the cortex.

By the 8th post-infection day the edema of the lungs had subsided and the cellular response in both the non-irradiated and irradiated animals had diminished. It was characterized by an increase in plasma cells. The irradiated immune group showed some necrotic foci with karyorrhexis and polymorphonuclear infiltration. The livers were similar to those described on the second post-infection day. An increase in the splenic red pulp had occurred in both groups at this time. This was due to a massive increase in hematopoietic cells: megakaryocytes, erythroid and myeloid cells, together with a reticuloendothelial hyperplasia. The lymph nodes of both immune groups contained large numbers of plasma cells in the inner cortex and medulla. There were only a few germinal centers in the nodes from irradiated animals, however.

DISCUSSION

The cellular response of the non-irradiated, non-immune mice reported here differed from that described by White, et al (1962) for Macaca irus exposed to an aerosol of P. tularensis, LVS. The predominant phagocytic cell found throughout the course of the infection in the mouse appeared to be the polymorphonuclear leukocyte. In the monkey, however, monocytes and macrophages were found to be the primary cell, although polymorphonuclear cells were also seen. The histological changes in the liver and spleen were minimal in the monkey, whereas in the mouse they were more extensive.

The greater and more extensive histological changes found in the mouse following exposure to aerosols of P. tularensis is reflected in the greater susceptibility of this species to pulmonary infection compared to man, monkey and guinea pig. Although LVS has a decreased virulence, it is not completely avirulent. The respiratory LD_{50} for LAF_1 mice has been found to be 1.5×10^3 inhaled organisms, a relatively low dose compared to that given to man and monkey (Hodge, et al, 1968). Eigelsbach, et al (1967), for instance, have reported that respiratory doses of 10^6 - 10^8 organisms were well tolerated by human volunteers with only minor symptoms resulting from the infection.

The prevalence of extracellular organisms and of polymorphonuclear cells in the lung washings throughout the course of the infection together with apparent destruction of macrophages, during the latter phases, suggested that the organisms were proliferating intracellularly in the macrophage. This was further suggested by the arrangement of some of the extracellular organisms. These had the appearance, shape and size of the cytoplasm of the macrophage, although no cellular material was observed. This indicated, perhaps, that the ingesting macrophage itself had been destroyed by the proliferating intracellular P. tularensis. Nutter and Myrvik (1966) have reported a toxic effect of P. tularensis, LVS, on alveolar macrophages from non-irradiated rabbits in vitro following intracellular growth.

The inability of alveolar macrophages obtained from irradiated mice to kill ingested P. tularensis may explain the apparent greater

phagocytic activity of these cells compared to those obtained from non-irradiated animals. The greater percent of macrophages which had phagocytized the organism, and the larger number of intracellular organisms may have resulted from intracellular growth of the bacteria rather than increased phagocytosis. Donaldson, et al (1956) and Nelson and Becker (1959) have shown that macrophages from acutely irradiated animals can not suppress intracellular growth or digest ingested foreign cells. The same may be true of cells from animals exposed continuously to low dose rate γ radiation. It has been reported previously that peritoneal macrophages from irradiated animals appeared to be more sensitive to bacterial endotoxins and/or bacterial metabolic products (Silverman, 1967).

The major histological differences between the irradiated and non-irradiated mice in these studies were quantitative rather than qualitative, i.e., an earlier appearance of lesions and, perhaps, more extensive pathological changes. The earlier appearance of lesions, again, may reflect the inability of the phagocytic cells to kill and digest the phagocytized bacteria.

Immunity against P. tularensis is considered to be due primarily to heightened activity of the macrophages rather than to humoral antibodies. Nutter and Myrvik (1966) have reported that immune rabbit alveolar macrophages in the presence of normal rabbit serum were as effective in suppressing intracellular growth as those suspended in immune serum.

Cells from non-immune rabbits together with immune serum did limit the growth of LVS, but to a lesser extent than the immune macrophages. In addition, they also found that rabbits immunized with LVS developed agglutinins against P. tularensis, but were not appreciably more resistant to challenge than were non-vaccinated animals. Our data would also indicate that a cellular immunity is of greater importance. Mice immunized subcutaneously with LVS developed only a low agglutination titer, 1:16 (Hodge, unpublished data). However, they were able to survive a respiratory challenge with the attenuated LVS strain, but not the virulent SCHU-S-5 Strain (Hodge, et al, 1968). Histological examination showed a less intense cellular destruction than that observed in the non-immune animals. A greater monocytic response in the immune group also indicated a heightened activity on the part of these cells.

The reasons for the greater susceptibility of the irradiated animals to the vaccine strain of P. tularensis still remain unclear. The major apparent differences between the low dose rate irradiated mice and non-irradiated mice are a smaller number of total cells in the lung washings of the former group and the more rapid development of lesions in the lungs, liver, spleen and lymph nodes. Although both non-irradiated and irradiated animals immunized with LVS can survive a respiratory challenge with the same strain of organism, the latter group did develope more extensive lung lesions. These, however, were much less severe than in either of the non-immune groups.

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UNCLASSIFIED
Security Classification

DOCUMENT CONTROL DATA - R & D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY *(Corporate author)*	2a. REPORT SECURITY CLASSIFICATION: UNCLASSIFIED 2b. GROUP
3. REPORT TITLE	
4. DESCRIPTIVE NOTES *(Type of report and inclusive dates)*	
5. AUTHOR(S) *(First name, middle initial, last name)* Myron S. Silverman, K. Hadley Vivian Greenman, Frederick A. Hodge Adam E. McKee, Colin G. Burriss	
6. REPORT DATE	7a. TOTAL NO. OF PAGES / 7b. NO. OF REFS
8a. CONTRACT OR GRANT NO. b. PROJECT NO. c. d.	9a. ORIGINATOR'S REPORT NUMBER(S): NRDL-TR-68-146 9b. OTHER REPORT NO(S) *(Any other numbers that may be assigned this report)*
10. DISTRIBUTION STATEMENT	
11. SUPPLEMENTARY NOTES	12. SPONSORING MILITARY ACTIVITY

13. ABSTRACT

(Abstract continued from another page)

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DD FORM 1 NOV 65 1473 (PAGE 1)
S/N 0101-807-6801

UNCLASSIFIED
Security Classification

UNCLASSIFIED

Security Classification

DOCUMENT CONTROL DATA - R & D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY *(Corporate author)*

Naval Radiological Defense Laboratory
San Francisco, California 94135

2a. REPORT SECURITY CLASSIFICATION

UNCLASSIFIED

2b. GROUP

3. REPORT TITLE

CELLULAR RESPONSE OF MICE TO INFECTION WITH PASTEURELLA TULARENSIS (LVS) FOLLOWING CONTINUOUS EXPOSURE TO LOW DOSE RATE γ RADIATION

4. DESCRIPTIVE NOTES *(Type of report and inclusive dates)*

5. AUTHOR(S) *(First name, middle initial, last name)*

Myron S. Silverman, K. Hadley
Vivian Greenman, Frederick A. Hodge
Adam E. McKee, Colin G. Burriss

6. REPORT DATE	7a. TOTAL NO. OF PAGES	7b. NO. OF REFS
(Distribution) 4 February 1969	30	25

8a. CONTRACT OR GRANT NO.	9a. ORIGINATOR'S REPORT NUMBER(S)
ARMY Contract No. 3A061102-B71-P-09.	NRDL-TR-68-146
b. PROJECT NO. BUMED Work Unit MF12.524.010-0005.	
c.	9b. OTHER REPORT NO(S) *(Any other numbers that may be assigned this report)*
d.	

10. DISTRIBUTION STATEMENT

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11. SUPPLEMENTARY NOTES	12. SPONSORING MILITARY ACTIVITY
	U. S. Army Research & Development Command, Washington, D. C. 20301 and Bureau of Medicine and Surgery, Washington, D. C. 20390.

13. ABSTRACT

Previous studies have shown that LAF_1 mice continuously exposed to radiation delivered from a Co^{60} source at 1.0-1.5 R/hr were more susceptible to subcutaneous and respiratory infections with the vaccine strain of Pasteurella tularensis, LVS, than were non-irradiated mice. Bacteriological studies indicated no significant difference in the growth or spread of the organisms in the two groups. Therefore, a histological study of lungs, liver, spleen and lymph nodes was undertaken to determine whether cellular injury in the irradiated animals might account for the increased susceptibility.

Lung washings suggested that a decrease in the number of phagocytic cells, both polymorphonuclear cells and macrophages, mobilized in the irradiated animals after infection might be one reason for the greater susceptibility. The phagocytes that were present, however, appeared to be more active as indicated by the higher percent of cells phagocytizing the organism and the greater number of bacteria ingested per cell. An alternative reason for the apparent increased activity could be that a decreased bactericidal capability of the cells permitted an increased intracellular bacterial proliferation.

Examination of sections of lung, spleen, liver and lymph nodes revealed only a more rapid appearance of lesions in the irradiated group compared to the non-irradiated mice. There was no obvious difference, however, in the nature of the lesions.

In contrast to studies on the histology of tularemia in monkeys, in which histiocytes were the predominant phagocytic cell, the polymorphonuclear leukocyte appeared to be more in infected mice.

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14 KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
Chronic irradiation						
Cellular immunity						
Respiratory infection						
Pathology						
Macrophage						