AN ABSTRACT OF THE THESIS OF

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Title: Relationship Between Macrophage Oxidative Metabolism and Antitularemia Activity Influenced by Inflammatory Agents

Abstract Approved: Helen McGhee Ph.D

A comparative study on the effects of inflammatory agents on oxidative metabolism as it relates to bactericidal function of murine peritoneal macrophages was conducted. Four groups of mice were used for the study. Three groups were injected with different inflammatory agents, peptone (PEP), thioglycollate (TG), and glycogen (GLY), 72 h prior to harvesting of macrophages from the peritoneal cavity. The fourth group served as the control group and provided resident macrophages. Macrophages were examined for *in vitro* intracellular killing of *Francisella tularensis*, phagocytic index, oxidative metabolism, and nitroblue tetrazolium (NBT) reduction. A relationship between oxidative metabolism and degree of resistance by macrophages was exhibited. PEP-elicited macrophages had the greatest increase in resistance to *F. tularensis*, greatest increase in superoxide production, and the most macrophages able to reduce NBT when compared to resident macrophages. TG-elicited macrophages had a significant increase in the susceptibility to *F. tularensis*, smallest increase in superoxide production and the fewest macrophages able to reduce NBT when compared to resident macrophages. GLY-elicited macrophages did not have a significant difference in the intracellular killing of *F. tularensis* from resident macrophages. Elicited macrophages had a significant increase in the uptake of *F. tularensis* when compared to the resident macrophages. Preliminary evidence indicates that *F. tularensis* is able to circumvent oxidative metabolism of murine macrophages.

RELATIONSHIP BETWEEN MACROPHAGE OXIDATIVE METABOLISM AND ANTITULAREMIA ACTIVITY INFLUENCED BY INFLAMMATORY AGENTS

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INTRODUCTION

Although phagocytosis and killing by macrophages is a first line of defense against many microorganisms, a number of microbial species survive and grow within macrophages. These intracellular pathogens include *Mycobacterium* (Draper and D'Arcy Hart 1975), *Listeria* (Cohn 1978), *Toxoplasma* (Jones and Hirsch 1972), *Francisella* (Stafanye *et al.* 1961), and *Trypanosoma* (Nogueria and Cohn 1978). The ability of macrophages to kill these organisms is primarily dependent upon the oxidative activity that results in reactive oxygen intermediates (ROI) during phagocytosis of the organism. The introduction of immunological stimuli, such as bacterial antigens, causes an increase in ROI (Badwey *et al.* 1983), which results in an increase in microbicidal activity (Murray 1984). The macrophages that are drawn into the area of the immunological stimulus are considered *activated* (Hahn and Kaufmann 1981). However, nonimmunological stimuli (inflammatory agents) such as peptone (PEP) and thioglycollate (TG) can also draw macrophages into the area (Cohn 1978). These macrophages are termed *elicited* because they are not a result of an immune response (Cohn 1978). The purpose of this investigation is to explore various inflammatory agents and the effects of these agents on the microbicidal function of murine peritoneal macrophages.

According to Nelson (1979) macrophages develop in the bone marrow as monoblasts and then mature into promonocytes. The promonocytes then travel to the blood where they are known as monocytes. These "immature macrophages" remain in the blood for a few hours then migrate into the tissues where they undergo additional differentiation and maturation to become mature resident macrophages. The mature macrophages are scattered throughout the host and include such populations as alveolar macrophages of the lung, Kupffer cells of the liver, and freely migrating macrophages of the peritoneal cavity (Cline *et al.* 1978).

Once these macrophages become established in the tissues, they are subjected to a barrage of signals for both stimulation and suppression. During instances of altered homeostasis, such as inflammation, their morphology, metabolism, and physiology change to an *activated* state in order to perform their numerous functions (Hahn and Kaufmann 1981). However, Mackaness (1960), and Jenkin and Benacerraf (1960) observed that some macrophages within a given population were less able to ingest and kill bacteria than others. This observation lead McIntyre *et al.* (1967) to observe the heterogenity of function at the single cell level, where some macrophages were shown to phagocytize bacteria in the presence of specific antibody and others did not. For these reasons, Hahn and Kaufmann (1981) state that the activation of a population of macrophages is not an all-or-none effect, but is an increase of physiological activities of a population of macrophages above the activities exhibited by resident macrophages.

The increase in macrophage antimicrobial activity was first described by Elie Metchnikoff (1905) when he observed that mononuclear phagocytes from animals resistant to certain infections had an increased capacity for ingesting and killing microbes. Lurie (1942) observed that macrophages from tuberculosis-vaccinated animals displayed an enhanced capacity for inhibiting the intracellular growth of the tubercle bacilli when cultured in the anterior chamber of the rabbit eye. It wasn't until George Mackaness (1964) elucidated the basis of cellular immunity to facultative and obligate intracellular pathogens, that macrophage activation was described. Mackaness observed mice that had acquired an immunity to the following intracellular pathogenic organisms: *L. monocytogenes*, *Brucella abortus*, and *M. tuberculosis*. The macrophages from the immune animals had an enhanced bactericidal activity towards pathogens other than the organism responsible for the immunization. He termed macrophages with such a nonspecific increase in bacteri-

cidal activity *activated*. Armstrong and Sword (1964) also demonstrated that the increased antimicrobial activity of macrophages activated by Listeriosis was nonspecific in that it was expressed against antigenically unrelated bacteria. The nonspecific increase in antimicrobial activity of macrophages was later shown to be associated with T-lymphocytes *in vivo* (Lane and Unanue 1972). This prompted Simon and Sheagren (1972) to demonstrate the enhanced bactericidal activity of macrophages when sensitized T-lymphocytes are incubated *in vitro* with normal macrophages and specific antigen. It was later demonstrated that the specifically sensitized T-lymphocytes released lymphokines that stimulated macrophages to produce the nonspecific microbicidal activity (Schmidt and Douglas 1977).

Other studies have shown that activated macrophages differ in several ways from non-activated resting macrophages. Characteristics of activated macrophages include an increase in size (Mackaness 1970), an increase in the content of hydrolytic enzymes (Saito and Suter 1965), and faster spreading on glass surfaces (Mackaness 1970). Also, they contain more mitochondria, lysosomes, and pinocytic vacuoles (North 1978).

North (1970) observed that the phagocytic capacity of macrophages increased with their activation. Along with this enhanced phagocytic activity there is an increase in O_2 consumption during the phagocytic process (Balbridge and Gerard 1933). This increase in O_2 consumption leads to another important characteristic of activated macrophages, and that is the respiratory burst (Babior *et al.* 1973). Edelson and Cohn (1976) and Unkeless *et al.* (1981) observed that activated macrophages respond with an increase in surface receptors (Fc receptor for IgG and the complement factor C3b) that are responsible for phagocytizing the opsonized antigen. Recently, phosphorylation of the Fc receptor for IgG has been shown to be necessary in the triggering of the intracellular events that lead to the respiratory

burst (Brozna *et al.* 1988). The respiratory burst results in the production of ROI beginning with the one electron reduction of the consumed O_2 (Babior *et al.* 1973). Rossi and Zatti (1964) first proposed that the electron donated to O_2 was provided by NADPH through the action of a membrane-bound enzyme, NADPH oxidase, in the reaction:

$$NADPH + 2O_2 + H^+ \text{---> } NADP^+ + 2O_2^- + H^+$$

The increased $NADP^+$ stimulates the oxidation of glucose through the hexose monophosphate shunt (HMPS) pathway which, in turn, converts $NADP^+$ back to NADPH (McCall *et al.* 1979). The superoxide anion (O_2^-) formed gives rise to other ROI products, hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radical ($OH\cdot$) (Berton *et al.* 1988). Hydrogen peroxide is formed spontaneously or by the dismutation of O_2^- by the enzyme superoxide dismutase (SOD) in the following reaction:

$$2O_2^- + 2H^+ \text{---> } H_2O_2 + O_2$$

The formation of the $OH\cdot$ radical occurs in an iron-catalyzed Haber-Weiss reaction, where O_2^- reduces H_2O_2 (Borregard 1988).

$$O_2^- + Fe^{3+} \text{---> } Fe^{2+} + O_2$$
$$Fe^{2+} + H_2O_2 \text{---> } Fe^{3+} + OH\cdot + OH^-$$

Recently, the role of ROI in macrophage-mediated intracellular killing has received considerable attention. It is evident that the formation of ROI contri-

butes to the antimicrobial activity of macrophages. For instance, peripheral blood monocytes from patients with chronic granulomatous disease (CGD) express a markedly decreased antimicrobial activity (Bridges *et al.* 1959). In this disease, characterized by recurrent intracellular infections, monocytes have a defective respiratory burst (Tauber *et al.* 1983). Patients with CGD lack the NADPH oxidase enzyme that catalyzes the reduction of 0_2 (Hohn and Lehrer 1975). Johnston *et al.* (1975) and Sagone *et al.* (1976) showed the involvement of ROI in bactericidal acitivity by demonstrating that the bactericidal activity decreased when the O_2^- was removed by SOD, the H_2O_2 by catalase, and the $OH^\cdot$ by other radical scavengers. Docampo and Moreno (1984) also demonstrated that H_2O_2 produced by the macrophages is toxic to *Trypanosoma cruzi* epimastigotes.

Recently, questions have arisen about macrophage activation when correlating it with microbicidal activity and the production of ROI. When macrophages are drawn to the peritoneal cavity following an injection of a sterile solution of such substances as caseinate, glycogen (GLY), PEP, and TG, they exhibit some of the same attributes as immunologically activated cells (Adams and Hamilton 1984). However, these *elicited* or *inflammatory* macrophages, as described by Cohn (1978), can also show characteristics that are very different from immunologically activated macrophages. For instance, Godfrey and Wilder (1984) observed that activated macrophages from *L. monocytogenes* injected mice produced significantly more 0_2^- and H_20_2 than did resident and TG-elicited macrophages.

Not only are there differences between activated and elicited macrophages, there are differences between macrophages which have been elicited with various substances. Leijh *et al.* (1984) observed that TG-elicited macrophages had a decreased antimicrobial activity towards *Staphylococcus epidermidis* compared to resident macrophages, whereas McElree and Downs (1961) observed that PEP-elicit-

ed macrophages manifested an enhanced bactericidal activity towards *Francisella tularensis*. Badwey *et al.* (1983) observed an increase in the amount of O_2^- released from macrophages elicited with PEP and caseinate compared to resident macrophages or macrophages elicited with TG.

The present investigation determined the differences among macrophages elicited by various inflammatory agents and assayed the effects of those agents on the macrophage's intracellular killing function against the facultative intracellular bacterial pathogen, *F. tularensis*. This investigation also provides preliminary evidence that *F. tularensis* evades the respiratory burst in normal macrophages.

MATERIALS AND METHOD

Animals: Adult white albino mice, bred in the Emporia State University animal room or at the Veterinary Diagnostics Laboratory in the College of Veterinary Medicine at Kansas State University, were used throughout the study. White albino rabbits housed in the Emporia State University animal room were used for the production of *F. tularensis* antiserum.

Elicitation of Peritoneal Exudate Cells: Four groups of mice, each group containing five animals, were used in the study. One group of mice was not injected prior to harvesting of the cells. This group provided the normal resident macrophages, which served as the control cell system. The other 3 groups were injected intraperitoneally (i.p.) with 1.5 ml of a sterile solution containing 0.05% sodium TG, 1% GLY, or 5% PEP for the induction of an inflammatory response. These reagents were obtained from Difco Laboratories (Detroit, MI.). Peritoneal exudate cells (PEC) were harvested 72 h after injection as described below. Normal resident PEC were harvested from the control group at the same time.

Harvesting of Peritoneal Exudate Cells: PEC were harvested as described by McElree and Downs (1961) with a few modifications. Mice were sacrificed by cervical dislocation and the skin over the abdomen was pulled back to expose the peritoneal wall. Three to 5 ml of a solution containing Eagle's Minimal Essential Media (MEM) (Sigma, St. Louis, MO.) supplemented with 200U/ml of penicillin G and 100U/ml of heparin was used as a harvesting medium and injected into the cavity. For measuring the O_2^- production and nitroblue tetrazolium (NBT) reduction, phenol red-free Hank's balanced salt solution (HBSS) was substituted for MEM. Following the injections, the peritoneal walls were massaged and the exudate was collected by cutting the peritoneal wall and removing the fluid with Pasteur pipets. The harvested cells were counted using a hemocytometer and ad-

justed to 3 x 10^6 cells/ml with MEM or HBSS depending on the assay. Cell counts from each system were recorded as per ml of total pooled exudate.

Intracellular Killing of Microorganisms: A portion of the PEC was dispensed in 1 ml aliquots into flying coverslip tubes and used as uninfected controls. The remaining PEC were infected with 10^4 bacteria/ml of an attenuated live vaccine strain of the facultative intracellular organism *F. tularensis* to obtain a final bacteria to macrophage ratio of 1:300.

Cultures were incubated for 2 h at 37° C to allow for complete phagocytosis. Following the incubation period, the coverslips were washed twice with MEM to remove nonadherent cells and nonphagocytized bacteria. Adherent cells were judged to be macrophages by microscopic examination. A complete culture medium (harvesting medium minus heparin with the addition of 10% fetal calf serum) was then added in 1 ml aliquots to the coverslips. At this time (0 h), and at 24 and 48 h, post-infection, 2 experimental tubes were sonicated for 3 min at a setting of 4 on a Heat Systems Ultrasonics sonicator to disrupt the macrophage membranes and release the bacteria. Ten-fold serial dilutions of the sonicate from each tube were plated on Cystine-heart agar (Difco) supplemented with 2% hemoglobin (Difco) and incubated at 37° C for 48-72 h and the resulting colonies counted. Averages of the 2 tubes were recorded for the number of viable bacteria. As a control, *F. tularensis* was incubated in the culture medium without macrophages and plated at the designated times to determine the growth in the culture medium alone. At each time, an uninfected control and an experimental coverslip were stained using the May-Grunwald-Giemsa method to determine the condition of the cells.

Measurement of Phagocytosis: The phagocytic index was measured using indirect immunofluorescence to intracellular *F. tularensis*. Antiserum was produced by immunization of rabbits to formalin-inactivated *F. tularensis* as described by Bur-

rell and Lewis (1987). Macrophages were harvested as described above and infected at a ratio of 8:1 (bacteria to macrophage) and incubated in a Lab-Tek 8-chamber culture slide for 2 h at 37° C. Macrophages were then thoroughly washed in PBS and fixed in acetone for 5 min and air dried. Macrophages were then incubated with the *F. tularensis* antiserum for 30 min at 37° C followed by washing with PBS and incubation with anti-rabbit IgG fluorescent conjugate for 30 min at 37° C. Macrophages were washed in PBS again and counterstained with Evans Blue. Percentages of positive cells were calculated by counting the number of phagocytes per field containing ingested organisms.

Measurement of O_2^- Production: O_2^- radical generation was determined by the SOD inhibitable reduction of ferricytochrome C in response to phorbol-myristate-13-acetate (PMA) (Sigma) as described by Fleming *et al.* (1990) with a few modifications. The cells were elicited and harvested as described. Eight-hundred microliters of the cell suspension was placed in a 2 ml microcentrifuge tube. The cells were mixed with 100 µl of 80 µM ferricytochrome C, 100 µl (200U) SOD and/or 10 µl of 100 ng/ml PMA. The mixtures were incubated for 15 min at 37° C and the 0_2^- was quantified by determining the amount of cytochrome C at 550 nm on a Gilson 240 spectrophotometer in supernatants centrifuged at 11,000 rpm for 30 sec. An absorbance coefficient of 21.1 mM^{-1} cm^{-1} was used for the calculation of 0_2^-.

Measurement of NBT Reduction: The qualitative reduction of the redox dye NBT was measured in response to PMA as described by Meuwissen *et al.* (1982) with a few modifications. After harvesting, macrophages were dispensed in 0.4 ml aliquots in a Lab-Tek 8-chamber culture slide and incubated for 2 h at 37° C to allow the macrophages to adhere to the slide. The cells were washed with HBSS to remove any nonadherant cells. The cells were incubated for 30 min at 37° C in HBSS containing 0.1 mg/ml NBT and 10 µl/ml of 100 ng/ml PMA. The cells were

washed in HBSS, fixed in acetone for 5 min, washed, and counterstained with safranin for 1 min. Cells were scored positive if they contained blue-black formazan deposits.

Statistical Analysis: All values represent the mean and the standard deviation from at least three trials in each experiment. The means of the elicited macrophages were compared to the mean of the normal resident macrophages by Student's *t* test. Other statistical tests performed are indicated where they were employed.

RESULTS

Effect of inflammatory agents on the number of PEC harvested from the peritoneal cavity. Intraperitoneal injection of three inflammatory agents (PEP, TG, GLY) led to significant increases in the cells harvested from the peritoneal cavity (Fig. 1). PEP caused the greatest increase with 6.59 x 10^6 cells/ml of exudate as compared to 4.48 x 10^6 cells/ml for the normal resident cells. TG-elicited cell counts were essentially the same as the PEP-elicited cells with 6.54 x 10^6 cells/ml. Counts for GLY-elicited cells were slightly lower at 6.06 x 10^6 cells/ml.

Effect of inflammatory agents on the intracellular killing capacity of macrophages. Macrophages were tested for the intracellular killing of *F. tularensis*. Figure 2 represents uninfected resident macrophages as they appear by the May-Grunwald-Giemsa staining method. Figure 3 represents the intracellular growth of *F. tularensis* in TG-elicited macrophages at 48 h. A difference was found in the intracellular growth of the bacteria over a 48 h period (Fig. 4). At 48 h, the numbers of *F. tularensis* within the culture medium without macrophages declined from 1.0 x 10^4 to 5.10 x 10^3. PEP-elicited macrophages incubated with *F. tularensis* had a significant decrease in the number of viable bacteria, 4.08 x 10^3 bacteria/ml, relative to the normal resident macrophages, which had 1.47 x 10^6 bacteria/ml at 48 h. However, TG-elicited macrophages incubated with *F. tularensis* had a significant increase in the number of viable bacteria, 5.63 x 10^7 bacteria/ml, relative to the normal resident macrophages. This would indicate that TG-elicited macrophages were different from PEP-elicited macrophages. Only the GLY-elicited macrophages incubated with *F. tularensis* did not have a significant difference in the number of viable bacteria, 2.58 x 10^6, from that of the normal resident macrophages at 48 h.

Effect of inflammatory agents on the phagocytic capacity of macrophages. Macro-

Figure 1. Number of peritoneal exudate cells harvested from the peritoneal cavity of mice. Resident (RES) cells are from mice not injected prior to harvest. Peptone-elicited (PEP), thioglycollate-elicited (TG), and glycogen-elicited (GLY) are cells from mice injected with the inflammatory agents. Results are the mean of 8 experiments $\pm$ SD. Elicited macrophages were significant ($p<0.05$) relative to RES control by Student's *t* test and a single factor Dunnett's test.

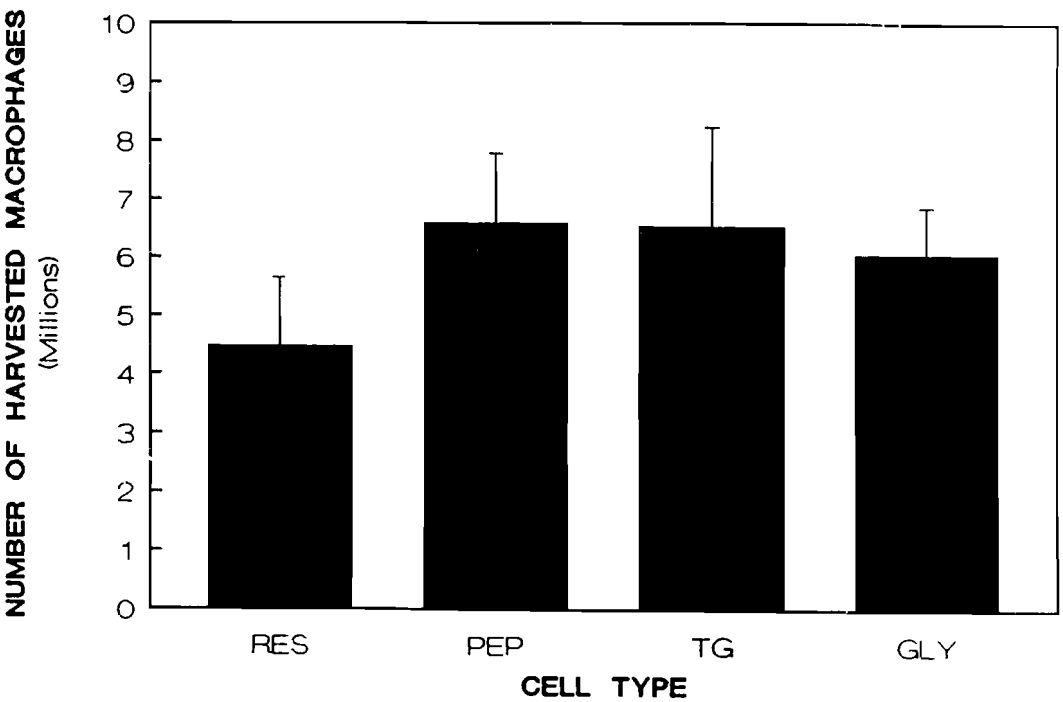

NUMBER OF HARVESTED MACROPHAGES
(Millions)
10
9
8
7
6
5
4
3
2
1
0
RES
PEP
TG
GLY
CELL TYPE

Figure 2. Normal resident macrophages as they appear by the May-Grunwald-Giemsa staining method (400x).

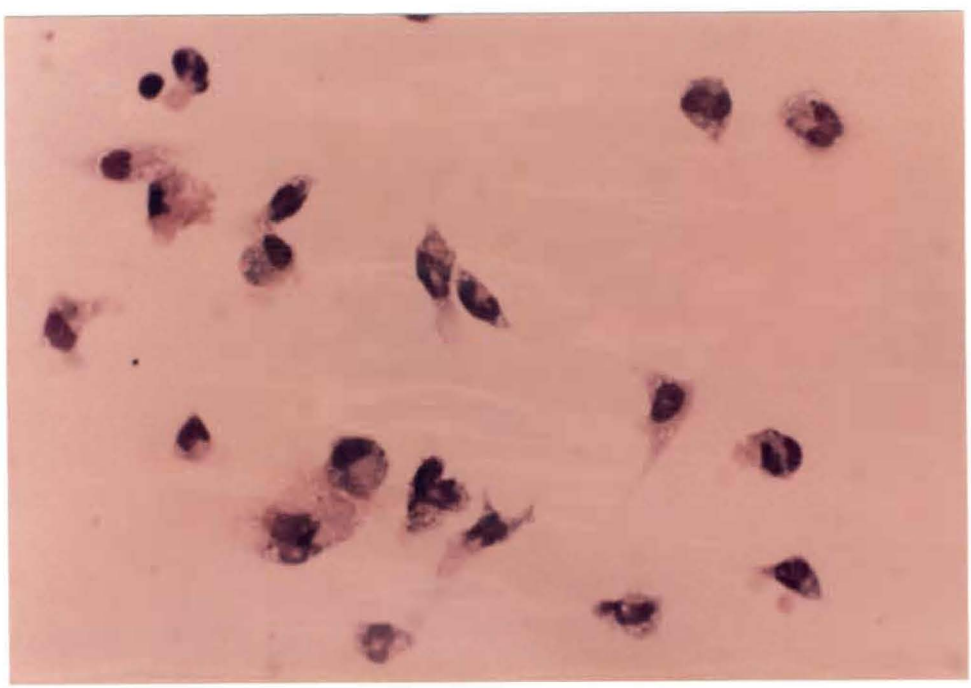


Figure 3. Cytopathogenic effect of *F. tularensis* on TG-elicited macrophages 48 h after infection (630x).

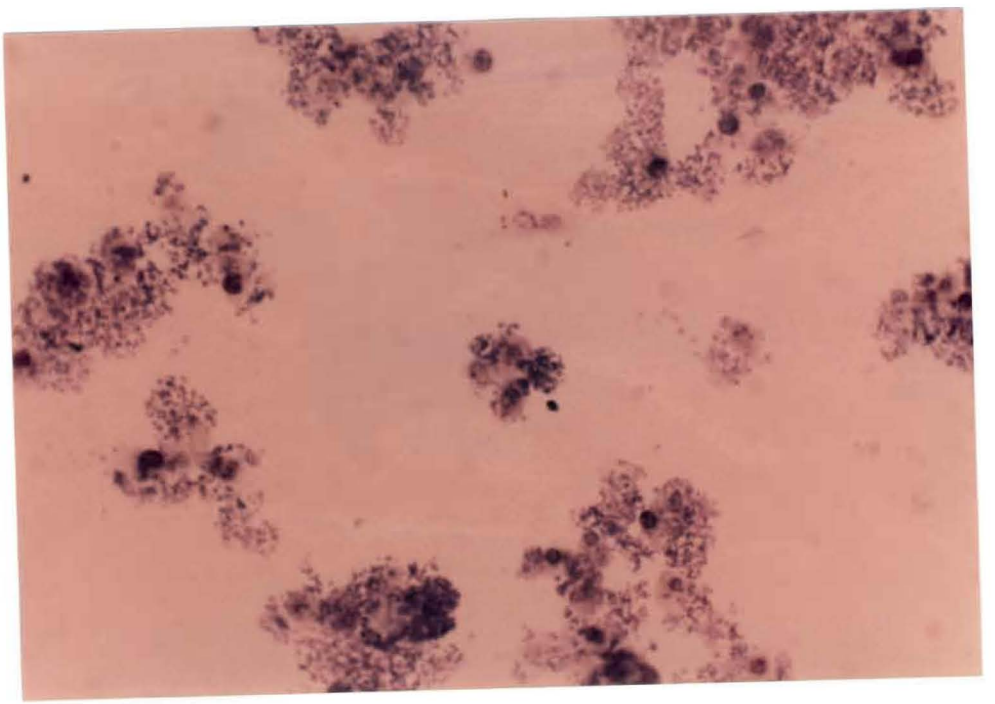
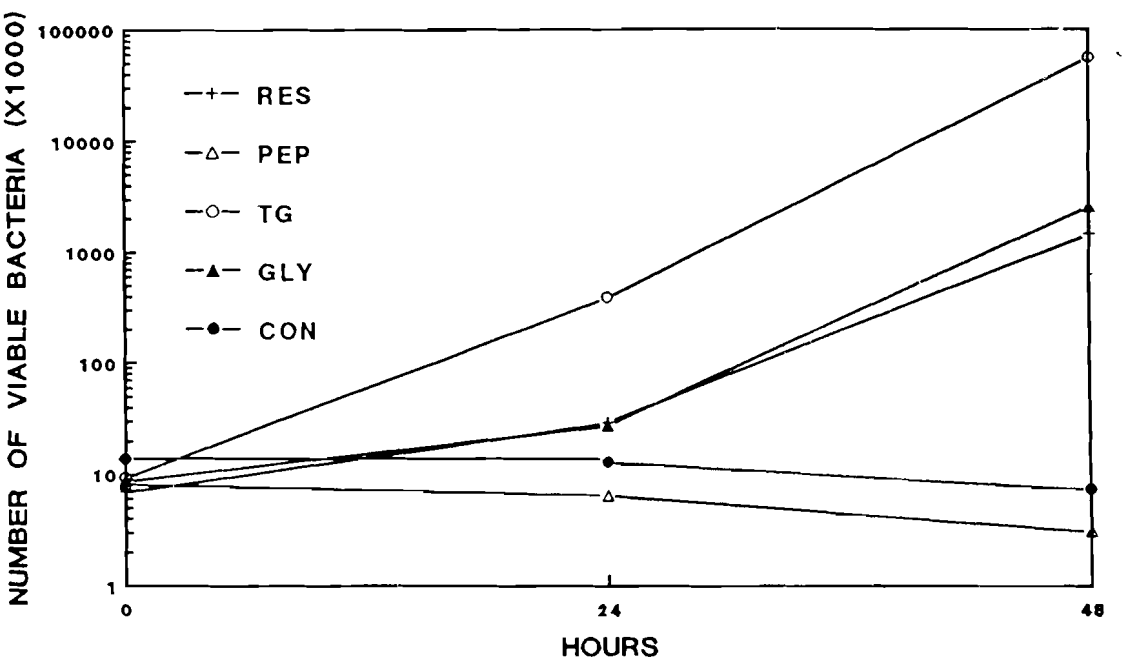


Figure 4. Number of viable *F. tularensis* over 48 h in resident (RES) peritoneal macrophages and macrophages elicited with thioglycollate (TG), peptone (PEP), and glycogen (GLY). Results are the mean of 3 experiments done in duplicate. Growth in TG and PEP elicited macrophages were significant ($p<0.05$) relative to RES control at 48 h by Student's *t* test and a single factor Dunnett's test. Noncellular control (CON) indicates a declining number of viable *F. tularensis*.


NUMBER OF VIABLE BACTERIA (X1000)
100000
10000
1000
100
10
1
RES
PEP
TG
GLY
CON
0
24
48
HOURS

phages were tested for differences in the phagocytosis of *F. tularensis*. To determine the phagocytic index, indirect immunofluorescence to *F. tularensis* was employed (Figs. 5 and 6). Figure 7 demonstrates the results on the phagocytic capacity of macrophages obtained from the peritoneal cavity of mice. Macrophages incubated with *F. tularensis*, obtained from the peritoneal cavity by the elicitation of all three inflammatory agents, had a significant increase in the percentage of phagocytically active macrophages. Again, PEP-elicited macrophages had the greatest increase, over 2-fold, with 44.6% of the macrophages containing ingested bacteria relative to the normal resident macrophages, in which only 20.5% contained bacteria. TG-elicited macrophages had close to a 2-fold increase in the phagocytic index, with 39.7% of the macrophages containing bacteria. GLY-elicited macrophages had a slightly lower, but significant, phagocytic index with 35.6% of the macrophages containing bacteria.

Effect of inflammatory agents on the growth of F. tularensis. To explore the possibility that the inflammatory agents affected the growth of the bacteria, *F. tularensis* was incubated in supernatants cleared of macrophages. After harvesting the macrophages, the supernatants were collected and centrifuged at 2800 rpm for 30 min to remove cells and inoculated with 10^4 org/ml. The same procedure was followed as for the intracellular killing assay for determining the growth pattern of the organism. Figure 8 demonstrates the growth pattern of *F. tularensis* in the supernatants of harvested macrophages. At 48 h no significant difference in the growth of *F. tularensis* in the supernatants of harvested macrophages was observed, indicating that the composition of the medium containing inflammatory agents or inflammatory products did not support the growth of the bacteria.

Effect of culture time on macrophages. To determine if the inflammatory agents had a prolonged effect on the macrophages in culture, the macrophages were in-

Figure 5. Uninfected control of PEP-elicited macrophages stained by the indirect immunofluorescent method. Macrophages counterstained with Evans blue. (1000x)

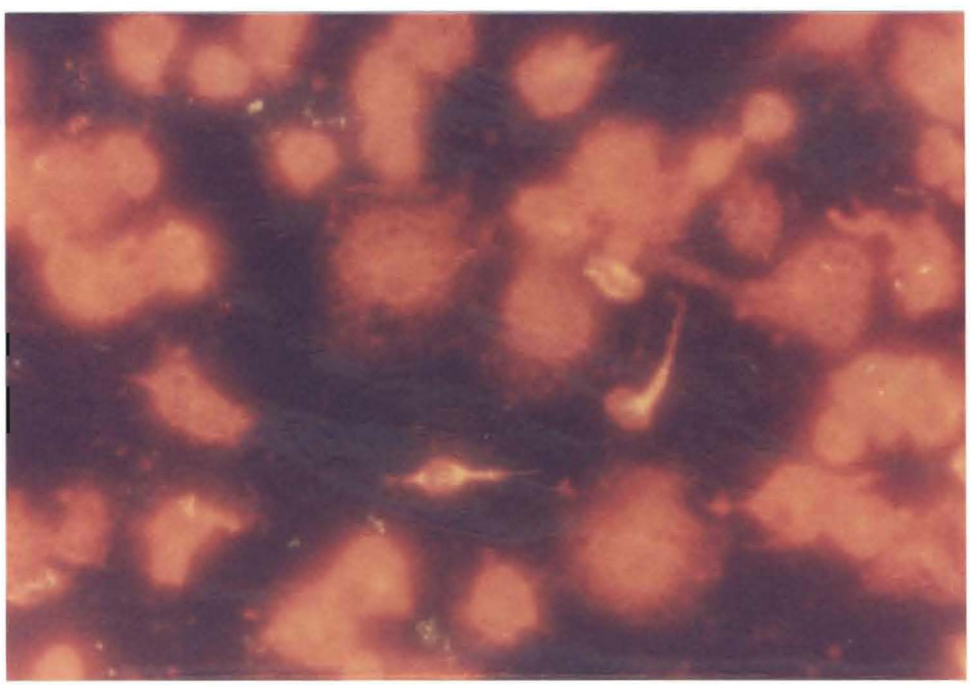


Figure 6. PEP-elicited macrophages infected with *F. tularensis* (A) stained by the indirect immunofluorescent method. Macrophages counterstained with Evans blue. (1000x)

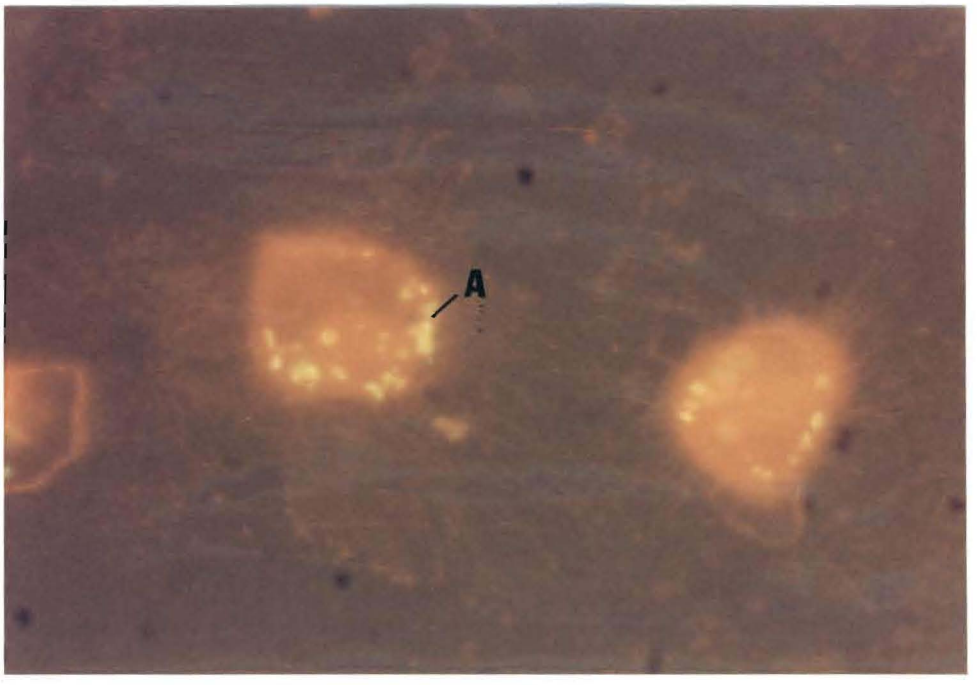

A

Figure 7. Resident macrophages infected with *F. tularensis* (A) after 24 h incubation at 37° C. Macrophages counterstained with Evans blue. (1000x)

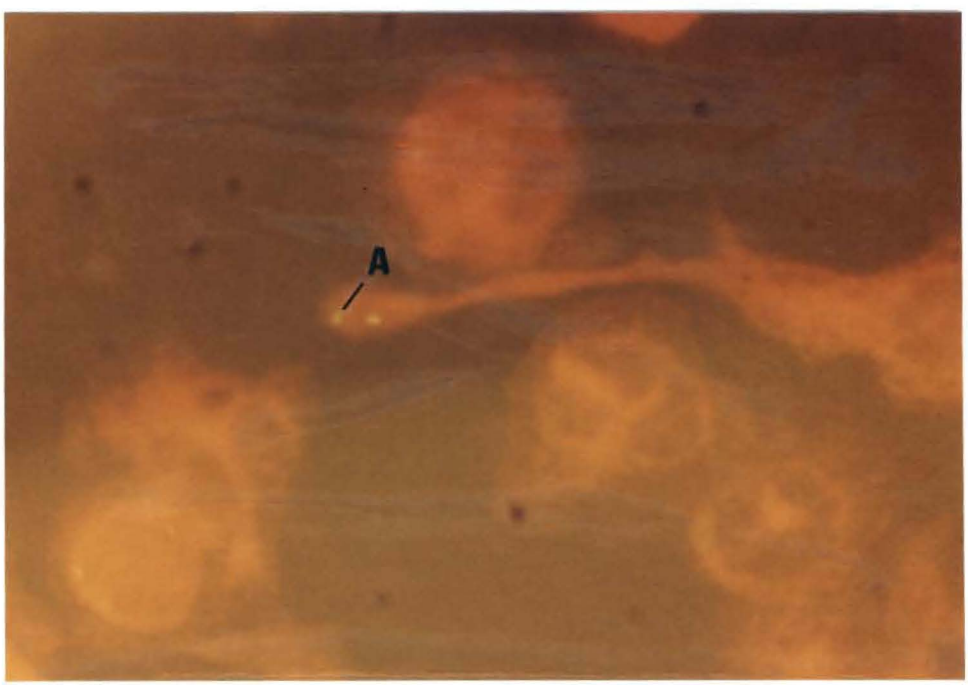

A

Figure 8. Index of phagocytized *F. tularensis* by resident (RES) macrophages and macrophages elicited with peptone (PEP), thioglycollate (TG), and glycogen (GLY). Results are the mean of 3 experiments $\pm$SD. Elicited macrophages were significant ($p<0.05$) relative to RES control according to Student's *t* test and a single factor Dunnett's test.

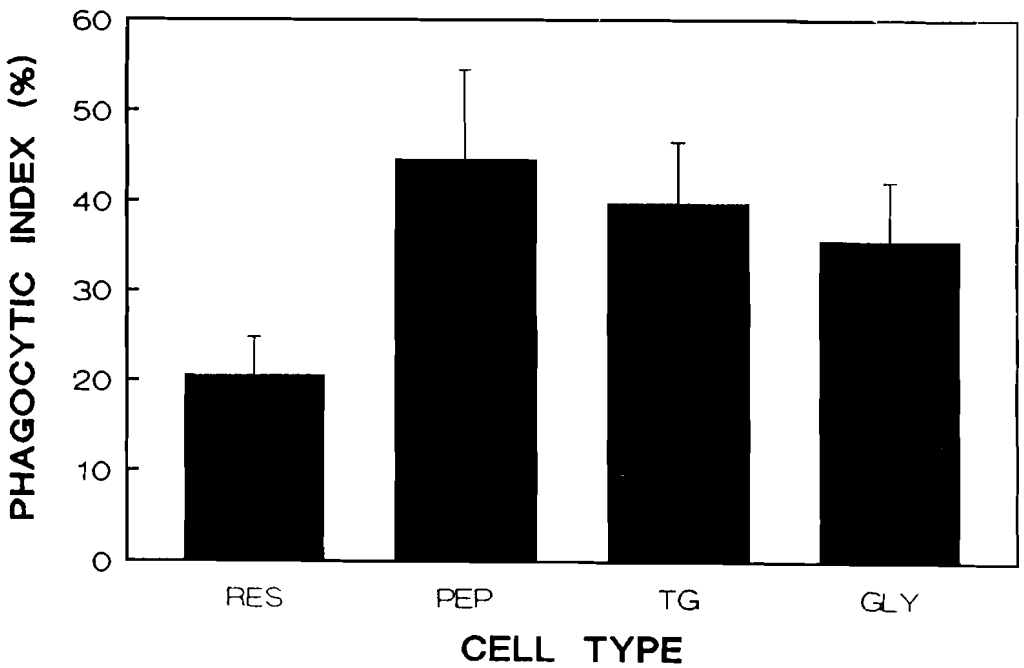
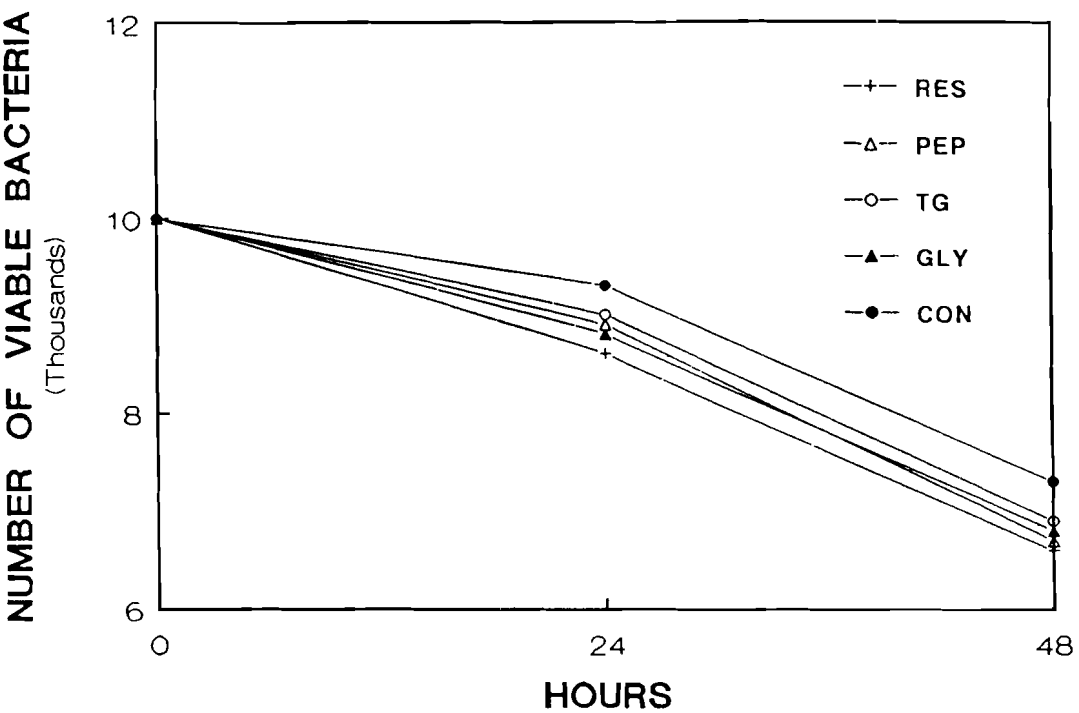

PHAGOCYTIC INDEX (%)
60
50
40
30
20
10
0
RES
PEP
TG
GLY
CELL TYPE

Figure 9. Number of viable *F. tularensis* over 48 h in the supernatants of harvested resident (RES) macrophages and macrophages elicited with peptone (PEP), thioglycollate (TG), and glycogen (GLY). Results are the mean of 3 experiments. Growth in supernatants were not significant ($p>0.05$) relative to RES control at 48 h according to Student's *t* test and a single factor Dunnett's test. Noncellular control (CON) indicates a decline in the number of viable *F. tularensis*.


NUMBER OF VIABLE BACTERIA
(Thousands)
12
10
8
6
0
24
48
HOURS
RES
PEP
TG
GLY
CON

fected with *F. tularensis* 48 h after harvest and the same procedure was followed as described in the Materials and Methods. Figure 9 demonstrates the intracellular growth pattern of *F. tularensis* in macrophages infected 48 h after harvest. At 48 h post-infection, no significant difference in the growth of the bacteria was observed among the elicited macrophages or when the growth of the organism in the elicited macrophages was compared to the normal resident macrophages.

Effect of inflammatory agents on the production of O_2^- by macrophages. The membrane-perturbating agent, PMA, was employed to stimulate the macrophages to produce O_2^-. Table 1 demonstrates the results obtained for the release of O_2^- from macrophages. PEP-elicited macrophages had the greatest significant increase in released O_2^- as compared to the resident macrophages. TG-elicited macrophages had the smallest increase of released O_2^- but was still significantly different from the normal resident macrophages. The Dunnett's test indicated that the amount of O_2^- released by PEP-elicited macrophages was significantly different from all other cell types.

Qualitative NBT reduction. Macrophages were tested for the ability to reduce the redox dye NBT in response to PMA stimulation. The reduction of NBT results in the formation of blue-black formazan deposits within the phagocyte by capturing hydride ions generated in the HMPS pathway (Figs. 10-12). Table 1 displays the results obtained from the macrophage reduction of NBT in response to PMA. A significant difference in the ability of the elicited macrophages was observed, indicating that oxidative activity of the elicited macrophages was enhanced.

Figure 10. Number of viable *F. tularensis* over 48 h in resident (RES) macrophages and macrophages elicited with peptone (PEP), thioglycollate (TG), and glycogen (GLY). Macrophages were infected 48 h post harvest. Results are the mean of 3 experiments. Growth in elicited macrophages were not significant ($p>0.05$) relative to RES control macrophages according to Student's *t* test and a single factor Dunnett's test. Noncellular control (CON) indicates a decline in the number of viable *F. tularensis*.

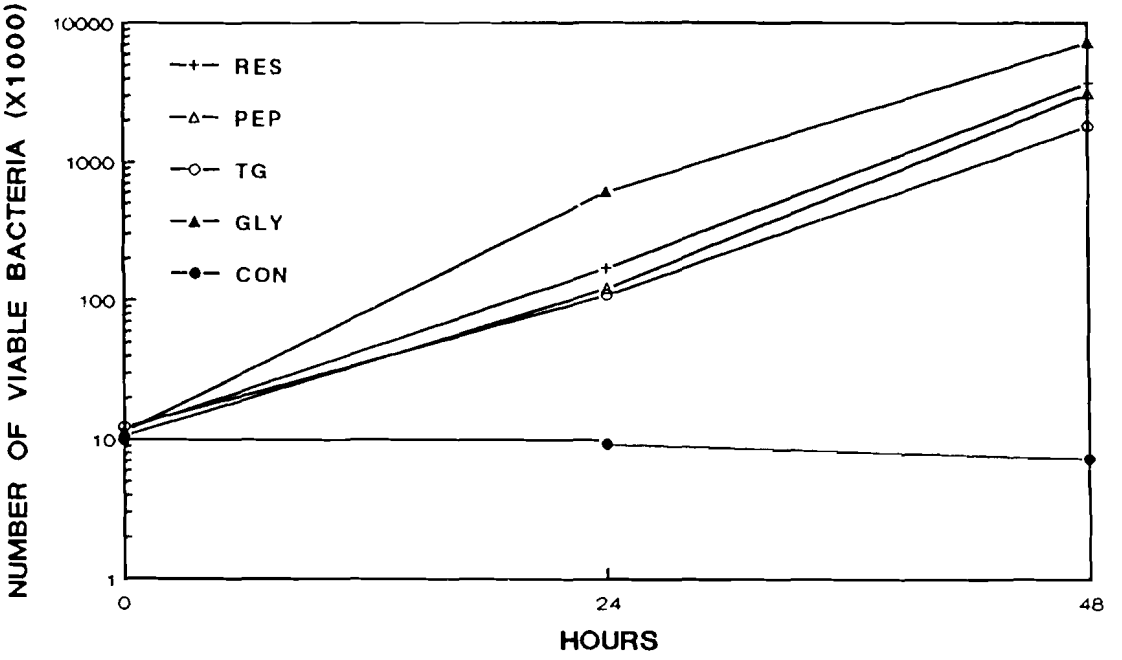

NUMBER OF VIABLE BACTERIA (X1000)
10000
1000
100
10
1
RES
PEP
TG
GLY
CON
0
24
48
HOURS

Figure 11. Photograph of peptone-elicited macrophages incubated with NBT and not stimulated with PMA showing no formation of formazan. Macrophages counterstained with Safranin. (200x)

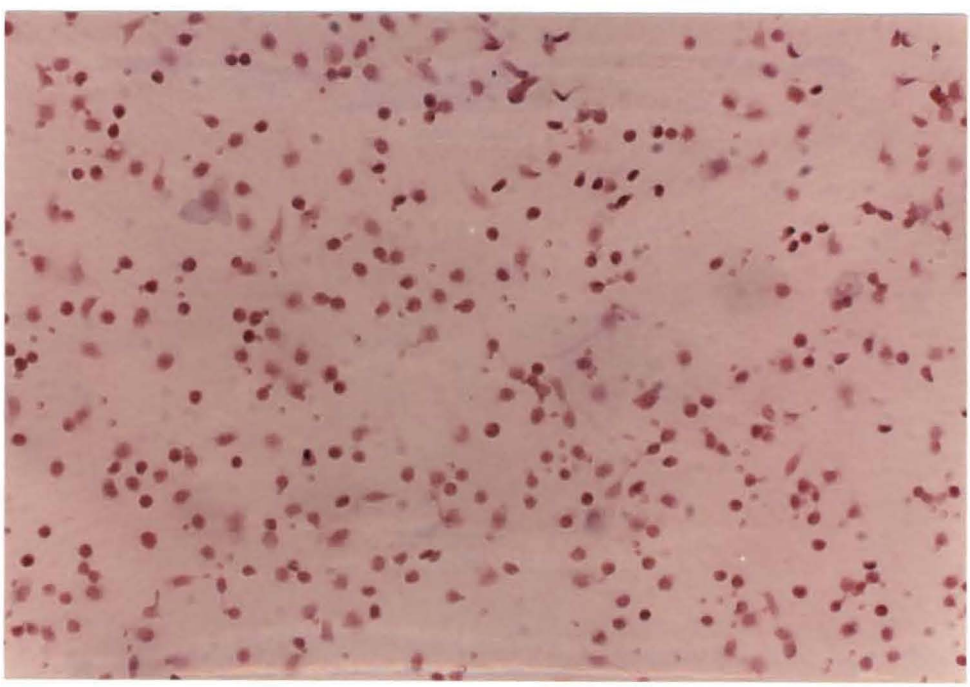


Figure 12. Photograph of resident macrophages incubated with NBT and stimulated with PMA showing moderate amount of formazan. Macrophages counterstained with safranin. (200x)

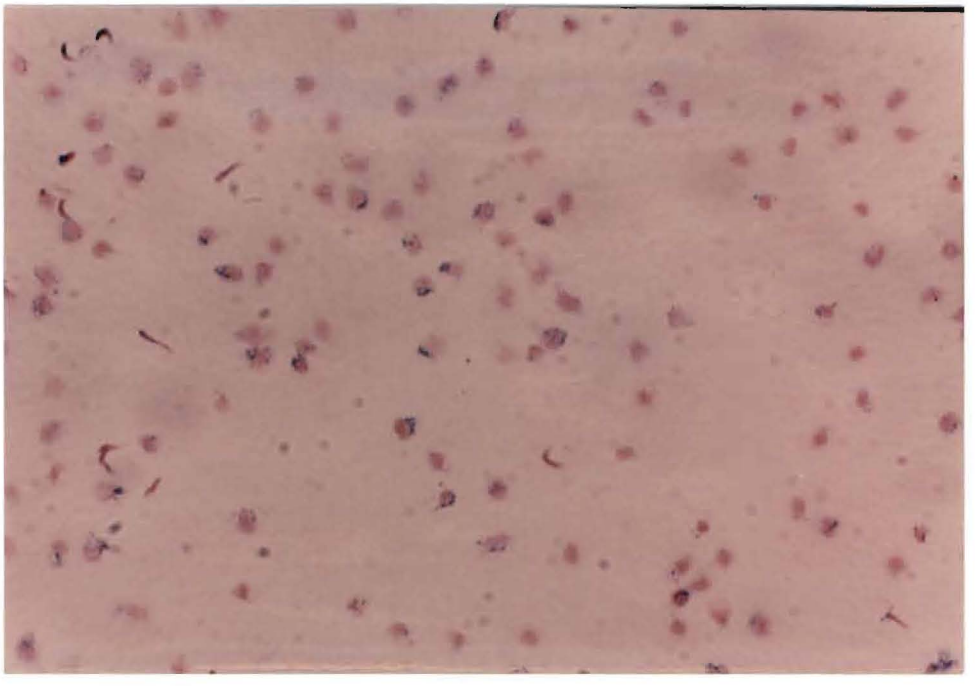


Figure 13. Photograph of peptone-elicited macrophages incubated with NBT and stimulated with PMA showing extensive amounts of formazan. Macrophages counterstained with safranin. (200x)

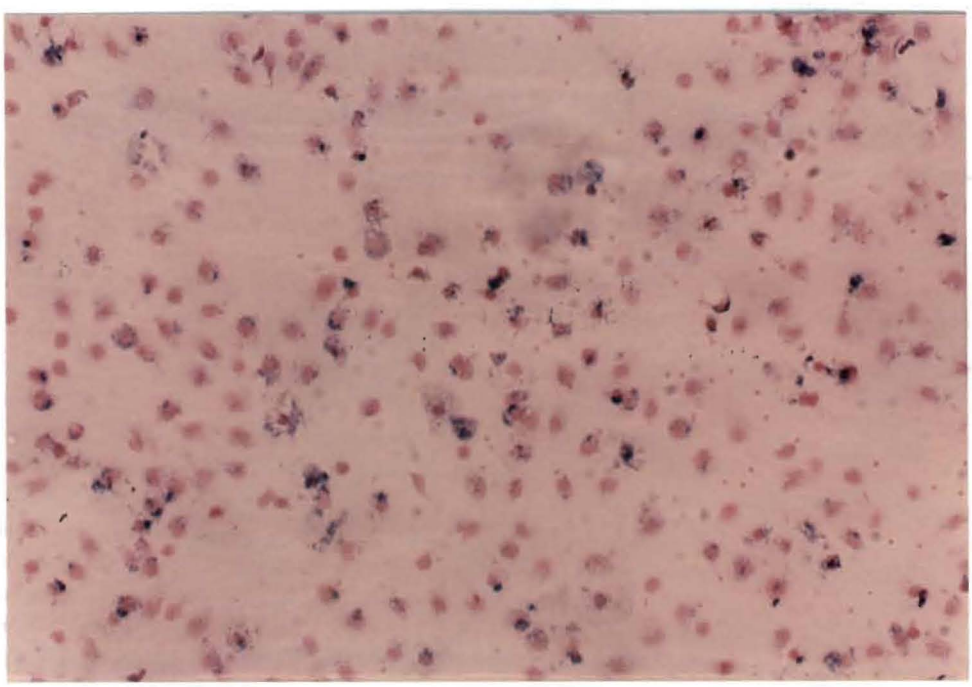


Table I.
Release of superoxide by murine peritoneal macrophages on contact with PMA

Cell Type	nM O_2^- Produced[a]	% NBT reduction[b]
Resident	1.46 ± 0.13	18.2 ± 3.0
TG	1.71 ± 0.13	38.6 ± 4.3
PEP[c]	5.09 ± 1.48	68.2 ± 5.6
GLY	2.07 ± 0.28	56.1 ± 4.4

[a]Nanomoles of released O_2^- by the stimulation of macrophages with PMA as indicated by the SOD inhibitable reduction of cytochrome c. Values are expressed as the mean of three trials run in duplicate ± SD. Elicited macrophages were significantly different from the resident macrophages according to a one-tailed Student's t test ($P<0.05$).

[b]Percent of NBT reducing macrophages by the stimulation with PMA as indicated by the production of formazan deposits. Values are expressed as the mean of three trials ± SD. Elicited macrophages were significantly different from the resident macrophages according to a one-tailed Student's t test ($P<0.05$).

[c]PEP-elicited macrophages were significantly different from all other cell types according to a single-factor Dunnett's test for both assays.

DISCUSSION

A variety of macrophage functions can be altered by the treatment of animals with various stimulating agents. These agents have been divided into two groups (i) inflammatory stimuli, such as TG and PEP that elicit large numbers of cells into the area (Cohn 1978) and (ii) immunologic stimuli, such as BCG, which elicit macrophages via lymphokines (Cohn 1978). Immunologically stimulated macrophages have been termed *activated* because they are capable of an increased antimicrobial activity after an immunological stimulus.

The primary purpose of injecting inflammatory agents into the peritoneal cavity is to obtain a greater yield of macrophages from the animal. The results of this investigation are consistent with previous studies (Conrad *et al.* 1977, Badwey *et al.* 1983) that have employed inflammatory stimuli to increase the number of macrophages harvested from the animal. However, a comparison of the effects of various inflammatory stimuli on the functions of macrophages, particularly intracellular killing, is limited. Therefore, it was the purpose of this investigation to compare how various inflammatory stimuli affect the intracellular bactericidal function of macrophages. This investigation also provides evidence which helps to explain the evasive strategies of *F. tularensis* which protect the bacteria from intracellular killing by normal macrophages.

First, an experiment was performed to evaluate the speculation that *F. tularensis* can grow in the extracellular environment of the supernatants of harvested macrophages. The bacteria was incubated in the culture medium as well as in the supernatants of the harvested macrophages. The decline in the number of the bacteria was essentially the same in the presence or absence of the inflammatory agents or inflammatory products in the supernatants compared to the growth of the bacteria in the culture medium alone, indicating that the inflammatory agents or

products do not influence the growth of the bacteria. Anthony *et al.* (1991) also demonstrated that the growth of *Francisella* spp. did not occur in the extracellular environment in the presence of growth-promoting factors secreted by macrophages. Therefore, the growth of the bacteria takes place intracellularly.

The present study revealed that the bactericidal activities of the elicited macrophages against *F. tularensis* varied depending on the inflammatory agent used. Bactericidal activity expressed by TG-elicited and GLY-elicited macrophages was poor. Other studies have also shown a decreased bactericidal activity from TG-elicited macrophages (Leijh *et al.* 1984, Miake *et al.* 1980). Bactericidal activity of PEP-elicited macrophages, however, was high compared with that of the normal resident macrophages. McElree and Downs (1961) also demonstrated that PEP-elicited macrophages have an increased bactericidal activity. Based on these results, macrophages accumulating in the peritoneal cavity of animals have different bactericidal activities depending on the inflammatory agents used.

In light of these findings, it was necessary to determine if differences in the phagocytosis of the bacteria could account for the differences in the bactericidal activity. In the present investigation, the elicited macrophages had a significantly higher capacity to phagocytize *F. tularensis* than the resident macrophages. Previous studies have also reported an enhancement of phagocytic activity from elicited macrophages (McElree and Downs 1961, Leijh *et al.* 1984).

Even though the elicited macrophages had a significant increase in the uptake of *F. tularensis*, the increase in the growth of the bacteria in TG-elicited and a decrease in the growth of the bacteria in PEP-elicited macrophages suggest that TG-elicited macrophages ingested significantly more bacteria than PEP-elicited macrophages. However, this was found not to be the case. Immunofluorescence revealed no difference in the uptake of the bacteria among the elicited macro-

phages. Hence, it was concluded that the resistance or susceptibility of elicited macrophages to *F. tularensis* was not due to a difference in the uptake of the bacteria. Similar results were found by Archinal and Wilder (1988) where *L. monocytogenes* activated macrophages and caseinate elicited macrophages had no difference in the uptake of the organism. Also, Huebner and Byrne (1988) found that resistance or susceptibility of macrophages to *Chlamydia psittaci* was not due to a difference in the phagocytic rate of the organism.

The observed differences in the amount of O_2^- released by the macrophages upon stimulation with PMA suggest that the bactericidal activity is relative to the oxidative metabolism of macrophages. The ROI that results from oxidative metabolism is required for optimal microbicidal function of macrophages. Its importance is demonstrated by CGD patients who lack the NADPH oxidase enzyme to catalyze the reduction of O_2 (Hohn and Lehrer 1975). CGD patients have an impaired microbicidal activity towards intracellular parasitic organisms (Quie *et al.* 1967). Inhibition of microbicidal activity of macrophages by scavengers of oxidative metabolism (Godfrey and Wilder 1984) and gamma irradiation of mice to impair oxidative metabolism (Kovarova *et al.* 1987) provide even more direct evidence that links ROI with microbicidal activity.

An increase in O_2^- generation was observed from PEP-elicited macrophages upon stimulation with PMA which correlates with the increase in bactericidal activity of the macrophages. Previous investigations have reported that PEP-elicited macrophages have increased HMPS activity, O_2 consumption (Karnovsky *et al.* 1975), and release as much O_2^- as activated macrophages (Karnovsky *et al.* 1988). Conversely, TG-elicited macrophages exhibited little increase in O_2^- generation, which correlates with the lack of bactericidal activity of these macrophages. TG-elicited macrophages have previously been reported to have diminished O_2^-

production (Badwey *et al.* 1983, Karnovsky *et al.* 1988). It is doubtful that TG-elicited macrophages produce less O_2^- as a result of less O_2 consumption during phagocytosis, since they have been shown to consume three times as much O_2 as resident macrophages (Cohen *et al.* 1981).

Tanaka *et al.* (1983) pointed out that the measurement of the extracellular concentration of O_2^-, which was employed in this study, may not be a reflection of the intracellular ROI concentration and as a result may not be an indication of ROI mediated intracellular killing. The reduction of NBT is, however, an intracellular event and can be interpreted as an indication of intracellular ROI (Baehner *et al.* 1976). In the present study, an increase of elicited macrophages to reduce NBT was observed upon stimulation with PMA, indicating that intracellular oxidative activity was enhanced. Although an increase in the ability of TG-elicited macrophages to reduce NBT was observed, a possible explanation for this increase would be that the NBT reduction assay employed is a qualitative determination of the ability to reduce NBT rather than a quantitative determination.

Johnston *et al.* (1981) pointed out that a *priming* mechanism exists for an enhanced oxidative metabolism and antimicrobial activity. He states that activated macrophages are similar at rest to normal resident macrophages and do not exhibit an enhanced oxidative metabolism until stimulated by phagocytosis or PMA. Once these cells are stimulated, an enhanced O_2^- release is observed. Macrophage priming has been demonstrated by the *in vitro* incubation of resident macrophages with lipopolysaccharides (Pabst and Johnston 1980) and lymphokines in association with γ-interferon (Belosevic *et al.* 1988). Recently, Interleukin-2 has been implicated as a cofactor for γ-interferon to increase macrophage antimicrobial activity (Belosevic *et al.* 1990). The priming of macrophages is a short-lived phenomenon and can only be sustained by the continual addition of lymphokines (Murray *et al.* 1979).

In the present investigation, the infection of macrophages 48 h post-harvest did not reveal a difference in the intracellular growth of *F. tularensis* among the elicited macrophages or when the elicited macrophages were compared to resident macrophages, indicating that the bactericidal activity of elicited macrophages is also a short-lived phenomenon. Activated macrophages have been shown to lose their ability to produce increased levels of O_2^- and antitoxoplasma activity by 48 h (Murray *et al.* 1979). These findings further support the correlation of the bactericidal activity to *F. tularensis* by elicited macrophages with oxidative metabolism.

It is important to note that the present investigation implicating O_2^- production as an indicator of antimicrobial activity was obtained using PMA-stimulated macrophages. PMA has been shown to directly activate protein kinase c (Kikkawa *et al.* 1983). This is consistent with previous investigations that have employed PMA as a membrane-perturbating agent in correlation with the production of ROI and antimicrobial activity (Murray *et al.* 1979, Mor *et al.* 1988, Fleming *et al.* 1990). Because the results for the quantification of O_2^- were obtained by the stimulation with PMA it should be noted that artificial stimuli are not comparable to the surface of bacteria or other microbes that might stimulate certain receptors on the macrophage membrane.

Although the biochemical basis for differences in the respiratory burst activity among elicited macrophages remains obscure, structural and functional modifications could be responsible for this phenomenon. These differences may be in the surface receptors or mechanisms which transfer signals from stimulant-receptor complexes to appropriate targets, since the activation of the macrophages with BCG or *Corynebacterium parvum* has been shown to be accompanied by changes in the expression of surface receptors (Ezekowitz *et al.* 1983). This is doubtful since no correlation has been demonstrated between expression of receptors for stimuli and

the triggering of the respiratory burst. These differences may be in the enzyme-mediated system that reduces the O_2 molecule. Berton *et al.* (1982) demonstrated that a correlation existed between NADPH oxidase activity and O_2^- production when the macrophages were stimulated with PMA. At present, it is not known what factors are involved in the activation of the NADPH oxidase, although phosphorylation of the Fc receptor for IgG and an increase in the activity of protein kinase c have been demonstrated prior to the respiratory burst (Brozna *et al.* 1988). Further research is required to elucidate the mechanisms responsible for the observed differences in O_2^- generation as it relates to bactericidal activity of elicited macrophages.

Even though a relationship exists between the oxidative metabolism and bactericidal activities of macrophages to *F. tularensis*, preliminary evidence indicates that, under normal circumstances, the bacteria may circumvent the triggering of the respiratory burst and grow intracellularly. The present data show that the normal resident macrophages generated very little O_2^- while a 100-fold increase in the growth of the organism by 48 h was observed. This is further supported by the fact that PEP-elicited macrophages were shown to be resistant to infection of *F. tularensis* while having an increase in O_2^- generation. Other microorganisms have also been found that circumvent the respiratory burst. McCabe and Mullins (1990) found that *T. cruzi* does not trigger the release of H_2O_2 or the reduction of NBT. Although Anthony *et al.* (1991) provide evidence that *F. tularensis* survives within membrane-bound vesicles that failed to fuse with lysosomes in normal macrophages which indicates that non-oxidative intracellular killing does not occur, the possibility that *F. tularensis* circumvents the triggering of the respiratory burst in normal macrophages can not be ignored. However, the mechanisms by which *F. tularensis* are able to resist the bactericidal activities and multiply within macrophages

awaits further investigation.

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APPENDIX

Abbreviations

BCG	Bacille Calmette-Guerin
CGD	Chronic Granulomatous Disease
GLY	Glycogen
HBSS	Hank's Balanced Salt Solution
HMPS	Hexose Monophosphate Shunt
MEM	Eagle's Minimal Essential Media
NADPH	Nicotinamide Adenine Dinucleotide phosphate
NBT	Nitroblue Tetrazolium
PEC	Peritoneal Exudate Cells
PBS	Phosphate Buffered Saline
PEP	Peptone
PMA	Phorbol Myristate-13-Acetate
ROI	Reactive Oxygen Intermediates
SOD	Superoxide Dismutase
TG	Sodium Thioglycollate

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Signature of Major Advisor

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