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Humoral and cellular aspects of resistance in mice to Francisella tularensis strain 425 Fb4G

M. Kay Luoma

The University of Montana

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HUMORAL AND CELLULAR ASPECTS OF RESISTANCE
IN MICE TO FRANCISELLA TULARENSIS STRAIN $425 \, F_4G$

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M. Kay Luoma
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Approved by:

C-ul S. Kernon
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Humoral and Cellular Aspects of Resistance in Mice to Francisella
Tularensis Strain 425 F_4C (88 pp.)

Director: Carl L. Larson

Until recently, studies and hypotheses of immunity to tularemia
and other intracellular organisms have dealt with either cellular
or humoral mechanisms. The immune response induced by ether-ex-
tracted antigen from Francisella tularensis in combination with
viable Mycobacterium bovis strain BCG offers an excellent model for
correlation of the two responses.

Immunity to F. tularensis strain 425 F_4G results from a combina-
tion of specific antibody and increased bacteriocidal capacities
of the reticuloendothelial system in cooperation with the T cell or
its products. Several lines of evidence support this hypothesis.
1) Immunization with ether-extracted antigen in combination with
BCG or heat-killed Bordetella pertussis protect mice from challenges
of greater than 10^{12} cells of F. tularensis strain 425 F_4G. This
is an increase of greater than 6 logs of protection over EEA
immunized mice. 2) Passive transfer of immune serum into BCG
stimulated animals protects mice from challenge with 10^8 (LD_{50}) cells
of F. tularensis strain 425 F_4G, whereas, immune serum alone protected
mice from a challenge of only 10^4 (LD_{50}) cells of the same organism.
3) The protective effects of the combined BCG-EEA antigen are
decreased by Min-U-Sil and anti-macrophage serum and immunosuppressive
agents which are effective in elimination of macrophages. 4) Immunity
induced by the combined BCG-EEA antigens is abolished by treatment
with cyclophosphamide, an immunosuppressive agent effective in elim-
ination of the antibody producing cell. 5) Treatment with anti-thymo-
cyte serum partially eliminated the immunity induced by the combined
vaccine, which possibly indicates participation of the T lymphocyte
and the lymphocyte products associated with these cells.

Immunosuppressive techniques demonstrate a two or perhaps three cell
interaction in immunity to tularemia. Cooperation of antibody from
B cells and phagocytic macrophages are primarily involved in protection
of mice against F. tularensis strain 425 F_4C. However, production of
lymphocyte products by the T cell could be of secondary importance and
might be considered as a secondary cell for the induction of resistance.

Delayed hypersensitivity to EEA and acquired resistance to tularemia
are thought to be independent phenomena. Supportive evidence for this
is as follows: 1) Resistance to high doses of F. tularensis strain
425 F_4G is induced with both BCG-EEA and B. pertussis-EEA; however,
only the BCG-EEA complex induces delayed hypersensitivity. 2) Both
BCG-EEA and LVS induce delayed hypersensitivity to EEA, but only the
live vaccine strain protects animals from challenge with the highly
virulent Schu strain. 3) Reduction of the delayed hypersensitivity
response with ATS reduces resistance only slightly (1-2 logs). 4)
In vivo MIF induction does not increase the protective effect of the
BCG-EEA complex in mice to challenge with F. tularensis strain Schu.

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ABBREVIATIONS

AMS	anti-macrophage serum
ATS	anti-thymocyte serum
BCG	Mycobacterium bovis strain bacille Calmette-Guerin
B-cell	bone marrow or bursal derived lymphocyte
C	degrees Centrigrade
CP	cyclophosphamide
EEA	ether-extracted antigen
FCS	fetal calf serum
gm	gram
g	gravities
i.p.	intraperitoneally
i.v.	intravenously
Klett xxx	suspension adjusted to a density of xxx Klett units on a Klett-Summerson photoelectric colorimeter fitted with #42 blue filter
LD_{50}	50% lethal endpoint
LPS	lipopolysaccharide; endotoxin from the cell wall of Gram negative bacteria
LVS	Francisella tularensis strain R2713 or EG-15, live vaccine strain
MAM	mean area of migration
MEM	minimal essential medium
MIF	macrophage migration inhibitory factor
MI	migration index
ml	milliliter
mm	millimeter

MTD	mean time of death
PIM	percent inhibition of migration
PPD	purified protein derivative (from tubercle bacillus)
RES	reticuloendothelial system
s.c.	subcutaneously
SGS	sterile gelatin saline
T-cell	thymus derived lymphocyte
v.u.	viable units

CHAPTER I

INTRODUCTION

There are two basic immunologic responses in a host: those mediated by humoral antibody and those mediated by specific cellular components of the circulatory and lymphoid systems (24). Humoral immunity results from the production and secretion of antibodies into the extracellular fluids. Antibody, immunoglobulin molecules capable of reacting with specific antigen, can influence the fate of bacteria in vivo by lysis (complement dependent) or opsonization (specific antibody enhancement of phagocytosis) (66). Cell-mediated immunity has been attributed to intrinsic properties of cells specifically altered by antigenic stimulation rather than to specific humoral factors (52,54). Cellular immunity is responsible for rejection of tissue grafts, immunity to tumors in man and other animals, and host resistance to infection by facultative intracellular organisms.

Facultative intracellular bacteria are parasites of the reticulo-endothelial system (RES). Examples of such microorganisms are found in widely diversified genera including Mycobacterium, Listeria, Brucella, Salmonella, Pasteurella, Yersinia, and Francisella. Host resistance to infection by facultative intracellular parasites necessitates an improvement in the ability of the host macrophages to phagocytize and inactivate bacteria. The degree to which specific antibody is involved is of considerable controversy among numerous investigators (reviewed by 22,41,52,66, 71).

Acquired resistance to facultative intracellular bacteria is accompanied by three conditions which are correlated with cell-mediated

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immune responses: 1) a consistant association with delayed-type hyper-sensitivity; 2) the inability to passively transfer this resistance with serum; and 3) a change in antibacterial activity of the host macrophages (52). The invariable association of these three conditions with resistance to certain agents gave rise to the idea of an immune mechanism distinct from and independent of antibody. Interest was, therefore, directed towards the population of lymphoid and phagocytic cells from immune and non-immune animals. In vivo and in vitro studies demonstrated that phagocytes from immune animals exhibited greater ability to ingest and kill bacteria than cells from non-immune donors. Lurie (46,47) made the initial observation that phagocytes from rabbits immunized with attenuated tubercle bacilli, inhibited growth of virulent tubercle bacilli, when both were placed in the anterior chamber of the eye of a normal rabbit. Suter (70) demonstrated that phagocytes from guinea pigs and rabbits immunized with viable Mycobacterium bovis strain BCG suppressed the multiplication of tubercle bacilli in vitro. A cellular basis of immunity has been hypothesized to explain resistance to infections by Brucella sp. (23, 37, 51), Listeria monocytogenes (3, 50), Salmonella enteritidis (58, 68), and S. typhimurium (5, 6, 53). The increased bacteriocidal capacity of macrophages from animals infected with these organisms, although resulting from a specific immunological response, is not specific for the organisms which induced the response (51). Activated macrophages from animals infected with one species of bacteria show no discrimination and are sometimes more efficient at phagocytizing bacteria of an antigenically unrelated species (6, 23, 38, 51). Non-specific stimulation of phagocytosis with bacterial lipopolysaccharides (9, 67)

and non-bacterial substances, such as simple lipids (16) and surfactants (17), has been interpreted as supportive evidence for the contention that antibody plays no part in resistance to facultative intracellular bacteria.

Infections with facultative intracellular bacteria lead to production of specific opsonins, antibodies which promote phagocytosis. This indicates that a concept of an immune mechanism independent of antibody need not be postulated. In vitro studies by Mackaness (48, 49) indicated that there was no difference in the ability of macrophages from immune or non-immune rabbits to inhibit the growth of tubercle bacilli. This may be explained by the absence of immune serum in the cell culture environment employed in these experiments. Fong, et al. (27, 28) demonstrated a requirement for a humoral component for expression of bacteriocidal capacities of the phagocytic cell. In vivo and in vitro experimentation on phagocytosis of Brucella and Salmonella species emphasized the importance of immune serum for the expression of bacteriocidal potentialities of macrophages. Immune serum was required for the prevention of cytotoxic degeneration of immune and normal phagocytes by virulent Brucella melitensis (22, 23). Virulent and avirulent Salmonella typhimurium incubated with specific antiserum were efficiently removed from the peritoneal cavity and digested. In the absence of immune serum, virulent organisms were not effectively removed even in the presence of lipopolysaccharide (LPS), a non-specific stimulant. Avirulent organisms, however, were eliminated (78). Subsequent experimentation substantiated these results (40, 65).

Although the host-parasite relationships involved with infection by facultative intracellular organisms have been studied extensively, the mechanism(s) of resistance to infection remains a point of considerable confusion. Host-parasite relationships in tularemia offer an excellent model for studying immunity to facultative intracellular organisms for several reasons. There is little, if any, opportunity for previous exposure of laboratory animals to the causative agent of tularemia; this is an important consideration in experimentation with Gram-negative bacteria. The role which endotoxin plays in the pathogenesis of the disease is not a variable since no endotoxin-like material has been isolated from Francisella tularensis (61, 62). In addition, bacterial strains with varying degrees of virulence can be obtained, as can experimental hosts differing in susceptibility.

Two types of immune responses have been associated with acquired resistance to tularemia in the mouse. The first type, which develops only after infection with avirulent strains capable of proliferation in vivo, allows survival of the host after challenge with strains of high virulence. This type of immunity has been considered to be dependent on a state of altered reactivity of the phagocytic cells and to be independent of extracellular antibody (69, 72, 73). Acquired resistance to tularemia is transferred adoptively with immune peritoneal exudate or spleen cells (1, 74, 79). Claflin and Larson (13) observed a correlation between resistance and delayed hypersensitivity in mice immunized with the live vaccine strain (LVS) of F. tularensis and challenged with a highly virulent strain (Schu). In addition, they detected a requirement for specificity in acquired resistance to the Schu strain and the absence of non-specific immunity.

A second type of immunity to tularemia in the mouse, developing after immunization with non-viable vaccines as well as after infection, protects only against strains of less than full virulence (4, 29, 43, 44). Immunity of the second type is explained partially by the action of humoral antibody directed against the somatic antigens.

Investigation of immune responses to facultative intracellular organisms has been aided by in vitro correlates of delayed hyper-sensitivity. Rich and Lewis (64) observed migration inhibition, in the presence of specific antigen, of cells from tuberculin sensitive spleen explants. This early work was supported by George and Vaughn (30) and David, et al. (20). Peritoneal cells from sensitized guinea pigs exhibiting delayed hypersensitivity were inhibited in migration from capillary tubes incubated in tissue culture medium supplemented with specific antigen. Peritoneal cells from normal animals did not exhibit this inhibited migration. Bloom and Bennett (7) suggested that the effector cells in the macrophage inhibition test were sensitized lymphocytes.

Sensitized lymphocytes secrete soluble protein, termed macrophage inhibitory factor (MIF) is non-dialyzable, heat stable ($56^{\circ}C$ for 30 min.) and has a molecular weight of 60,000-70,000 (7,60). It is produced by stimulation of sensitized lymphocytes with specific antigen and not with unrelated antigens (60). However, once produced, MIF exerts its effects in a non-specific manner. Another result of interaction between sensitized lymphocytes and specific antigen is the transformation of the lymphocytes into blast cells (21). This seems to be a non-specific reaction and not necessarily associated with delayed hypersensitivity.

A hypothesis set forth by Waksman (77) attempts to explain delayed reactions in vivo, by correlation of the data obtained from in vitro observations. The antigen eliciting the response comes into contact with sensitized lymphocytes circulating in the vicinity of the injection site. The interaction between lymphocytes and antigen induces the cells to produce MIF which in turn hinders the flow of monocytes. These monocytes adhere to the vessel wall, differentiate into macrophages, and migrate through the wall into the area of injection. The macrophages attack or ingest the antigen, which is then destroyed through the action of its hydrolases.

It has been proposed that acquired resistance to facultative intracellular parasites could be attained through delayed-type hypersensitivity (18, 52). Mackaness (50) and Collins and Mackaness (15) observed consistant association between occurrence of resistance and delayed hypersensitivity in listeriosis and salmonellosis, respectively. Additional correlation between resistance and delayed hypersensitivity has been observed in tuberculosis (71) and brucellosis (51). In these infections, the onset of delayed hypersensitivity correlates with the appearance of activated macrophages. It is well known, however, that to induce delayed hypersensitivity without accompanying resistance is possible (22, 23, 31, 52).

Evaluation of the importance lymphocytes and other cellular components of the immune response to facultative intracellular bacteria provided a means for defining the mechanism(s) involved. Cellular cooperation in the genesis of cellular and humoral immunity has been well documented (14, 55, 56). At least three cell types have been

implicated in these responses: a thymus derived, theta positive,
lymphocytic cell (T-cell (56); a bone marrow-derived thymus-independent
lymphocytic cell (B-cell), a precursor of high rate antibody synthesizing
cells (56); and a surface-adherent, phagocytic cell (macrophage) (57).
The first two are believed to be antigen specific and clonally pre-
determined (11), presumably via cell surface receptors that are antigen
specific (12, 25). The third cell type has a non-specific role necessary
in both the efferent and afferent arcs of the immune response (33)
(prior to antibody production or altered cellular reactivity). Only
recently has the morphology of lymphoid tissue been clarified in terms
of cell population dynamics and migration streams. Gowans and Knight
(32) described a population of small rat lymphocytes which recirculated
between the blood stream and lymphatics. These cells populated the
cortex of the lymph nodes and the periarteriolar white pulp of the
spleen and were termed thymus-dependent since depletion of the area
occurs in neonatally thymectomized animals. Two other areas were
distinguishable in lymph nodes. The medulla consisted of a sessile
population rich in plasma cells and macrophages. In the outer cortex
of the lymph node and adjacent to the periarteriolar white sheath of
the spleen were areas composed of densely packed small lymphocytes,
the primary follicles. These cells appeared to be thymus-independent and
important in production of humoral antibodies.

Immunosuppressive techniques, used to eliminate one or more
resistance factors, have been correlated with deactivation and/or
elimination of cellular components involved in production of an immune
response. Allison (2) has noted that the immune state transferred via

immune sera was reduced dramatically in animals treated with cyclophosphamide. Histological examination (75) of tissues from animals treated with cyclophosphamide showed marked depletion of the lymphocyte population from the lymph follicles, the cortex, and the cortico-medullary junction, areas associated with B-cell populations and antibody production. In addition, relatively little effect on the lymphocytes in the pericortical areas of the lymph nodes and the thymus-dependent areas of the spleen was noted. Antithymocyte serum (ATS) used as an immunosuppressive agent affects primarily the cells responsible for cell-mediated immunity. Levey and Medawar (45) first proposed the use of ATS as an immuno-suppressive agent effective in inhibiting homograft rejection. The effect of ATS on induction of delayed hypersensitivity was first noted when treatment with ATS was found to have an effect on the pericortical area of the lymph node (59). Anti-thymocyte serum had little effect on the lymph node follicles, the cells of the germinal centers, or on the proliferation of plasma cells in the medullary cords and the cortico-medullary junction (59). Depletion of small lymphocytes caused a reduction in size of the pericortical areas and replacement by active phagocytes of the RES, effects observed in neonatal thymectomy or athymic mice (59). Mackaness, et al. (54) have observed suppression of resistance in listeriosis in animals treated with ATS.

Anti-macrophage serum (AMS) and silica are immunosuppressive agents acting against the macrophage. Unanue (76) first demonstrated that a specific AMS (to mouse macrophages) may be obtained in rabbits. Anti-macrophage serum effectively decreases the phagocytic activity of macrophages in vivo and interferes with antibody responses to particulate antigens when treatment precedes immunization (39). The potentiating

effect of silica is due to the destruction of macrophages. Silica particles are destructive to macrophages because the particles are efficiently taken up by these cells and react relatively rapidly with the membranes surrounding the secondary lysosomes. Reaction with these membranes results in destruction of the permeability barrier allowing escape of enzymes into the cytoplasm, producing extensive general damage and death of the cell.

Acquired resistance to facultative intracellular parasites occupies an ambiguous position in immunology. Mechanisms regulating acquired resistance to these parasites may be clarified by investigation at the cellular level. The Francisella tularensis system provides an excellent model for correlating humoral and cellular immunity and for showing whether the resistance to facultative intracellular organisms occurs at the cellular level, humoral level, or both levels.

Statement of problem

Immunological studies to date, dealing with facultative intracellular bacteria, have employed organisms of low virulence, such as Listeria monocytogenes or organisms that are technically difficult to study as is Mycobacterium tuberculosis. Francisella tularensis is an excellent organism for the study of immunological responses involved in resistance to facultative intracellular bacteria. This organism is highly virulent for mice, the time of onset of disease in these animals is well established, and standard cultural methods are available which allow for an effective study of the immune apparatus. The purpose of this investigation was to examine the cellular components involved in resistance (both humoral and cellular) in mice to tularemia. As part of

this study, a delayed hypersensitivity response stimulated by killed

F. tularensis organisms in combination with viable BCG vaccine was

examined.

CHAPTER II

MATERIALS AND METHODS

Experimental animals

Swiss-Webster mice, 5 to 7 weeks old from the colony maintained at Rocky Mountain Laboratory, Hamilton, Montana, were used throughout this investigation.

Hartley strain guinea pigs, obtained locally, were used for in vivo and in vitro demonstration of delayed hypersensitivity.

Adult, New Zealand white rabbits, obtained locally, were employed for production of antisera for passive transfer and immunosuppressive studies.

All animals were allowed food and water ad libitum.

Challenge organisms

Strains of Francisella tularensis were obtained in lyophilized form from Dr. J.F. Bell, Rocky Mountain Laboratory, Hamilton, Montana These organisms were maintained on glucose-cysteine heart agar (Difco) supplemented with whole rabbit blood to a final concentration of 5 percent. Francisella tularensis strain 425 F_4G, a strain of high virulence for guinea pigs and mice (LD_{50} 1-10 organisms) but of low virulence in rabbits, was used in the majority of the experiments in this investigation. Francisella tularensis strain Schu, a strain of high virulence in higher mammals, has an LD_{50} of 1-10 organisms in mice, guinea pigs, and rabbits and was used as the challenge organism in some of the experiments.

Challenge doses were prepared by washing cells from a 24 hour culture of F. tularensis with sterile gelatin saline (SGS) (0.1% gelatin

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+ 1% NaCl). The cells were washed once in SGS and centrifuged at 8,000 x g (at 4 C) in an RC-2B Sorval centrifuge. The cells were resuspended and diluted serially in SGS from an appropriate concentration which was based on readings of turbidity in the Klett-Summerson photo-electric colorimeter fitted with a #42 blue filter. The number of viable organisms used for challenge was determined by the plating technique of Fenner (26). Three 0.1 ml drops of appropriate dilutions were placed on each of 2 plates, which were incubated at 37 C for 2-4 days, and then counted.

Vaccines

Ether-extracted antigen (EEA) was prepared according to the method of Larson (43). Washings from 48 hour cultures of F. tularensis strain 425 F_4G or strain Schu were used to inoculate flasks of peptone broth (0.1% glucose, 2% peptone, 1% NaCl) which were incubated 48 hours at 37 C on a shaker. The cells were harvested by centrifugation at 8,000 x g for 30 minutes. After washing cells 3 times in SGS, the resuspended cells were treated with equal volumes of ethyl ether and allowed to stand for 24 hours. The aqueous phase was drawn into a sterile serum bottle, incubated at 80 C until residual ether had evaporated, and stored at 5 C. Immunizing doses were made by dilution of the antigen with SGS to a Klett reading of 100, and 0.2 ml of the diluted antigen was injected into the tail veins of mice.

Viable BCG vaccine was prepared as described by Larson, et al. (unpublished data). Flasks of Dubos medium (Difco) were inoculated with Mycobacterium bovis strain BCG, maintained on Hohn's medium (Difco)

and incubated for 5 to 7 days at 37 C. The culture was transferred an
additional time and allowed to incubate another week. The cells were
harvested by centrifugation at 5000 x g and washed three times with
Dubos medium. The cells were resuspended in Dubos medium and adjusted
to a density of 200 Klett units and stored at -20 C. Mice were immunized
i.v. with 0.2 ml of suspension adjusted to a density of 200 Klett units
(Klett 200). Viable counts were done according to the method of
Fenner (26).

A Klett 200 suspension of viable BCG vaccine was diluted 1:1 with
a Klett 200 suspension of EEA to investigate the combined effects of
a specific and a non-specific vaccine. A 0.2 ml injection of the
BCG-EEA suspension was made into the tail vein of the mouse.

Bordetella pertussis vaccine prepared by Dr. A. C. Blaskett,
Rocky Mountain Laboratory, Hamilton, Montana, was used in studies
comparing the non-specific immune response involved with a live and
killed antigen. The vaccine was prepared by heating B. pertussis
strain 3379-L_2S_4 for 1 hour at 56 C. The heat-killed suspension of
cells was dialyzed against saline and stored at 5 C. An immunizing
dose of 2.5 x 10^5 cells was given in the tail vein.

Bacterial agglutination

A standard suspension of 10^9 formalin killed cells of F. tularensis
strain 425 F_4G was used as the agglutinogen. Serum was collected by
pooling blood from 10 to 15 previously immunized mice. A constant
volume of 0.5 ml of the agglutinogen was added to two-fold dilutions
(0.5 ml) of serum.

Preparation of immunosuppressive agents

Anti-thymocyte serum (ATS) - Adult, New Zealand white rabbits were given intravenous injections of $2.4 \times 10^7 - 3.2 \times 10^7$ mouse thymocytes at weekly intervals. Thymus glands, suspended in minimal essential medium (MEM) supplemented with 5 units of heparin, were sieved through 40 mesh screens to obtain a single cell suspension. The thymocytes were washed three times in MEM + 3% fetal calf serum (FCS), incubated for 4 hours at 37 C to eliminate contaminating glass adherent cells, counted, and injected into the rabbits. Two weeks following the final stimulation, the rabbits were exsanguinated by cardiac puncture. The serum was separated, adsorbed eight times with mouse liver powder (acetone dried), and stored at -20 C. The leukoagglutination titer was 128. One fourth ml of ATS was given into the tail vein of the mouse 24 hours preceding challenge, simultaneously with the challenge, and 24 hours after the challenge with F. tularensis.

Anti-macrophage serum (AMS) was prepared by the same method described above. However, glass adherent cells from the peritoneal cavity of the mouse were used as the stimulant. Anti-macrophage serum was administered intraperitoneally (ip) at the same time intervals as the ATS.

Silica - The silica preparation consisted of a saline suspension of # 216 Min-U-Sil (Whittaker, Clarke, and Daniels, Inc.). The Min-U-Sil was suspended at a concentration of 250 mg/ml, and 0.2 ml was injected ip into mice 4 hours before challenge.

Cyclophosphamide - Cyclophosphamide (CP) (Cytotoxan, Mead-Johnson) was suspended in saline at a concentration of 30 mg/ml. Treatment was

begun 14 days after initial immunization. Two doses of 150 mg/kg were given ip 7 days apart.

Protection tests

Protection against challenge infections was measured in terms of survival of mice after subcutaneous (s.c.) or ip injection with serial dilutions of organisms. Immunized or control mice in groups of 6-12 were inoculated s.c. or ip with 0.2 ml of each dilution. Deaths in challenged mice were followed daily for 14 days at which time the LD_{50} (63) and the mean time of death were calculated. The degree of protection afforded by a particular vaccine was determined by subtracting the log LD_{50} value of the control group from that of the experimental group.

Test for hypersensitivity

Groups of 5-10 mice each were injected in one hind footpad with 0.03 ml of a Klett 50 suspension of EEA in saline. Equal volumes of saline alone were injected into the contralateral footpad. At 4, 12, 24, and 48 hours, the footpad thicknesses were measured with a dial gauge caliper (Schnelltäster, Kroplin). Significance in the differences in thickness of saline and EEA inoculated feet in each group was determined by Student's t test.

Groups of four guinea pigs each were injected with 0.1 ml of Klett 10 to Klett 200 suspensions of EEA. Measurement of the hypersensitive area and comparable non-hypersensitive areas on the guinea pigs was done with the dial gauge caliper at 2, 4, 8, 12, 24 and 48 hours.

In vitro correlates to delayed hypersensitivity

Macrophage inhibition test - The macrophage inhibition test

employed in this investigation was a modification of that described by

David (19) and George and Vaughn (30). Animals immunized previously

and shown to develop cutaneous delayed hypersensitivity after injection

with antigen were used in these experiments. In addition, guinea pigs

immunized with EEA alone and exhibiting only immediate hypersensitivity

were used as controls.

Each animal under study was injected ip with 30 ml of sterile

light mineral oil 96 hours prior to harvest of the peritoneal cells.

Following sacrifice of the animal, cells were harvested by injection

of 30 ml of MEM supplemented with 5 units of heparin into the peritoneal

cavity of the guinea pig. The peritoneal fluid was withdrawn from the

cavity with a syringe, transferred to a sterile, cold, separatory funnel,

and allowed to separate into oil and aqueous phases for 30 min at 4 C.

The aqueous phase was drawn off into centrifuge tubes, and the cells were

centrifuged at 500 x g for 15 min. Following centrifugation, the

supernatant fluid was discarded and the cells resuspended in MEM. The

cells were washed twice and resuspended to a concentration of 10% in

MEM supplemented with 15% normal guinea pig serum.

The cells were drawn into sterile hematocrit tubes, one end of

which was heat-sealed, then centrifuged at 500 x g for 10 min. The

hematocrit tubes were cut at the cell-supernatant fluid interface, and

the fragments containing the cells were placed in sterile Mackaness

tissue culture chambers. Each chamber received MEM or MEM containing

30 ug/ml of EEA. In addition, to eliminate bacterial growth, 50 ug/ml

of streptomycin and 50 units/ml of penicillin were incorporated into the MEM. The chambers were incubated at 37 C for 24 to 48 hours. Migration patterns were traced and recorded using a Bausch and Lomb camera lucida. Areas of migration in square millimeters (mm^2) were determined by measurement of the area of the tracing with a Gelman planimeter.

The percentage of macrophage migration in the presence of antigen is defined as the migration index (MI).

$$M.I. = \frac{\text{mean area of migration with antigen}}{\text{mean area of migration without antigen}} \times 100$$

The percent inhibition of migration (PIM) is calculated by: PIM = 100-M.I.

Passive transfer of immunity

Passive transfer of serum and adoptive transfer of cells was done by a modification of the method of Allen (1). Mice immunized and boosted with BCG-EEA were anesthetized with ether and exanguinated by cardiac puncture. The spleens were excised and washed in cold MEM, placed in Petri dishes with MEM + 10% FCS, and minced and teased apart with sterile forcepts. Large pieces of debris were removed by filtering the suspension through a wire mesh screen. The resulting filtrate containing individually suspended cells was counted, centrifuged at 500 rpm for 15 min, and resuspended in MEM to a concentration of 5.0×10^8 cells/ml. Recipient mice, 6-8 weeks old, were injected ip with 1 ml of the cell suspension. Serum from the same mice was separated and injected into the peritoneal cavity of the recipient mice simultaneously with the transferred spleen cells. Challenge of the recipient mice was done 4 hours after transfer of the cells and serum.

Attempt to produce non-specific immunity to F. tularensis strain 425 F_4G.

Non-specific stimulation of the reticuloendothelial system (RES), referred to as non-specific immunity, has been shown to be effective in preventing diseases caused by facultative intracellular organisms. Immunization with viable BCG protects mice subsequently challenged with a strain of Listeria monocytogenes exhibiting low virulence (50, 51). Remington, et al. (42), produced protection in mice to a moderately virulent strain of Toxoplasma gondii by immunization with Freund complete adjuvant (FCA).

An experiment was performed to determine if mice immunized with viable BCG vaccine were protected against challenge with F. tularensis strain 425 F_4G. Groups of mice were immunized intravenously with viable BCG (4.2×10^7 [viable units]) three weeks previous to challenge with serial tenfold dilution of F. tularensis strain 425 F_4G. Table I and Figure 1 show that immunization with BCG resulted in an increase of 0.4 to 1.3 days in the mean time of death (MTD), but that the mortality ratios and LD_{50} values were the same for normal and BCG immune mice. The non-specific stimulation of the RES with BCG gave virtually no protection against challenge with F. tularensis.

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Table I. Attempt to induce non-specific resistance to subcutaneous challenge with F. tularensis strain 425 F$_4$G in mice immunized 3 weeks previously with viable BCG (4.2 x 10^7 v.u.) administered i.v.

Challenge dose of F. tularensis (v.u.)	BCG immunized mice		Control mice	
	d/t[a]	MTD[b]	d/t	MTD
10^6	14/14	4.7	16/16	4.0
10^5	14/14	5.3	16/16	4.0
10^4	14/14	5.6	16/16	5.0
10^3	14/14	5.4	16/16	5.0
10^2	14/14	6.0	16/16	5.1
10^1	14/14	6.0	16/16	5.1
LD$_{50}$	< 17.7 organisms		<17.7 organisms	

[a] Deaths/total challenged.

[b] Mean time of death in days.

Specific immunity to F. tularensis strain 425 F$_4$G.

Although immunity to facultative intracellular parasites is considered to be a cellular process independent of antibody, some evidence exists which demonstrates a requirement for a specific humoral factor in immunity to tularemia. Larson (43) demonstrated that passive transfer of hyperimmune serum protected rats from challenge with fully virulent F. tularensis. Bell, et al. (4) showed that mice immunized with an antigen (ESA) released after ether treatment of F. tularensis exhibited protection (3.82 logs) to infection with F. tularensis strain 425 F$_4$G. Experiments were performed to determine the degree of protection by mice specifically immunized and challenged with F. tularensis strain
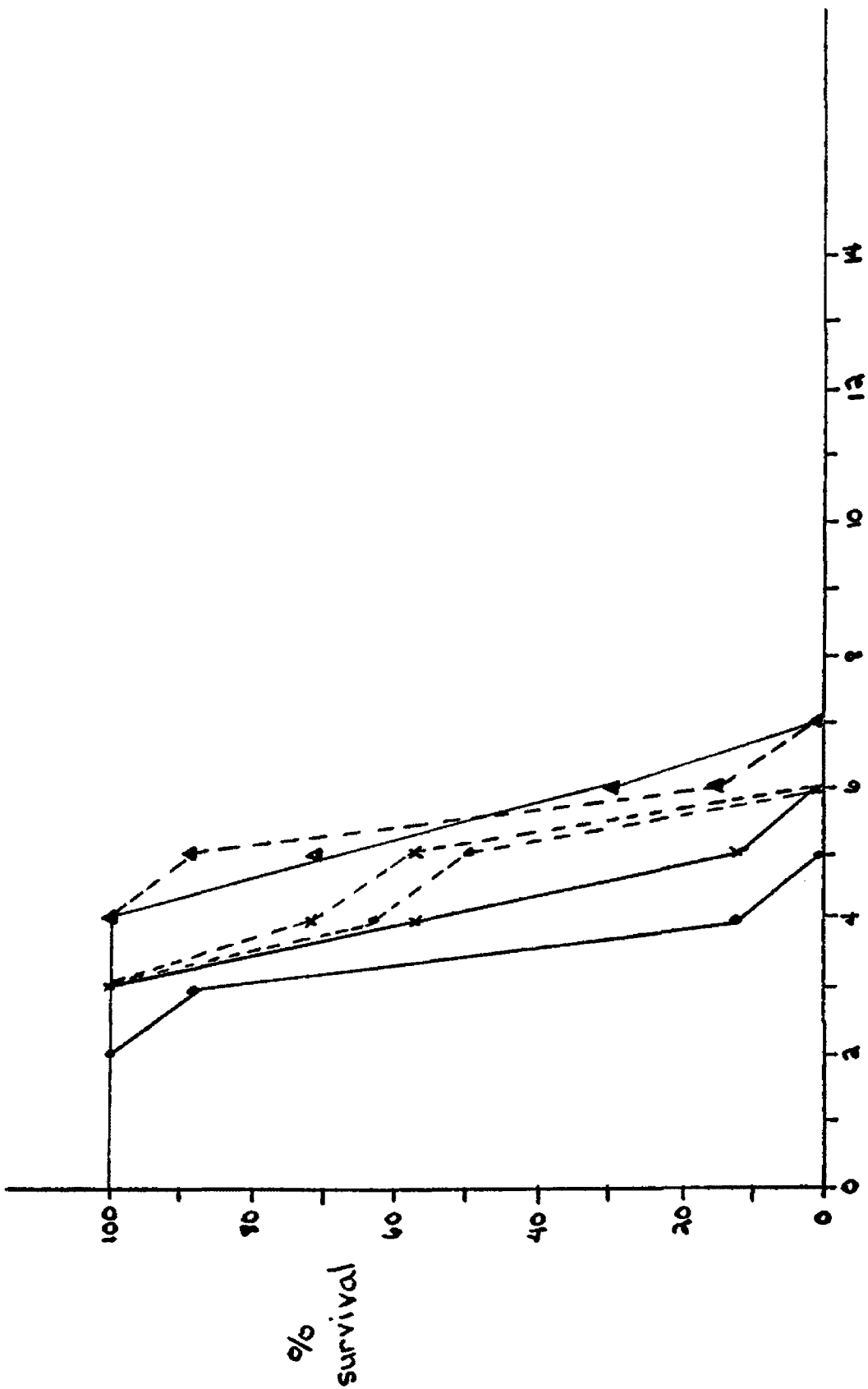
Fig. 1-The effect of BCG immunization (4.2×10^7 v.u.) in mice challenged with serial-dilutions of F. tularensis 425 F_4G. —non-immune 10^6 v.u. challenge —; non-immune 10^7 v.u. challenge •—•; immune 10^6 v.u. challenge x—x; immune 10^5 v.u. challenge x—x; immune 10^4 v.u. challenge —; immune 10^3 v.u. challenge •——•.

425 F_4G.

Experiment 1 - Passive transfer of specific immune serum.

Four hours prior to challenge with serial ten-fold dilutions of F. tularensis strain 425 F_4G, groups of mice were injected with 0.25 ml of adsorbed normal or immune rabbit anti-tularensis serum. Experimental results are shown in Table II and Figure 2. Passive transfer of hyper-immune serum increased the MTD by at least two days and afforded four logs of protection compared to that obtained with normal rabbit serum. This demonstrates a requirement for specific antibody in immunity to tularemia.

Table II. Passive transfer of immunity by i.v. injection of rabbit anti-tularense serum to mice subsequently challenged s.c. with F. tularensis strain 425 F_4G.

| Challenge dose of F. tularensis (v.u.) | Results obtained in mice treated with | | | |
| | Immune Serum | | Normal Serum | |
	d/t[a]	MTD[b]	d/t	MTD
10^6	10/12	5.2	12/12	3.5
10^5	8/12	5.7	12/12	3.5
10^4	6/12	9.2	12/12	4.3
10^3	2/12	8.0	12/12	5.2
10^2	2/12	9.0	12/12	6.1
10^1	2/12	11.0	12/12	6.3
10^0	0/12	-	12/12	6.5
LD_{50}	10^4 organisms		< 1 organism	

[a] Deaths/total challenged.

[b] Mean time of death in days.
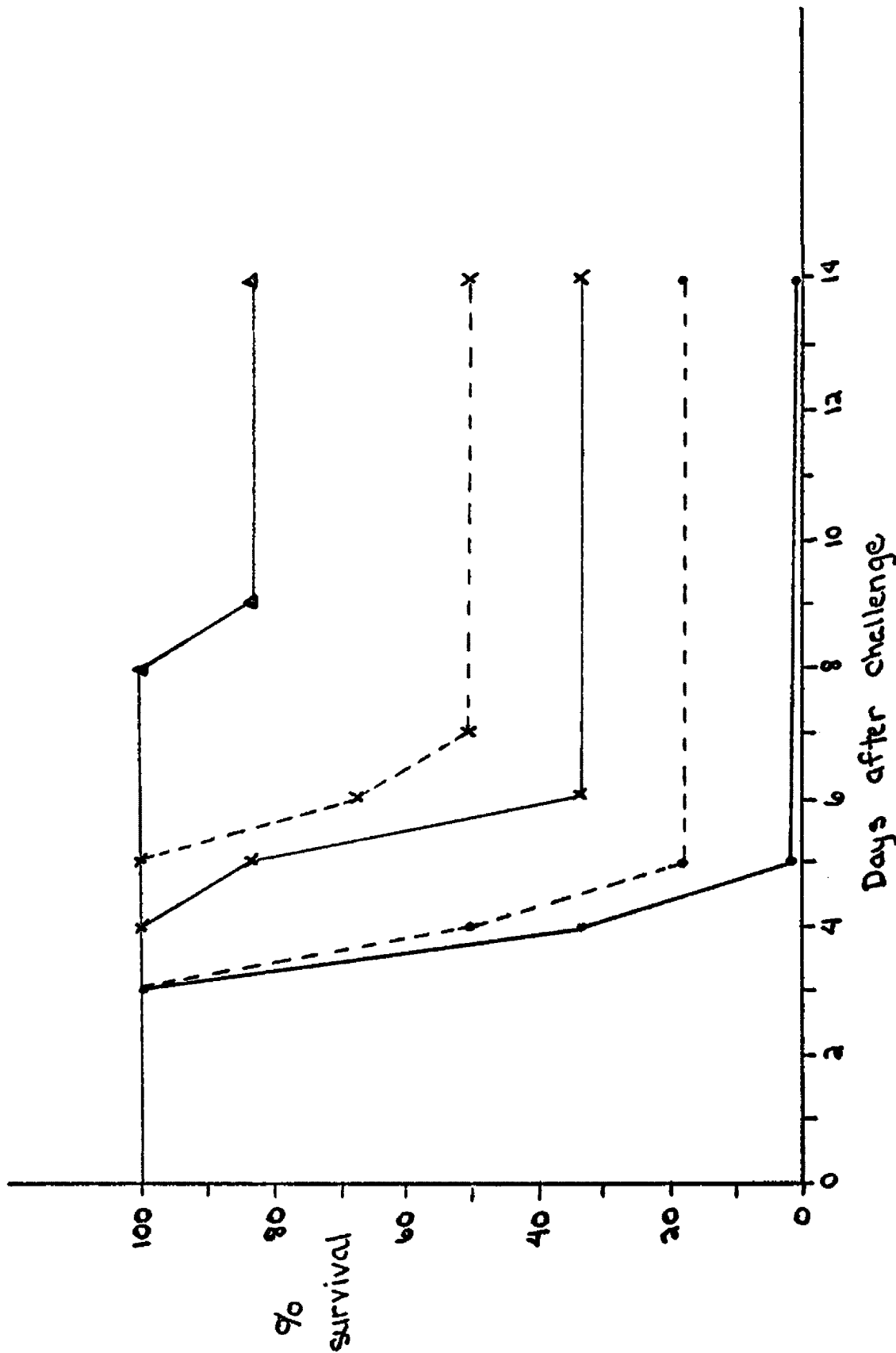
Fig. 2—The effects of passive transfer of 0.25 ml of rabbit anti-tularensis serum into mice challenged s.c. 4 hours after with F. tularensis strain 425 F4G.

Non-immune - 10^4 v.u. challenge ——; immune - 10^5 v.u. challenge ·—·; immune- 10^5 v.u. challenge x——x; immune - 10^4 v.u. challenge x---x; immune - 10^3 v.u. challenge ▲——▲.

Experiment 2 - Active immunization with ether-extracted antigen (EEA).

This experiment was performed to determine the degree of protection afforded by immunization with EEA administered either by the intravenous or intraperitoneal routes. Groups of mice were injected with 0.2 ml of a Klett 100 suspension of EEA from $\underline{F}$. $\underline{tularensis}$ strain 425 F_4G. Two weeks later groups of immune and nonimmune mice were challenged subcutaneously with $\underline{F}$. $\underline{tularensis}$ strain 425 F_4G. The results, shown in Table III and Figures 3 and 4, indicated that immunized mice were resistant to at least 10^3 more organisms than were normal mice. Intraperitoneal immunization appeared to protect mice better than intravenous immunization but the increase was not significant.

Combined specific and non-specific immunity in protection against F. tularensis strain 425 F_4G.

' Allen (1) and Thorpe and Marcus (72, 74) maintained that resistance to tularemia in mice is dependent entirely upon enhancement of the bacteriocidal capacity of immune macrophages. However, Claflin and Larson (13) demonstrated a requirement for a specific live vaccine that produced both an antibody and a cell-mediated response to protect mice from tularemic infection caused by $\underline{F}$. $\underline{tularensis}$ strain 425 F_4G.

Table III. Protective effects of immunization with EEA in mice challenged subcutaneously with serial 10-fold dilutions of F. tularensis strain 425 F_4G.

Challenge dose of F. tularensis $\frac{\text{(v.u.)}}{}$	EEA (i.p.)		EEA (i.v.)		Control	
	d/t[a]	MTD[b]	d/t	MTD	d/t	MTD
10^9	12/16	7.2	16/16	5.8	16/16	4.6
10^8	10/16	5.8	16/16	6.2	16/16	5.4
10^7	10/16	5.9	8/14	6.5	16/16	5.2
10^6	10/16	5.8	8/14	8.0	16/16	5.5
10^5	8/16	7.4	12/18	7.8	16/16	5.6
10^4	6/16	8.0	6/14	9.0	16/16	6.0
10^3	6/16	8.2	4/14	9.2	16/16	6.1
10^2	2/16	7.0	2/14	9.4	16/16	6.1
LD_{50}	10^5		$10^{4.27}$		$< 10^2$	
Logs of protection	$> 10^3$		$10^{2.27}$		-	

Results obtained in mice immunized with

[a] Deaths/total challenged.

[b] Mean time of death in days.

Experiment 1 - The combination of passive immunity and non-specific immunity to tularemia

This experiment was performed to determine if the combined effects of passive transfer of specific immune serum and non-specific stimulation to the RES would be more effective in protecting mice against tularemic
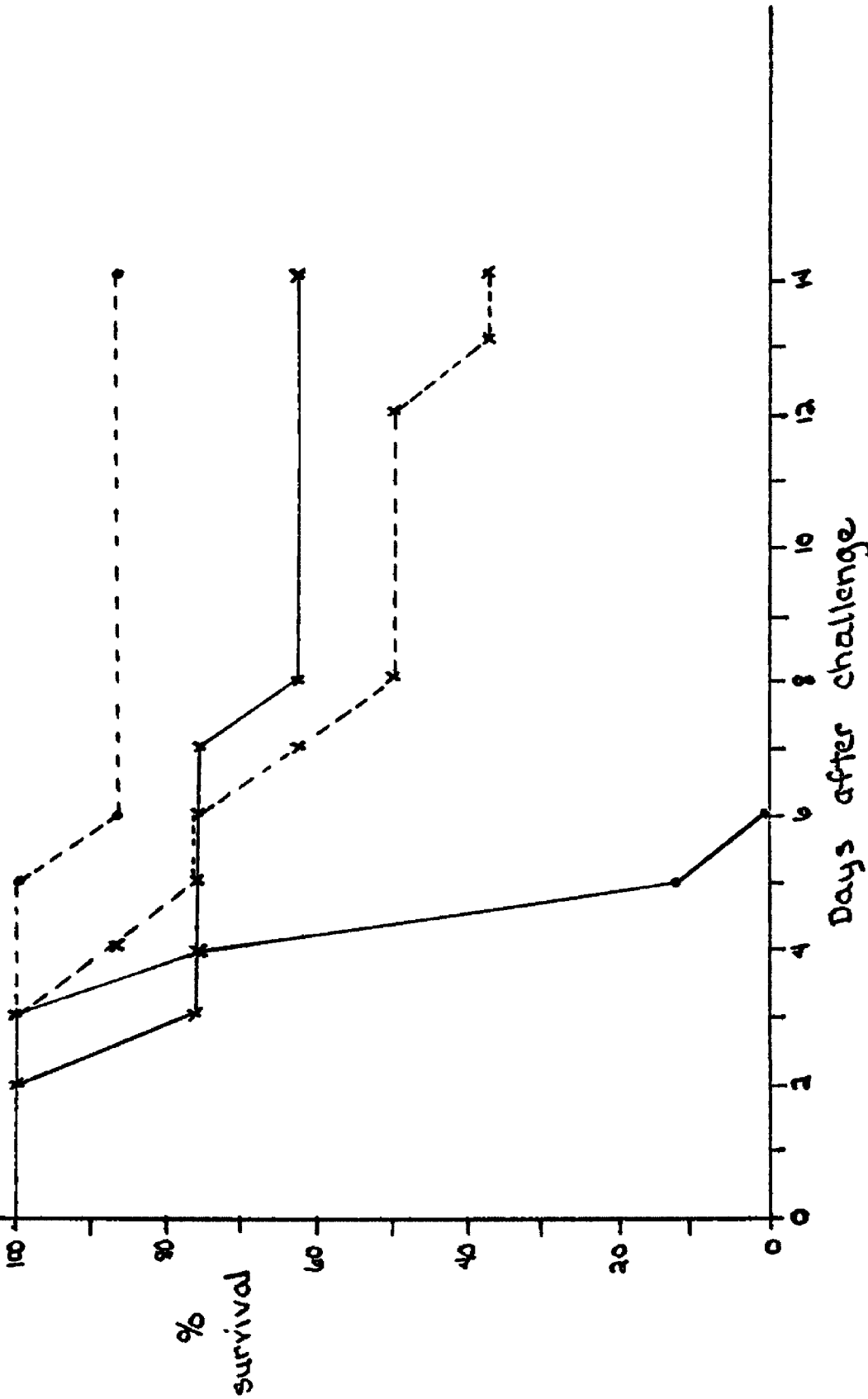
Fig. 3–Protective effects of EPA (0.2 ml-Klett 100) given ip to mice challenged 14 days later with serial dilutions of F. tularensis strain 425 F.G.
Non-immune – 10^2v.u. challenge ———; EPA immune 10^8v.u. & 10^6v.u. challenge x---x;
EPA immune – 10^4v.u. challenge x——x; EPA immune – 10^2v.u. challenge ·—·—·

Fig. 4—Protective effects of EEA (0.2 ml-Klett 100) given iv to mice challenged
14 days later with serial dilutions of F. tularensis strain 425 F4G. Non-immune -
10^v.u. challenge ———; EEA immune - 10^8v.u. challenge ●---●; EEA immune - 10^6v.u.
challenge x——x; EEA immune - 10^4v.u. challenge x——x; EEA immune - 10^2v.u. challenge
●----●.

infection. Groups of normal mice and mice immunized 14 days previously with viable BCG were injected with 0.25 ml of normal-or rabbit-anti-tularensis four hours before challenge with serial ten-fold dilutions of F. tularensis strain 425 F_4G. Results shown in Table IV and Figures 5-8 demonstrated that enhanced RES activity in combination with passive transfer of hyperimmune serum increased the LD_{50} by greater than 2 logs over that attained with immune serum alone.

Experiment 2 - Specific active immunization in combination with non-specific immunization with viable BCG.

Groups of normal mice and mice immunized with EFA, BCG, or both were challenged subcutaneously with serial ten-fold dilutions of F. tularensis strain 425 F_4G. The results from typical experiments are shown in Table V and Figures 9-12. The combined vaccines increase the LD_{50} in mice challenged with F. tularensis strain 425 F_4G by 5 logs to greater than 10^{11} cells.

Table IV. Protective effect of administration of hyperimmune serum and immunization with BCG in mice challenged subcutaneously with serial 10-fold dilutions of F. tularensis str. 425 F_4G.

| Challenge dose (v.u.) | Results obtained from mice treated with | | | | | | | |
| | Normal serum | | BCG | | Immune serum | | BCG + immune serum | |
	d/t[a]	MTD[b]	d/t	MTD	d/t	MTD	d/t	MTD
10^6	12/12	3.5	12/12	5.0	10/12	5.2	0/12	-
10^5	12/12	3.5	12/12	5.5	8/12	5.7	0/12	-
10^4	12/12	4.3	10/12	6.7	6/12	9.2	0/12	-
10^3	12/12	5.2	10/12	7.6	2/12	8.0	0/12	-
10^2	12/12	6.1	12/12	7.6	2/12	9.0	0/12	-
10^1	12/12	6.3	12/12	7.3	2/12	11.0	0/12	-
10^0	12/12	6.5	12/12	6.5	0/12	-	0/12	-
LD_{50}	< 1 organism		< 1 organism		10^4		$> 10^6$	

[a] Deaths per total mice challenged.

[b] Mean time of death.
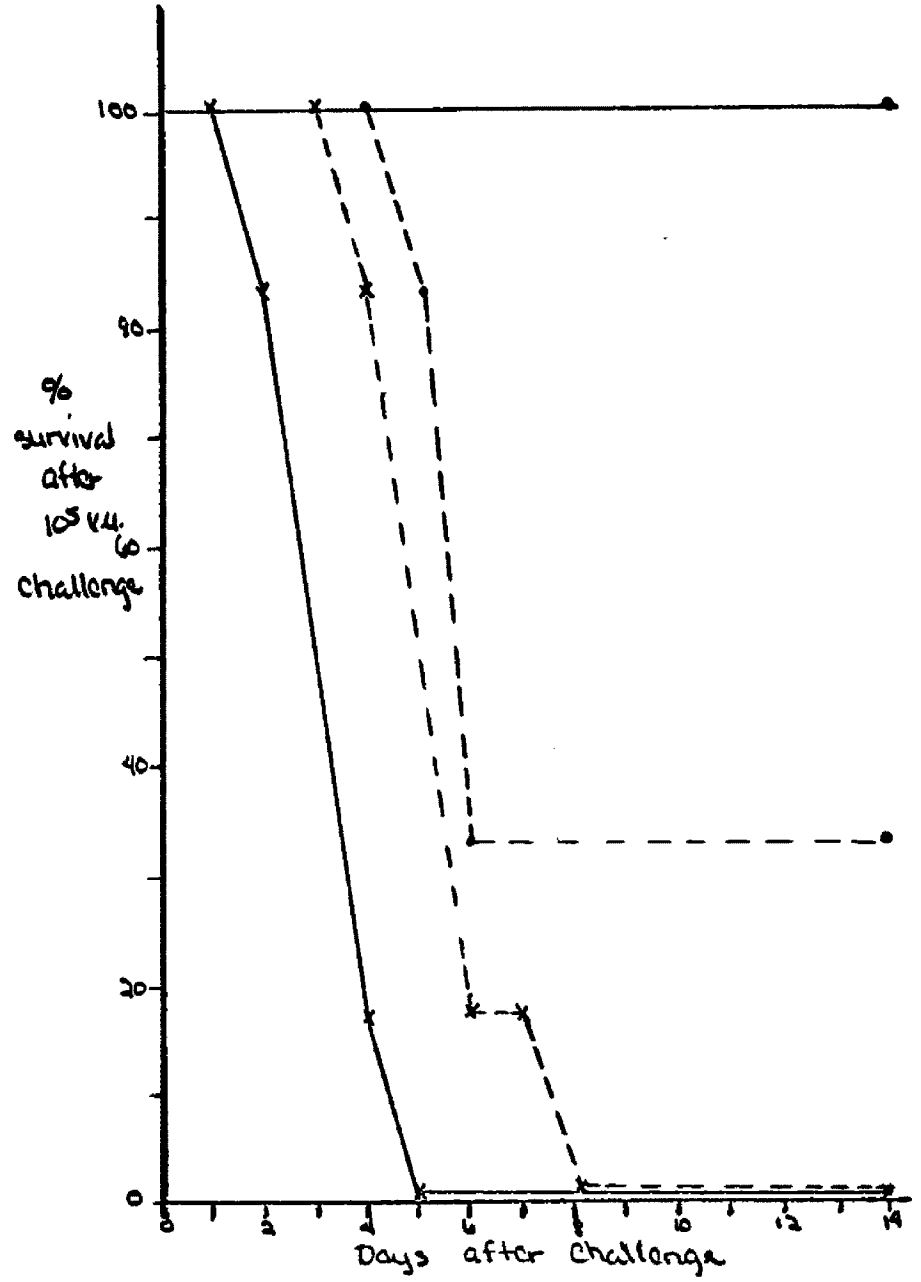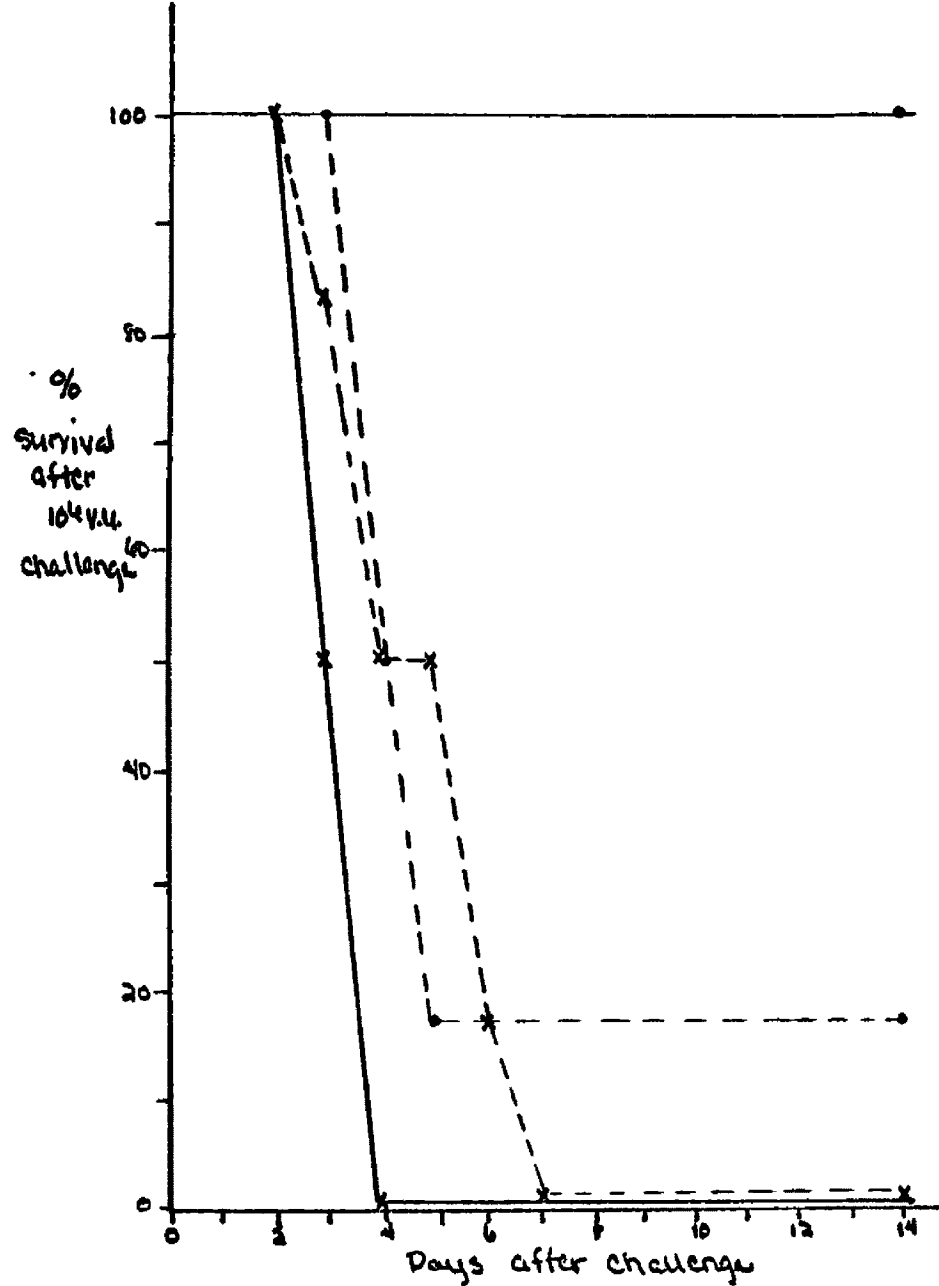
Fig. 5-6 - Effects of passive transfer of 0.25 ml of anti-tularensis serum (iv) to BCG immune mice subsequently challenged with serial dilutions of F. tularensis strain 425 F_4G. BCG+ immune serum ————; immune serum •——•; BCG immune x---x; non-immune x——x.

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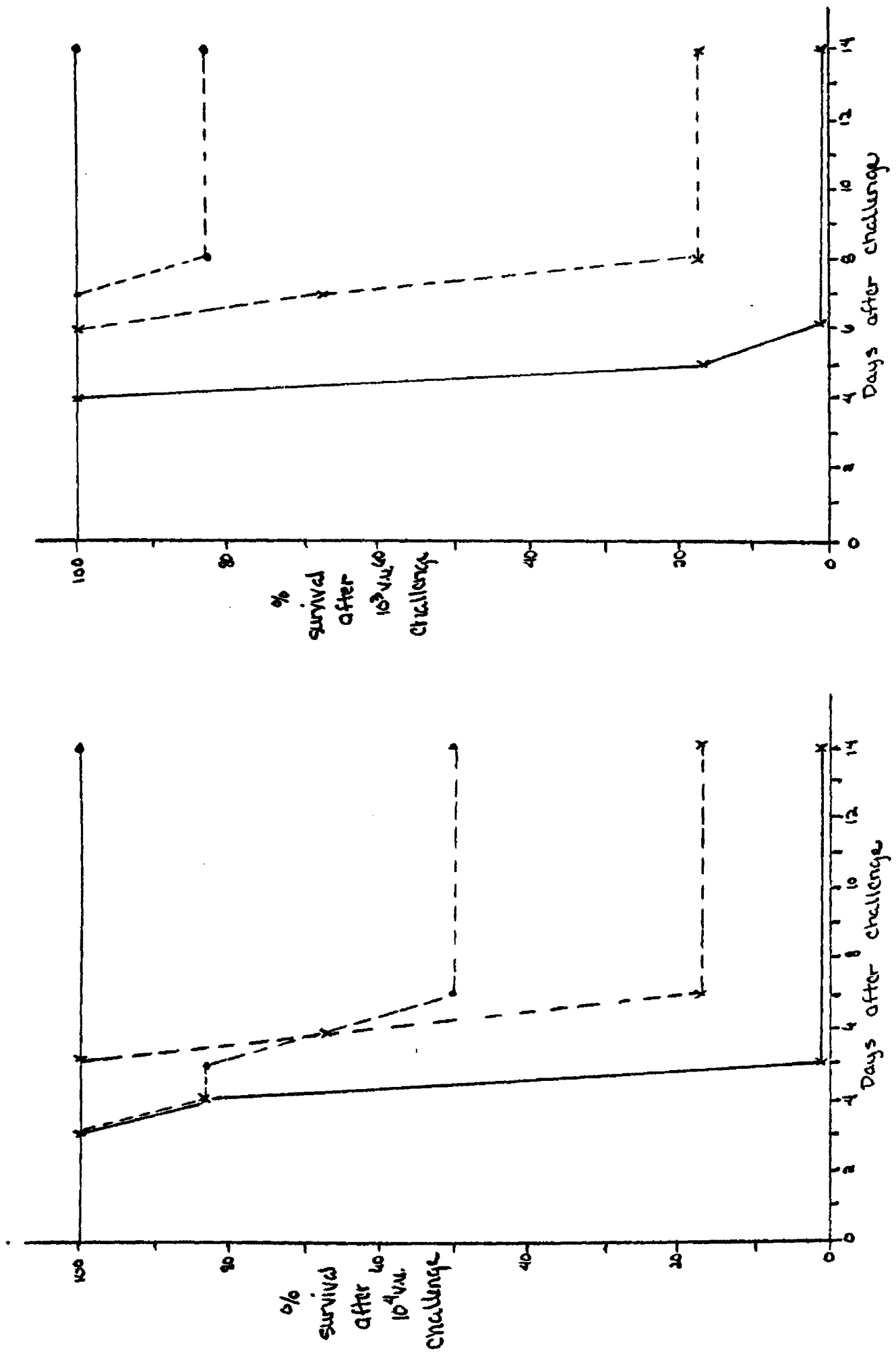
Fig. 7-8 - Effects of passive transfer of 0.25 ml of anti-tularensis serum (iv) to BCG immune mice subsequently challenged with serial dilutions of F. tularensis strain 425 F_4. G. BCG+ immune serum •——•; immune serum •——•; BCG immune x——x; non-immune x——x.

Table V. The effectiveness of specific immunization with ether-treated F. tularensis (EEA) and non-specific immunization with viable BCG[c] on mice challenged subcutaneously with serial 10-fold dilutions of F. tularensis str. 425 F_4G.

Challenge dose (v.u.)	Results obtained from mice treated with							
	Control		BCG + EEA		BCG		EEA	
	d/t[a]	MTD[b]	d/t	MTD	d/t	MTD	d/t	MTD
10^{11}	16/16	3.4	2/16	7.0	16/16	3.9	16/16	5.0
10^{10}	16/16	3.6	2/16	7.2	16/16	3.9	16/16	5.4
10^9	16/16	4.6	2/16	7.2	16/16	4.0	16/16	5.8
10^8	16/16	5.4	0/16	-	16/16	4.3	16/16	6.2
10^7	16/16	5.2	0/16	-	16/17	4.2	9/16	6.5
10^6	16/16	5.5	0/16	-	16/16	4.7	8/16	8.0
10^5	16/16	5.6	0/16	-	16/16	5.3	6/16	7.8
LD_{50}	$<10^5$		$>10^{11}$		$<10^5$		10^6	
Logs of protection	0		$>10^6$		0		10^1	

[a] Deaths per total mice challenged.

[b] Mean time of death.

[c] Klett 200 suspensions of BCG + EEA combined; 0.2 ml injected i.v. 2 weeks before challenge.

Experiment 3 - Effects of different routes of BCG injection on EEA immunized mice.

Hibbs (34) and Hibbs, et al. (35, 36) have observed a greater non-specific protective effect if both BCG and the challenge organisms are injected by the same route. An experiment was performed to determine the effect of different routes of BCG immunization in mice immunized simultaneously with EEA and challenged subsequently with F. tularensis
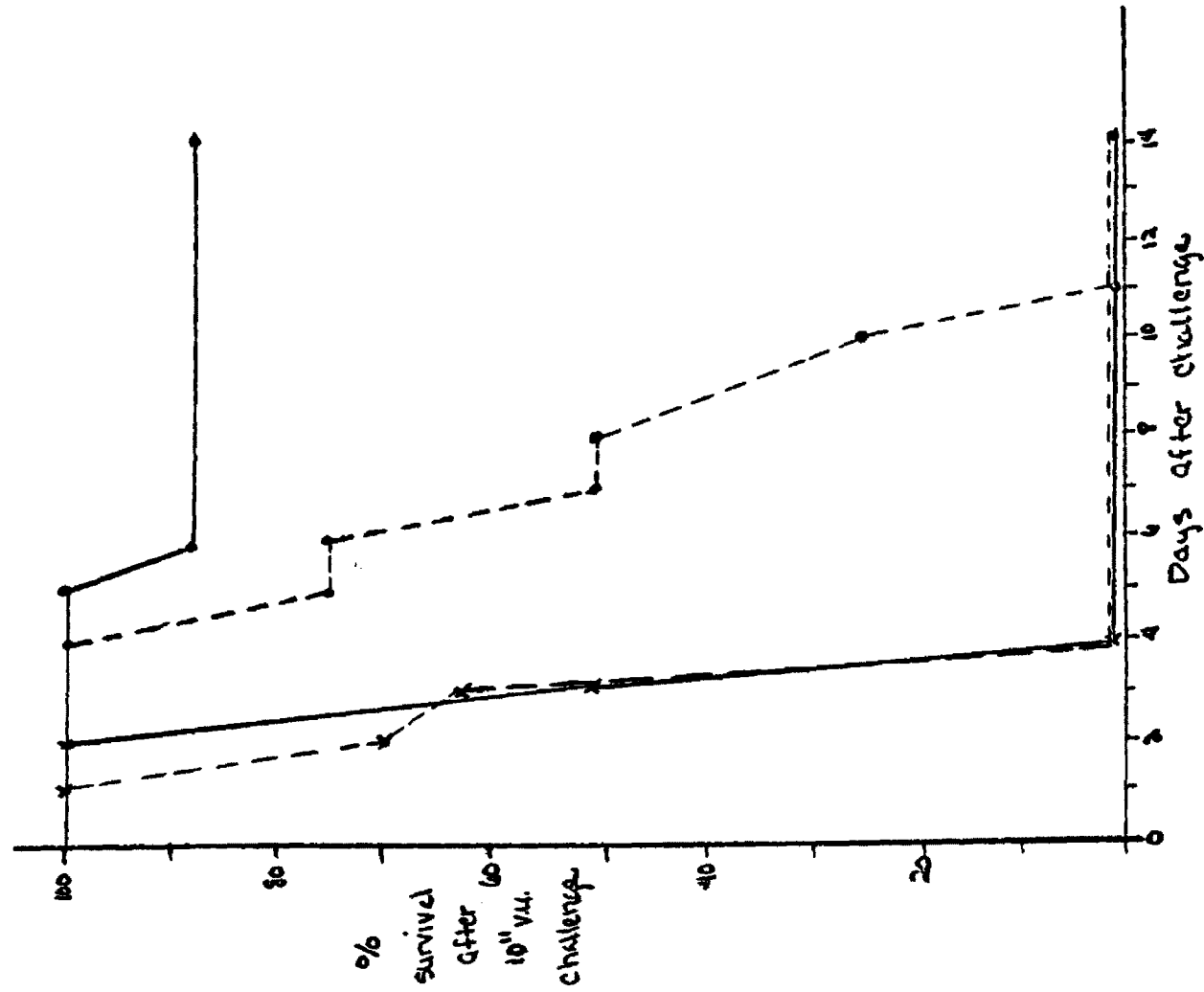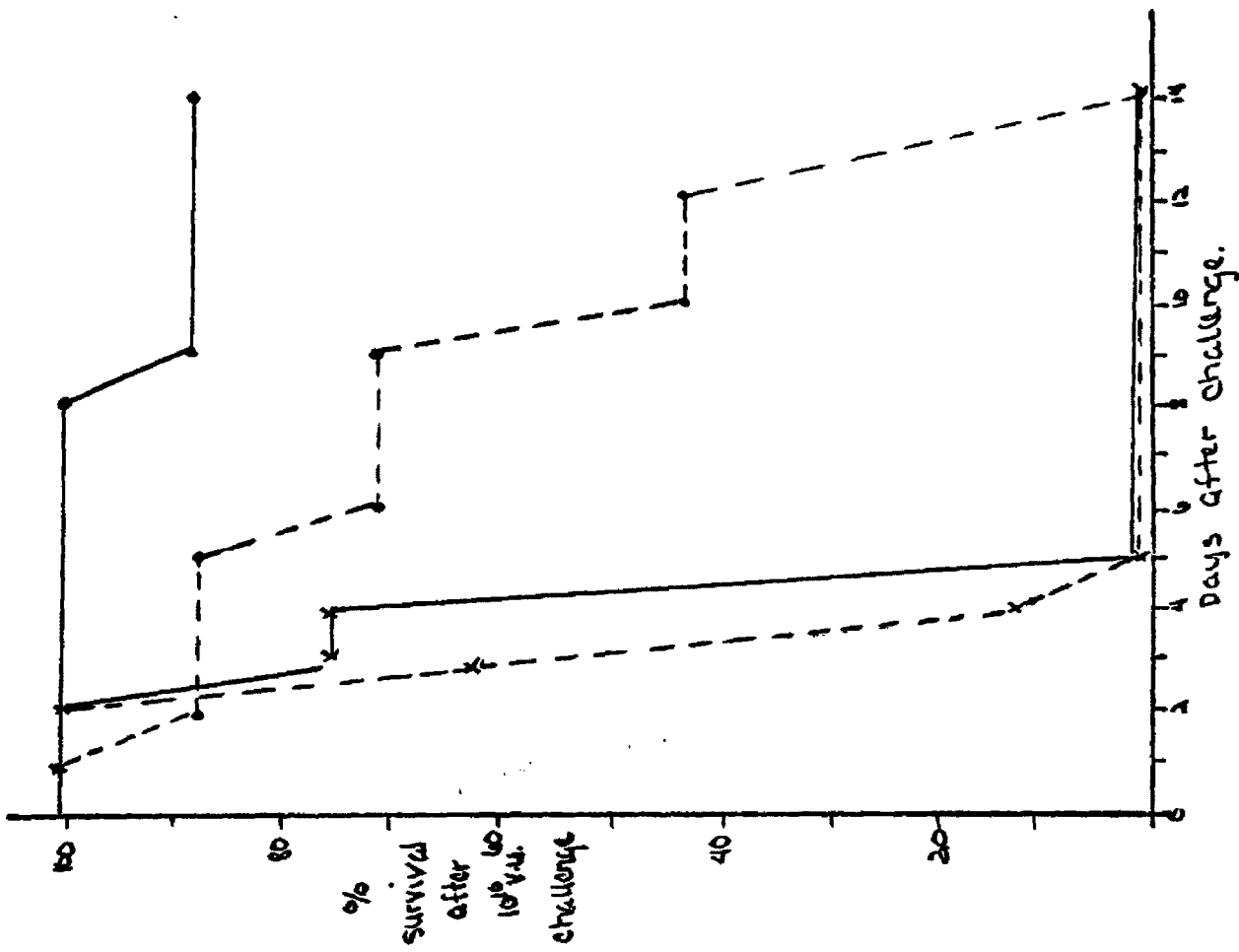
Fig. 9-10 - Effects of a combination of BCG and EEA immunization (0.2 ml Klett 100 iv) in mice challenged with F. tularensis strain 425 F₄G. BCG +EEA ——; EEA immune ·——·; BCG immune x-—-x; non-immune x——x.
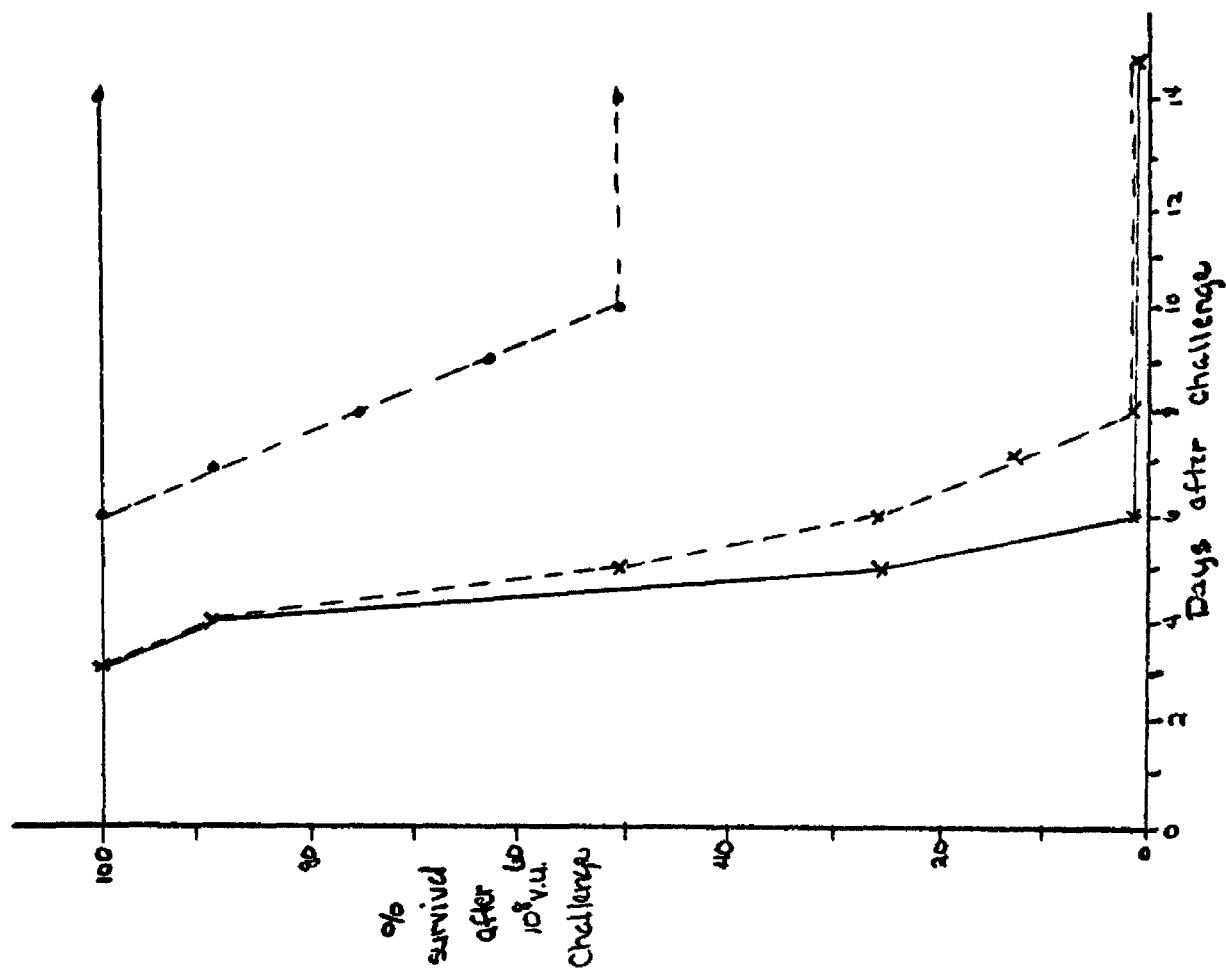
Fig. 11-12 - Effects of a combination of BCG and EEA immunization (0.2 ml Klett 100 iv) in mice challenged with F. tularensis strain 425 F_4G. BCG + EEA immune ——; EEA immune ——·—·; BCG immune x---x; non-immune x——x.

33
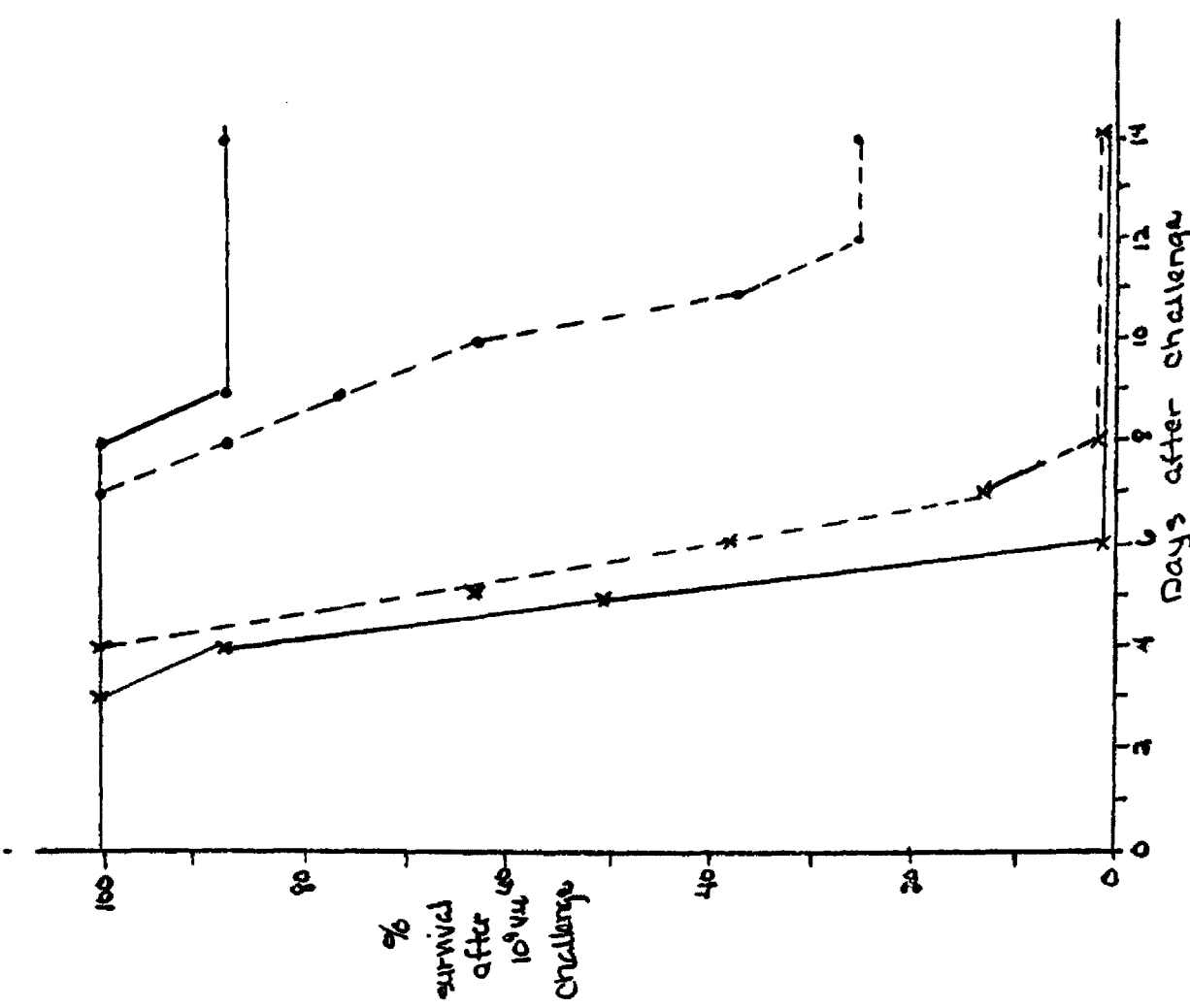
strain 425 F_4G. Groups of mice were injected i.v., i.p., or s.c. with 0.2 ml of a Klett 100 suspension of BCG. At the same time, all groups of mice were immunized i.p. with 0.2 ml of a Klett 100 suspension of EEA. Table VI and Figure 13 represent the data obtained from a typical experiment. Mice immunized intravenously with BCG elicited a greater degree of protection to all routes of challenge, demonstrated by increased LD_{50} values and decreased mortality ratios. Subcutaneous challenge produced the highest LD_{50} values in comparison to other routes of infection. Animals immunized with either BCG or EEA alone did not survive challenges of 10^7 cells of F. tularensis. Mean time of death values from groups of mice challenged with 10^{11} cells demonstrated increased survival times and possibly a greater degree of protection when BCG immunization and F. tularensis challenge was done by the same route.

Table VI. Effects of different routes of BCG immunization on EEA immunized mice challenged i.v., s.c., i.p. with F. tularensis strain 425 F_4C.

mls EEA (i.p.)	Route of BCG Injection[a]	Route of Challenge	Challenge Dose	d/t[b]	MTD[c] (days)
0.2	i.v.	i.v.	10^{13}	8/8	2.0
0.2	i.v.	i.v.	10^{11}	8/8	4.0
0.2	i.v.	s.c.	10^{13}	3/8	5.0
0.2	i.v.	s.c.	10^{11}	0/8	-
0.2	i.v.	i.p.	10^{13}	6/8	2.5
0.2	i.v.	i.p.	10^{11}	4/8	4.3
0.2	s.c.	i.v.	10^{13}	8/8	2.0
0.2	s.c.	i.v.	10^{11}	8/8	2.1
0.2	s.c.	s.c.	10^{13}	5/8	6.8
0.2	s.c.	s.c.	10^{11}	4/8	6.5
0.2	s.c.	i.p.	10^{13}	8/8	2.4
0.2	s.c.	i.p.	10^{11}	8/8	3.8
0.2	i.p.	i.v.	10^{13}	5/5	2.8
0.2	i.p.	i.v.	10^{11}	5/5	3.6
0.2	i.p.	s.c.	10^{13}	2/6	5.0
0.2	i.p.	s.c.	10^{11}	0/6	-
0.2	i.p.	i.p.	10^{13}	7/8	3.4
0.2	i.p.	i.p.	10^{11}	5/8	5.8
0.2	-	i.v.	10^7	4/5	2.8
0.2	-	s.c.	10^7	5/5	4.0
0.2	-	i.p.	10^7	5/5	4.0

[a] 0.2 ml Klett 100.

[b] Death/total challenged.

[c] Mean time of death.
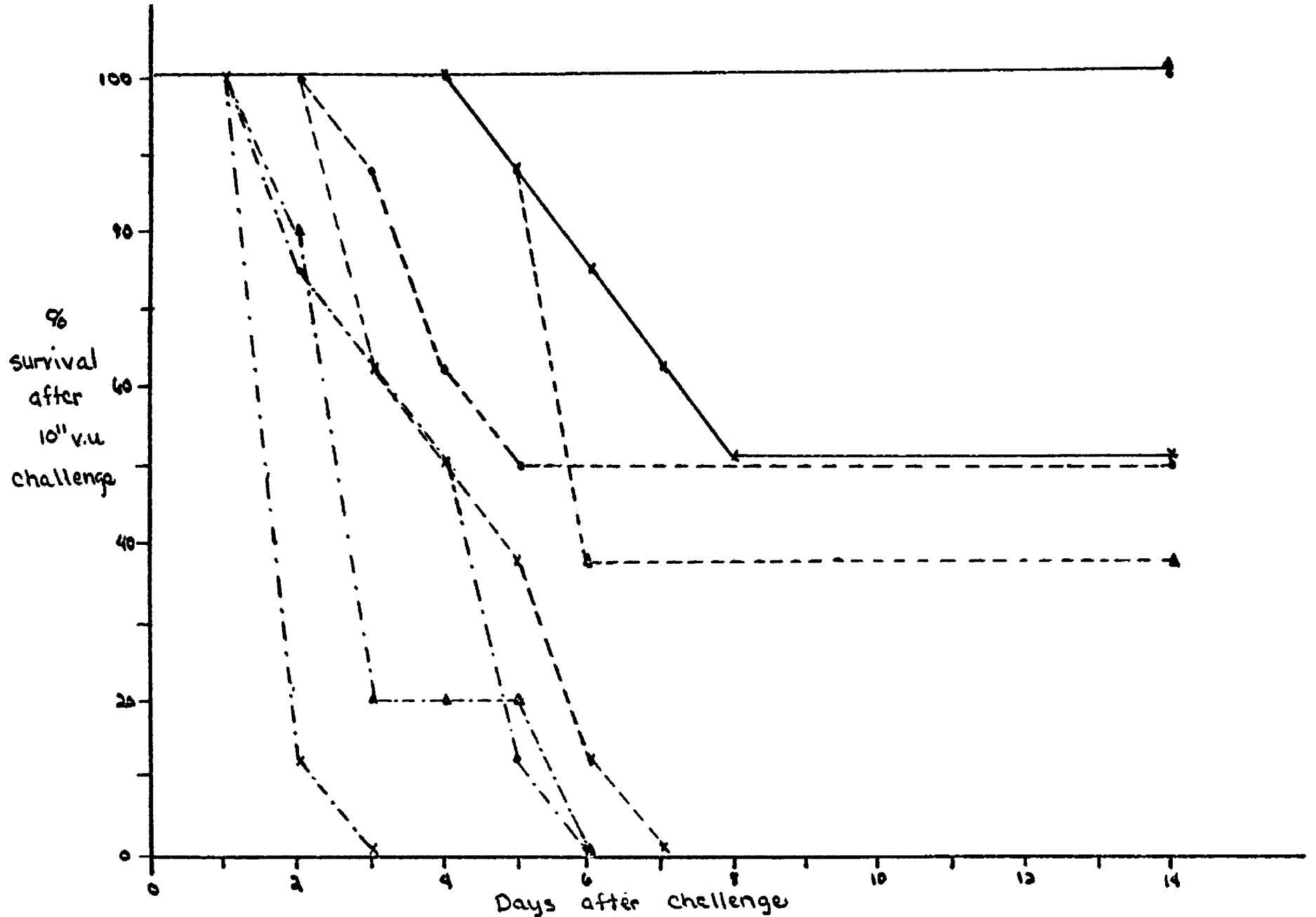
Fig. 13-Effects of different routes of BCG immunization (0.2 ml Klett 100) in EEA mice challenged iv, ip, and sc with F. tularensis strain 425 F$_4$G. BCG sc, challenge x-·-x, ip x- -x, sc x——x; BCG ip, challenge iv ▲-·-▲, sc ▲——▲; BCG iv, challenge iv ⌣·⌣, ip ⌣ ⌣, sc ·——·.

Experiment 4 - Active immunization with EEA in combination with non-specific immunization with heat-killed Bordetella pertussis.

This experiment was performed to determine if the protective effect observed from the combination of BCG and EEA immunization was unique to that non-specific stimulant or if stimulation of the RES by other substances would produce similar results. Groups of mice were immunized with heat-killed B. pertussis, EEA or both and challenged with F. tularensis strain 425 F_4G in the manner described in Experiment 2 of this section. Experimental results shown in Table VII and Figures 14-17 indicate that specific antibody in combination with stimulation of the RES by either a viable or a killed stimulant are responsible for immunity to tularemia caused by F. tularensis strain 425 F_4G.

Table VII. The effectiveness of sepcific immunization with ether-treated F. tularensis (EEA) and non-specific immunization with heat-killed Bordetella pertussis on mice challenged with serial 10-fold dilutions of F. tularensis 425 F_4G.

| Challenge dose (v.u.) | Results obtained from mice treated with | | | | | | | |
| | Control | | B. pertussis + EEA | | B. pertussis | | EEA | |
	d/t[a]	MTD[b]	d/t	MTD	d/t	MTD	d/t	MTD
10^{10}	-	-	2/10	6.5	-	-	4/7	6.3
10^8	-	-	0/8	-	10/10	3.5	4/7	8.4
10^6	8/8	4.5	0/8	-	6/8	3.6	6/9	7.7
10^4	8/8	4.5	0/8	-	8/8	4.9	3/7	9.0
10^2	8/8	4.5	-	-	7/8	5.0	3/7	9.2
LD_{50}	$< 10^2$		$> 10^{10}$		$< 10^2$		$10^{4.3}$	

[a] Deaths per total mice challenged.

[b] Mean time of death.
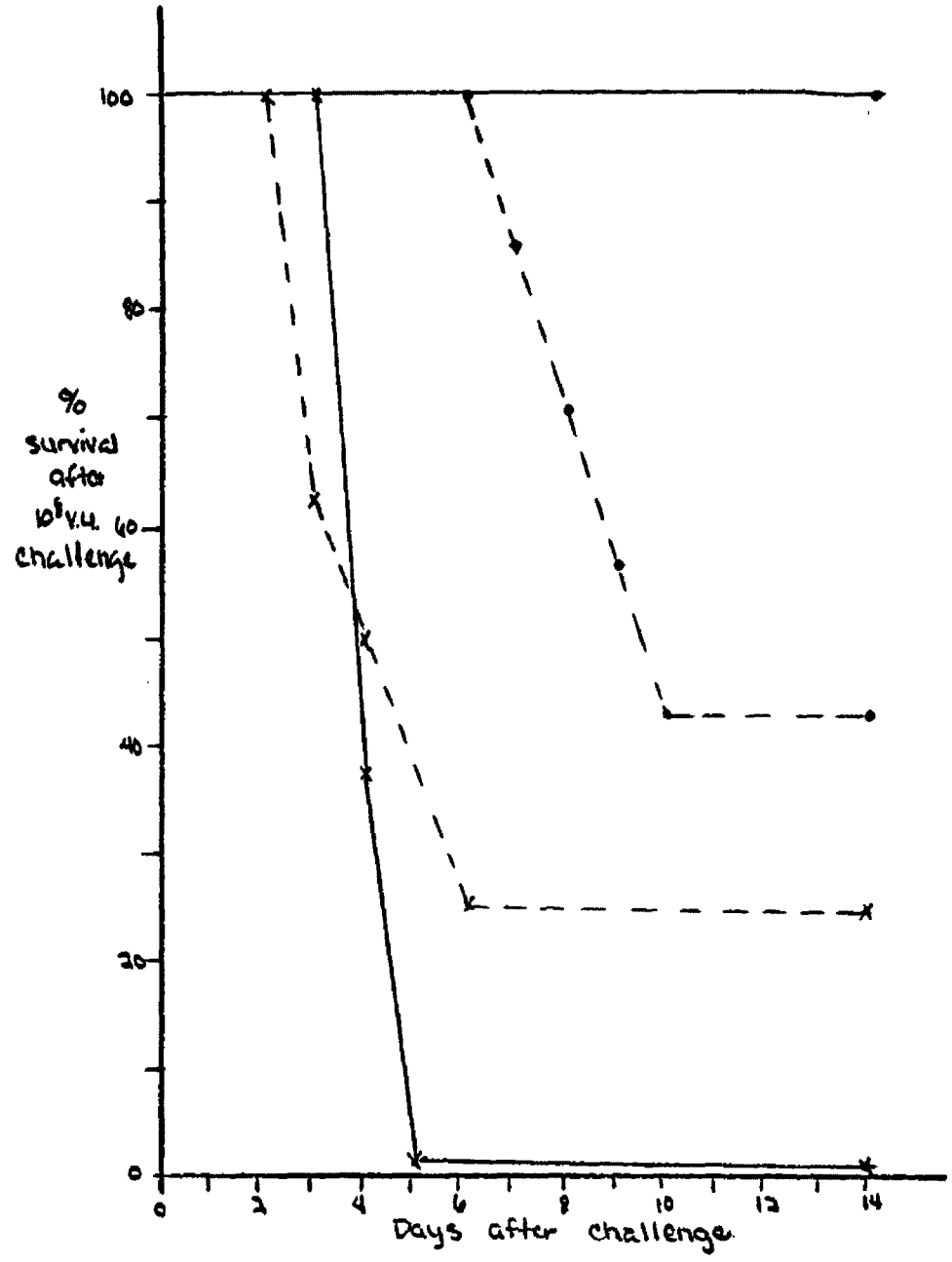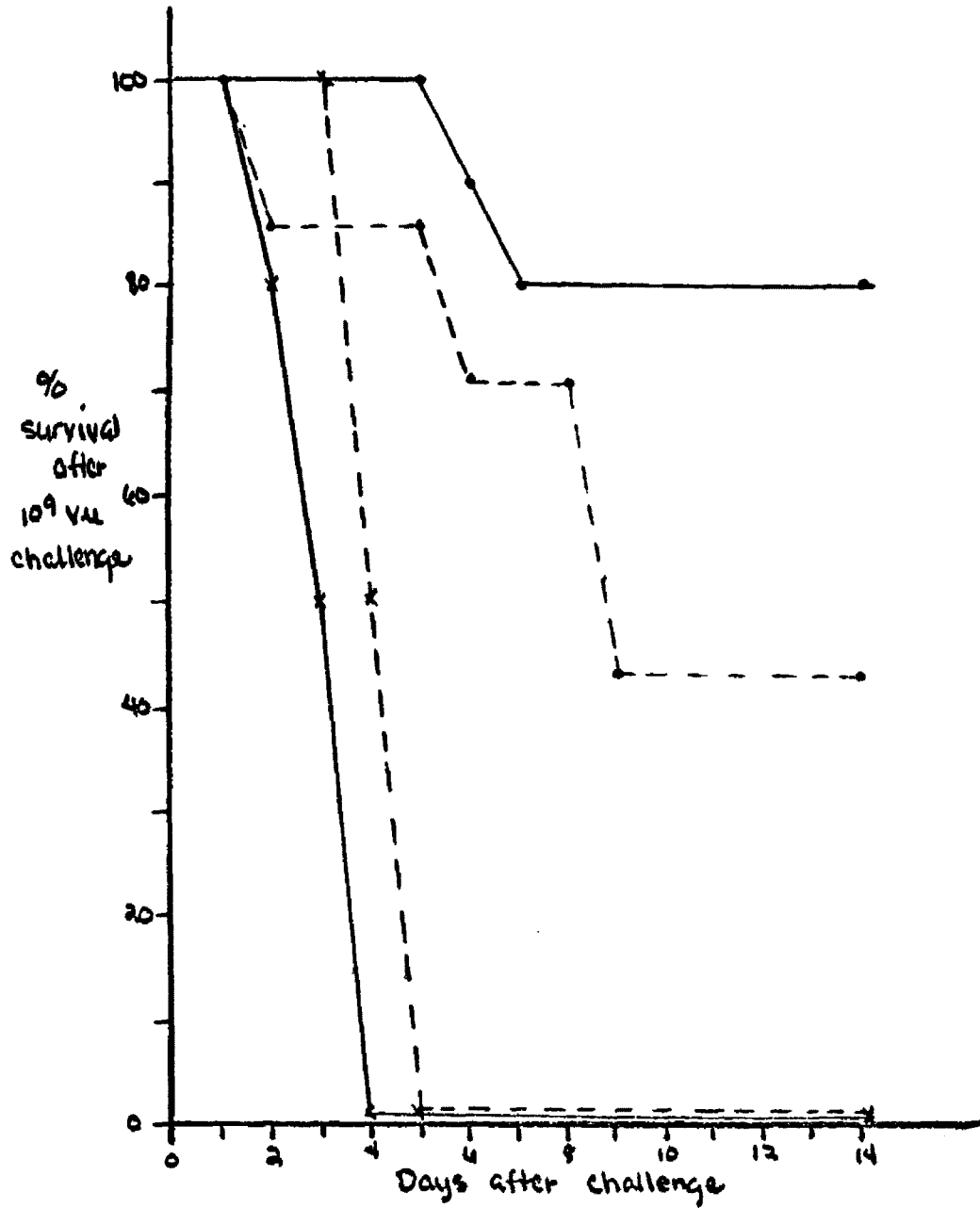
Fig. 14-15 - Protection induced by the combination of <u>B. pertussis</u> + EEA in mice challenged with F. tularensis strain 425 F$_4$G. <u>B. pertussis</u> + EEA immune ——— ; EEA immune —— —— ; <u>B. pertussis</u> immune x— —x; non-immune x——x.

38
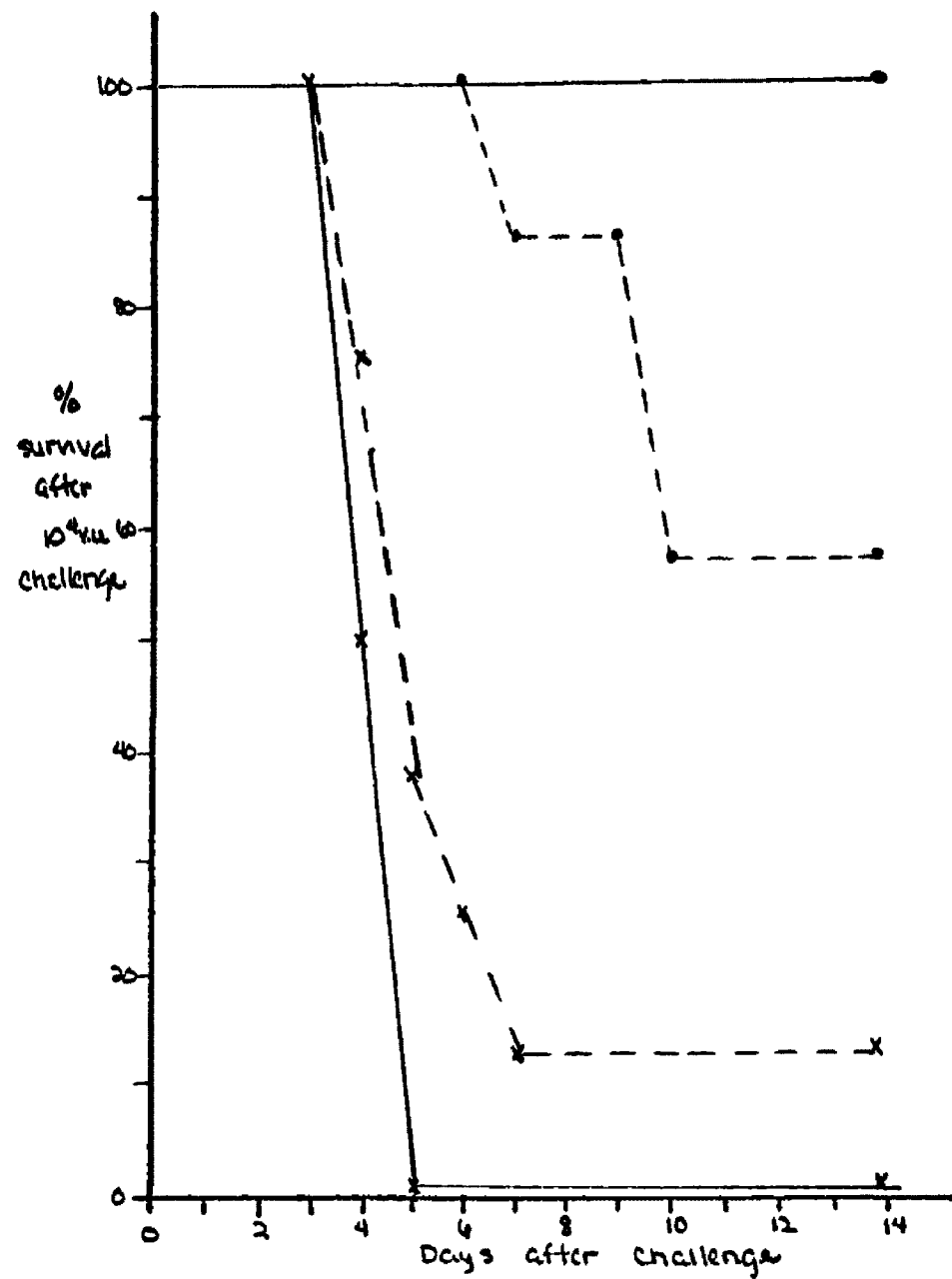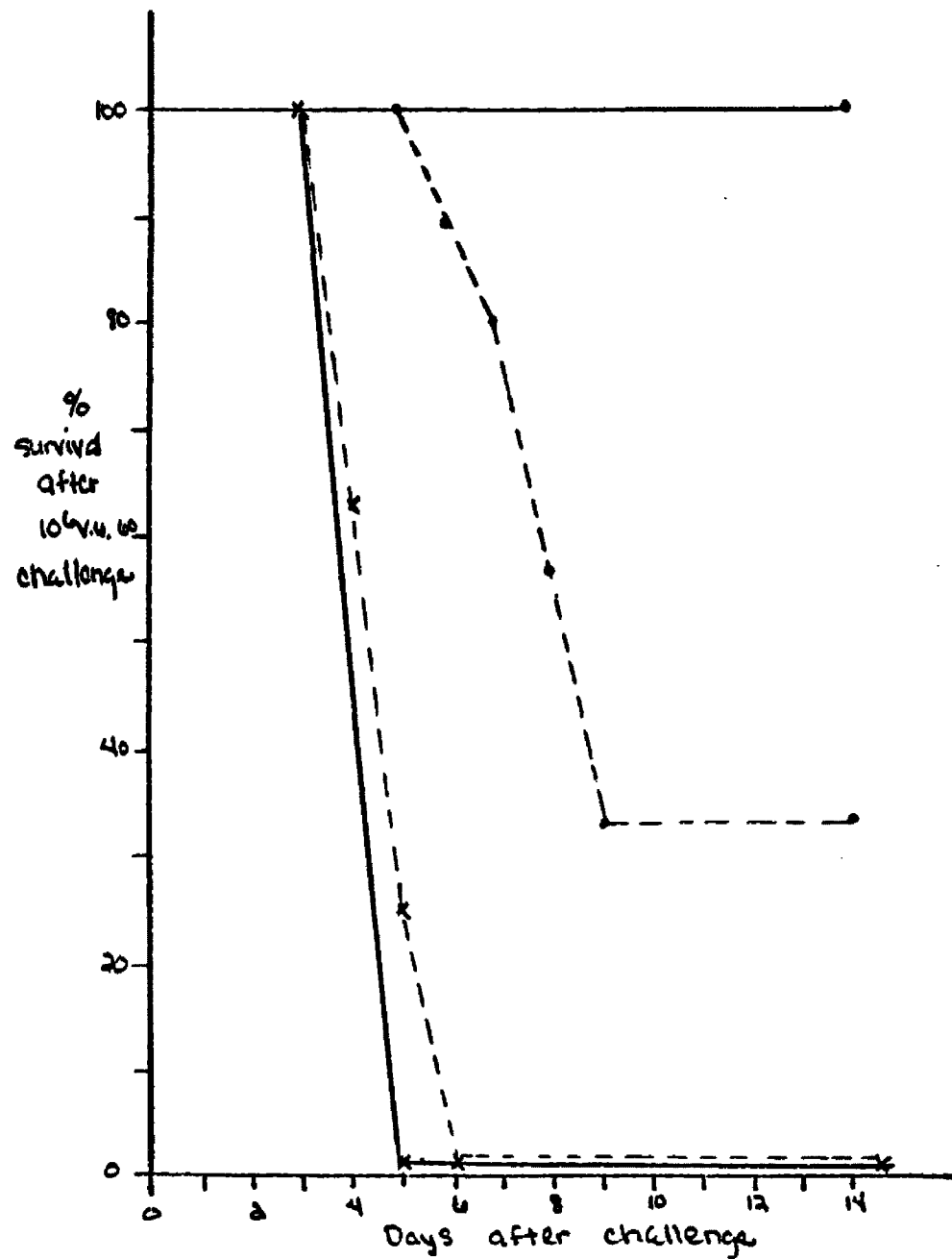
Fig. 16-17 - Protection induced by the combination of B. pertussis and EEA in mice challenged with
F. tularensis strain 425 F₄C. B. pertussis + EEA immune ———; EEA immune -----; B.
pertussis immune x---x; non-immune x——x.

Adoptive transfer of immunity to F. tularensis strain 425 F_4G.

Adoptive transfer of immunity has been used classically to demon-strate cell-mediated immunity. Allen (1), Thorpe and Marcus (74), and Claflin and Larson (13) demonstrated adoptive transfer of immunity to tularemia. Transfer of BCG-EEA immune spleen cells with and without immune serum did not transfer immunity to F. tularensis strain 425 F_4G. A modification of the method of Allen (1) was employed. Cells from the spleens of animals immunized i.v. with 0.2 ml Klett 100 suspension of BCG-EEA 2 weeks earlier were injected i.p. into normal mice. These mice were treated i.p. with either immune or normal serum and were challenged 4 hours later. The results obtained from the adoptive transfer experi-ment are shown in Table VIII. Extension in MTD is observed in mice receiving immune cells and serum, but the mortality ratios are similar in all groups. The results of this experiment are over-shadowed by the fact that heterogeneic, rather than allogeneic animals were used. Also, in many cases mice must be irradiated to create areas in the spleen for the transferred cells to locate.

Table VIII. Adoptive transfer of immunity to F. tularensis strain 425 F_4G with spleen cells.

Cells transferred (5.0 x 10^8 i.p.) to recipient mice	Serum transferred (0.25 mls i.v.)	Challenge dose (v.u.)	d/t[a]	MTD[b]
immune spleen cells	Immune	10^4	8/8	5.4
immune spleen cells	Immune	10^2	8/8	5.7
immune spleen cells	Normal	10^4	8/8	4.4
immune spleen cells	Normal	10^2	8/8	4.4
normal spleen cells	Immune	10^4	8/8	3.4
normal spleen cells	Immune	10^2	8/8	3.5
normal spleen cells	Normal	10^4	8/8	3.0
normal spleen cells	Normal	10^2	8/8	3.0
immune spleen cells	---	---	3/8	5.0
normal spleen cells	---	---	2/8	5.0
BCG-EEA immune donor mice	---	10^4	0/8	---

[a] Deaths/total challenged.

[b] Mean time of death.

Evaluation of the immune response produced by vaccination with viable BCG and EEA.

Experiment 1 - Production of antibody

Bacterial agglutination tests were performed on serum from immune mice to determine if the antibody titer correlated with the degree of protection afforded by the various vaccines previously mentioned. Serum was collected by cardiac puncture or from the axial vein from groups of mice (10) immunized i.v. with BCG, EEA or both. The mice were boosted with EEA 7 days after primary immunization and were bled 14 days after boosting. The results shown in Figure 18 indicated no difference in

the antibody titers of mice immunized with EEA or the BCG-EEA complex, indicating a lack of correlation between antibody titer and the degree of protection.

Guinea pigs also, were immunized with the three antigens. Antibody titers of EEA immunized and BCG-EEA immunized animals were the same (Fig. 19).

Experiment 2 - Induction of immediate and delayed hypersensitivity.

States of hypersensitivity have been shown to correlate closely with types of immune responses, i.e. humoral or cellular. Immediate hypersensitivities have been shown to result from effects of antigen-antibody reactions whereas, delayed hypersensitivities correlate closely with cell-mediated immunity. Studies of such hypersensitivities could reveal factors responsible for the immune response in question.

Groups of unimmunized mice and mice immunized with BCG, EEA and both were tested by injection of 0.03 ml (Klett 50) of EEA into the hind footpad. Increases in the thickness of the footpad injected with the skin test antigen over the other hind footpad injected with 0.03 ml of saline were recorded. Results are indicated in Figure 20. Mice immunized with both BCG and EEA gave positive skin tests of both the immediate and delayed types. The reaction increased in size at two hours, peaked at 4 hours, was reduced in size at twelve hours, but increased again at 24 hours. This reaction had decreased in size by 48 hours and was negligable by 72 hours. Mice injected with EEA alone showed an immediate reaction but a delayed reaction was not observed.

In order to define more satisfactorily the skin reactions observed in mice, groups of four guinea pigs were injected with BCG, EEA, or both.

Agglutination titer

256
128
64
32
16
8
4
0

Days after second booster
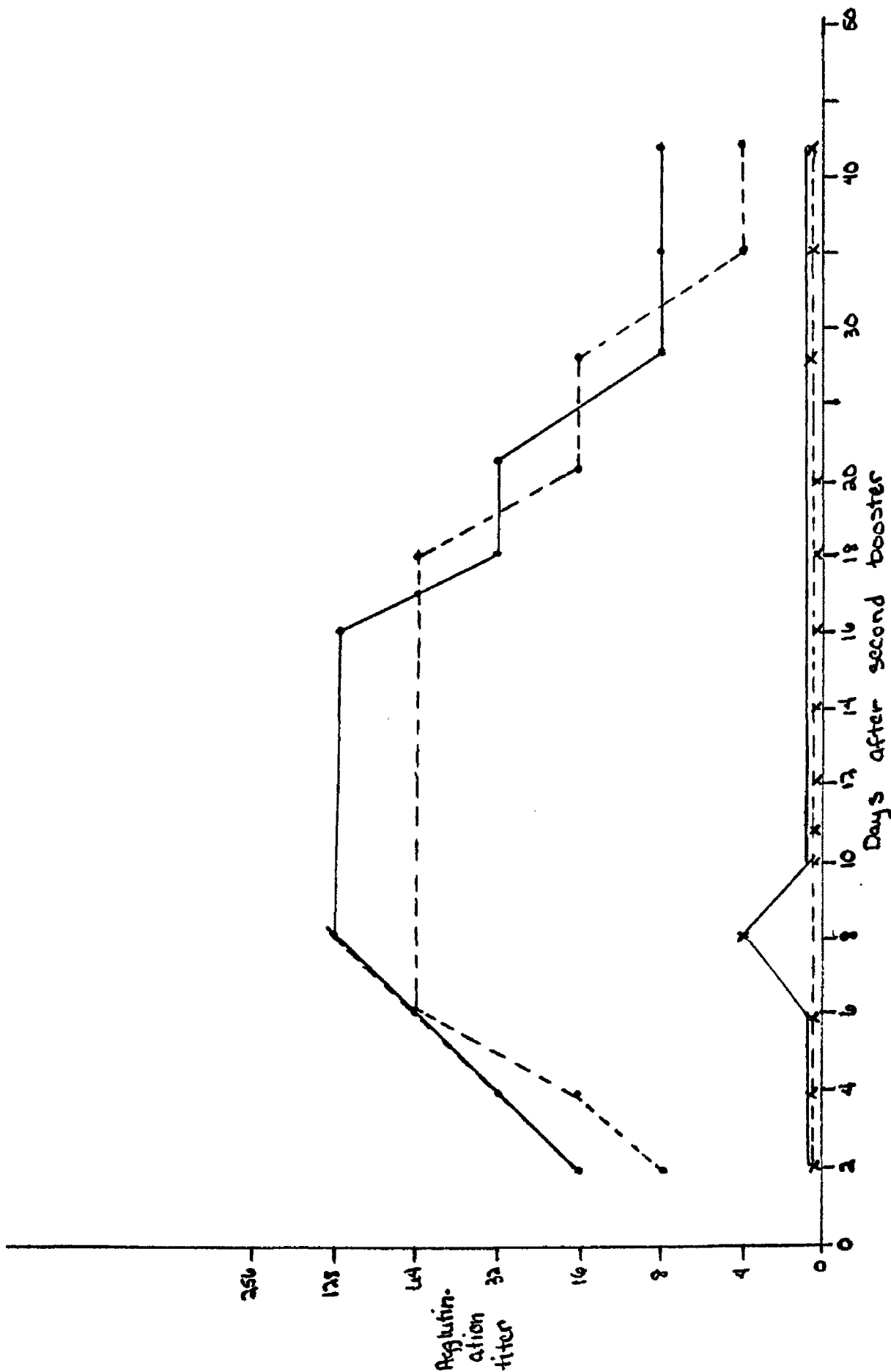
Fig. 18—Temporal production of antibody mice immunized with 0.2 ml of a Klett 100 suspension (iv) of BCG, EEA or both. BCG-EEA immune ———; EEA immune +———; BCG immune x———x; non-immune x---x.
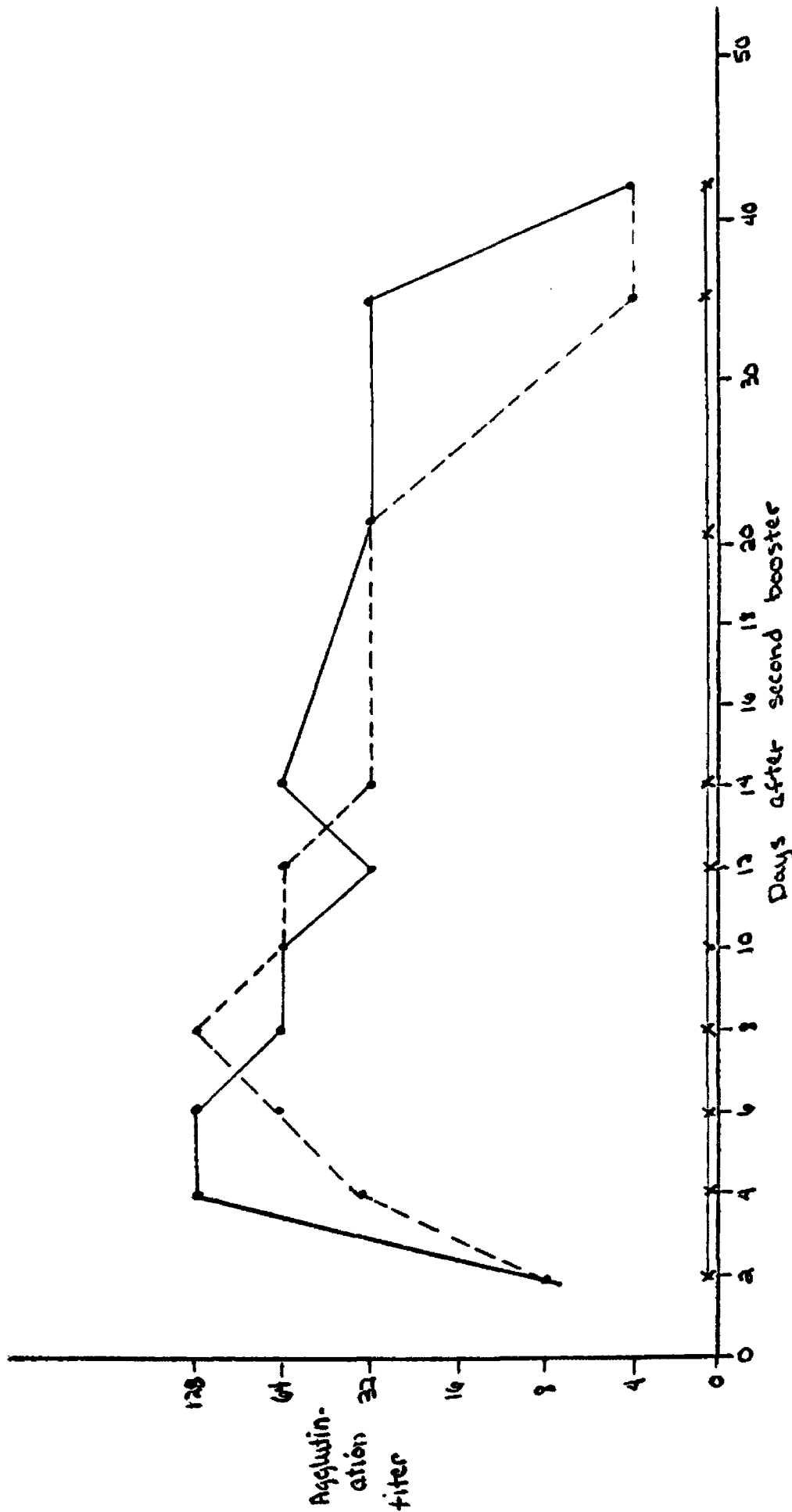
FIG. 19—Temporal production of antibody in guinea pigs immunized with 0.2 ml of a Klett 100 suspension of BCG, EEA, or the combination. BCG-EEA ———— ; BCG *———*; EEA *– – –* ; non-immune *———*.
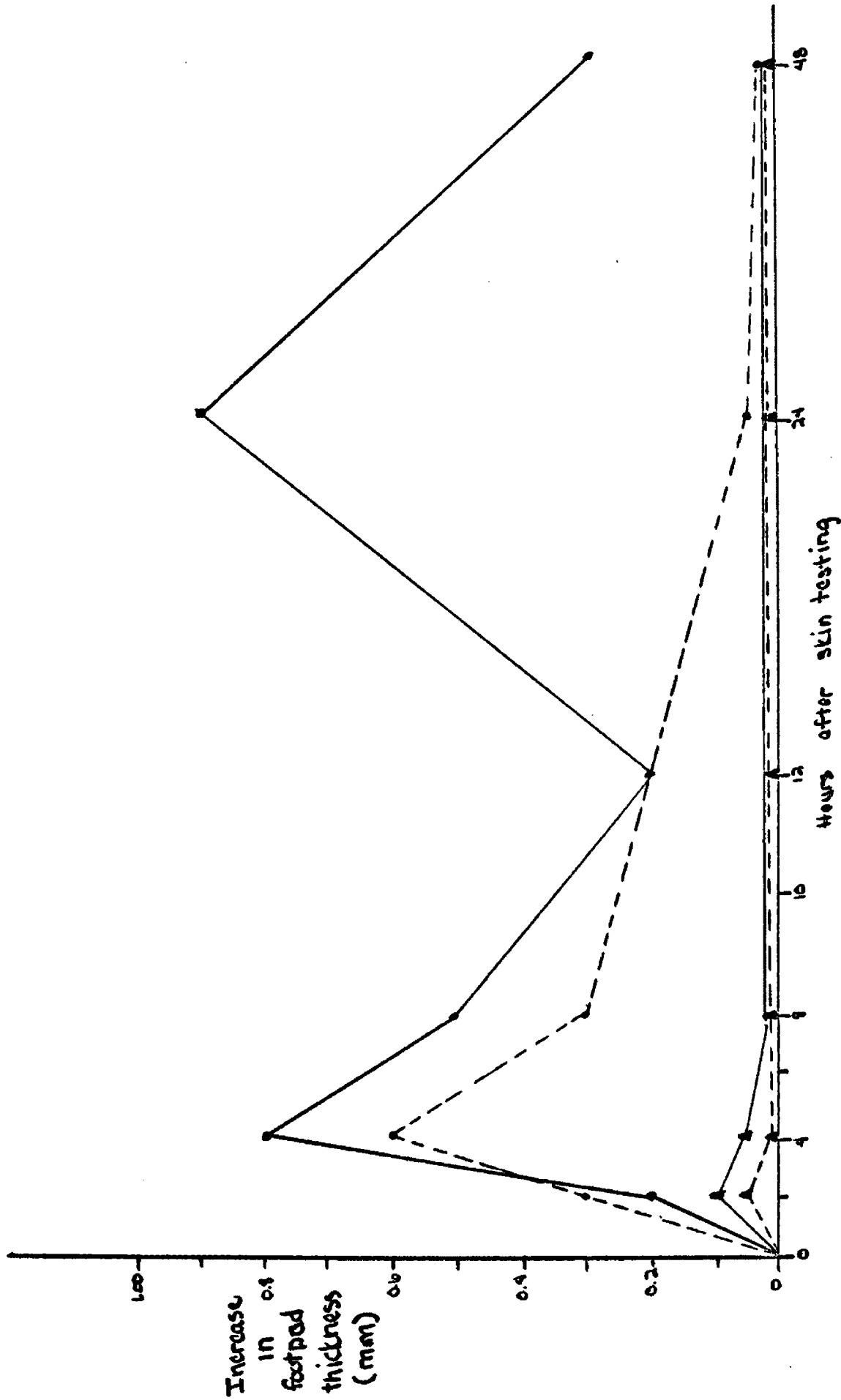
Fig. 20 – Time course of foot pad reactivity in mice immunized with 0.2 ml of a Klett 100 suspension of BCG-EEA, BCG, or EEA. Each point represents the mean of 10 mice after injection of 0.03 ml of EEA (Klett 50) BCG-EEA immune ———— ; EEA immune ————— ; BCG immune ————— ; non-immune ————— .

The skin reactions observed in mice correlated to those observed in guinea pigs. Table IX indicates the data obtained from 24 hour skin tests in groups of guinea pigs immunized with the three antigens. Delayed skin tests were observed following injection of as little as a Klett 10 suspension of EEA.

Table IX. Twenty-four hour skin tests in guinea pigs immunized with viable BCG, ether-treated F. tularensis or both.

1° Antigen	Skin test Antigen	Width (mm)	Mean Lesion Dimensions Length (mm)	Thickness (mm)	Volume (mm^3)
BCG-EEA	EEA	18	18	25	303.8
BCG only	EEA	2	2	0.1	0.2
EEA	EEA	4	4	0.3	1.8
None	EEA	2	2	0.1	0.2

The efficacy of heat-killed B. pertussis and viable BCG in combination with EEA as inducers of delayed-type hypersensitivity was studied in mice. Groups of mice were immunized with B. pertussis, BCG, EEA, and B. pertussis and EEA, or BCG and EEA. Fourteen days after immunization groups of ten mice were skin tested. The results are shown in Fig. 21. The BCG and EEA combination was the only vaccine tested that induced a delayed hypersensitivity skin reaction, however B. pertussis-EEA and EEA produced immediate hypersensitivity only.

MIF Analysis

To elucidate further the delayed hypersensitivity reaction involved in the BCG-EEA system, in vitro correlations of delayed hypersensitivity were performed. David (19) and David, et al. (20) and Bloom and

Increase in footpad thickness (mm)

Hours after skin test
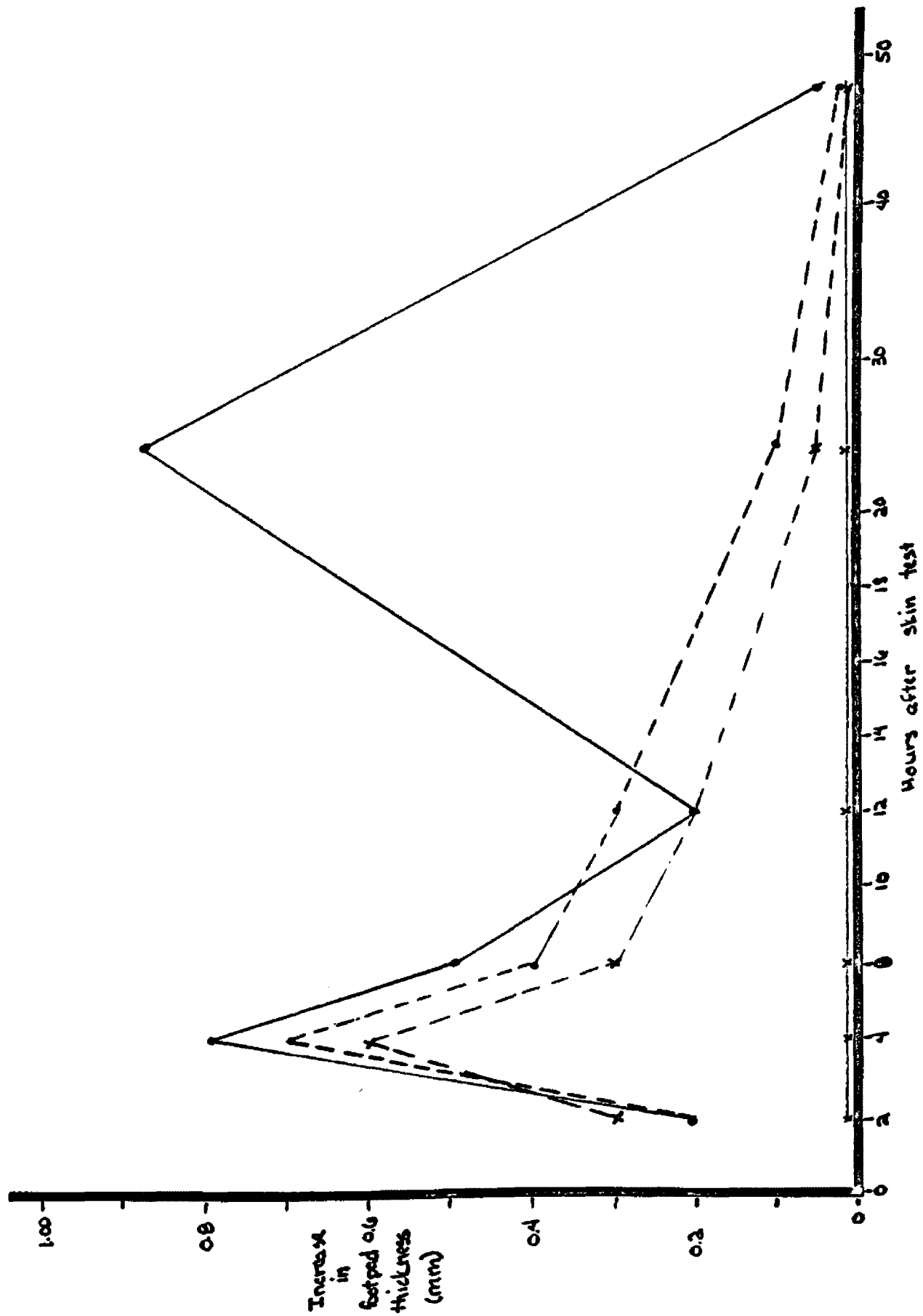
Bennett (7) developed the capillary tube method for detection of macro-phage inhibition factor (MIF). Groups of guinea pigs were immunized with BCG, EEA, or both. Peritoneal exudate cells were collected and capillary tube MIF assays were performed. Table X shows results obtained from the average values of 10 Sykes-Moore chambers.

Table X. In vitro demonstration of macrophage inhibition factor in guinea pigs sensitized with BCG, EEA or both.

Source of Peritoneal Exudate Cells	Incubation Medium	M.A.M.[a]	M.I.[b]	Percent Inhibition
Norm. Guinea Pigs	no antigen	1.54	100	0.00
	Klett 50 EEA	1.43	94	6.00
Guinea Pigs Stimulated with BCG and EEA	no antigen	2.10	100	0.00
	Klett 50 EEA	0.45	20	80.00
Guinea Pigs Stimulated with BCG	no antigen	1.57	100	0.00
	Klett 50 EEA	1.45	92	8.00
Guinea Pigs Stimulated with EEA	no antigen	1.48	100	0.00
	Klett 50 EEA	1.62	100	0.00

[a] Mean Area of Migration.

[b] Migration Index = $\dfrac{\text{Mean area of migration with antigen}}{\text{Mean area of migration without antigen}} \times 100.$

Migration of peritoneal cells from guinea pigs stimulated with BCG-EEA was inhibited 80% when cells were incubated with EEA. In contrast, cells from animals immunized with BCG or EEA alone were inhibited 8% or less. This substantiates the in vivo studies and demonstrates delayed hypersensitivity to EEA in mice immunized with BCG-EEA.

Immunosuppression of protective effects exhibited by combined BCG and EEA immunization.

Several immunosuppressive agents have been shown to eliminate one of the cellular components important in the immune mechanism; this makes

it possible to study the importance of a single cell in resistance to tularemia.

Experiment 1 - Effects of anti-thymocyte serum (ATS).

Anti-thymocyte serum (ATS) has been shown to eliminate the thymus-derived (T) lymphocyte and to be effective in elimination of the cell-mediated immune response. Mackaness and Hill (54) observed loss of immunity to Listeria monocytogenes in animals treated with anti-lympho-cyte globulin. Claflin and Larson (13) used F. tularensis strain LVS as a vaccine and showed a decrease in protective effect in ATS-treated immune mice challenged with F. tularensis strain Schu. Experiments were performed to determine the effects of ATS treatment and probable importance of the T-cell in mice immunized with BCG and EEA and challenged with F. tularensis strain 425 F_4G. Groups of normal and BCG-EEA immune mice were treated i.v. with a single dose of 0.25 ml of ATS 24 hours prior to challenge, simultaneously with challenge, or 24 hours after challenge with F. tularensis strain 425 F_4G. Treatment with ATS at this time eliminates a pre-existant immune state. Challenges were made by the subcutaneous route. Table XI and Figure 22 show the effect of ATS treatment. At high challenge doses, a decrease of at least one log in the protective effects of the combined vaccine.

Experiment 2 - Immunosuppressive effects of cyclophosphamide.

Allison (2) observed an increase in pathogenicity of Coxsackie B virus in adult mice treated with cyclophosphamide. Treatment with cyclophosphamide has been observed to be effective in reducing numbers of antibody producing B-cells (75). Experiments were performed to detect the effect of cyclophosphamide on the immune response provoked by BCG

Table XI. Immunosuppressive effects of i.v. ATS treatment on mice immunized with BCG-EEA and challenged s.c. with _F_. tularensis strain 425 F_4G.

Immunizing Antigen	Time of ATS Treatment (0.25 ml - i.v.)	Challenge dose	d/t[a]	MTD[b]	LD$_{50}$
BCG-EEA[c]	24 hours prior to challenge	10^{10}	5/8	7.0	$10^{9.35}$
		10^9	2/8	7.0	
		10^8	3/8	10.0	
BCG-EEA	with challenge	10^{10}	4/8	7.1	10^{10}
		10^9	1/8	10.0	
		10^8	0/8	-	
BCG-EEA	24 hours post challenge	10^{10}	6/8	7.0	10^9
		10^9	4/8	6.5	
		10^8	1/8	11.1	
BCG-EEA	-	10^{10}	0/8	-	$>10^{10}$
None	24 hours prior to challenge	10^8	8/8	3.5	$<10^7$
		10^7	8/8	3.7	
None	with challenge	10^8	8/8	3.6	$<10^7$
		10^7	8/8	3.5	
None	24 hours post challenge	10^8	8/8	3.5	$<10^7$
		10^7	8/8	3.0	
None	None	10^7	8/8	4.0	$<10^7$
None	0.24 ml	-	0/8	-	-

[a] Deaths/total challenged.

[b] Mean time of death.

[c] 0.2 ml Klett 100 i.v.
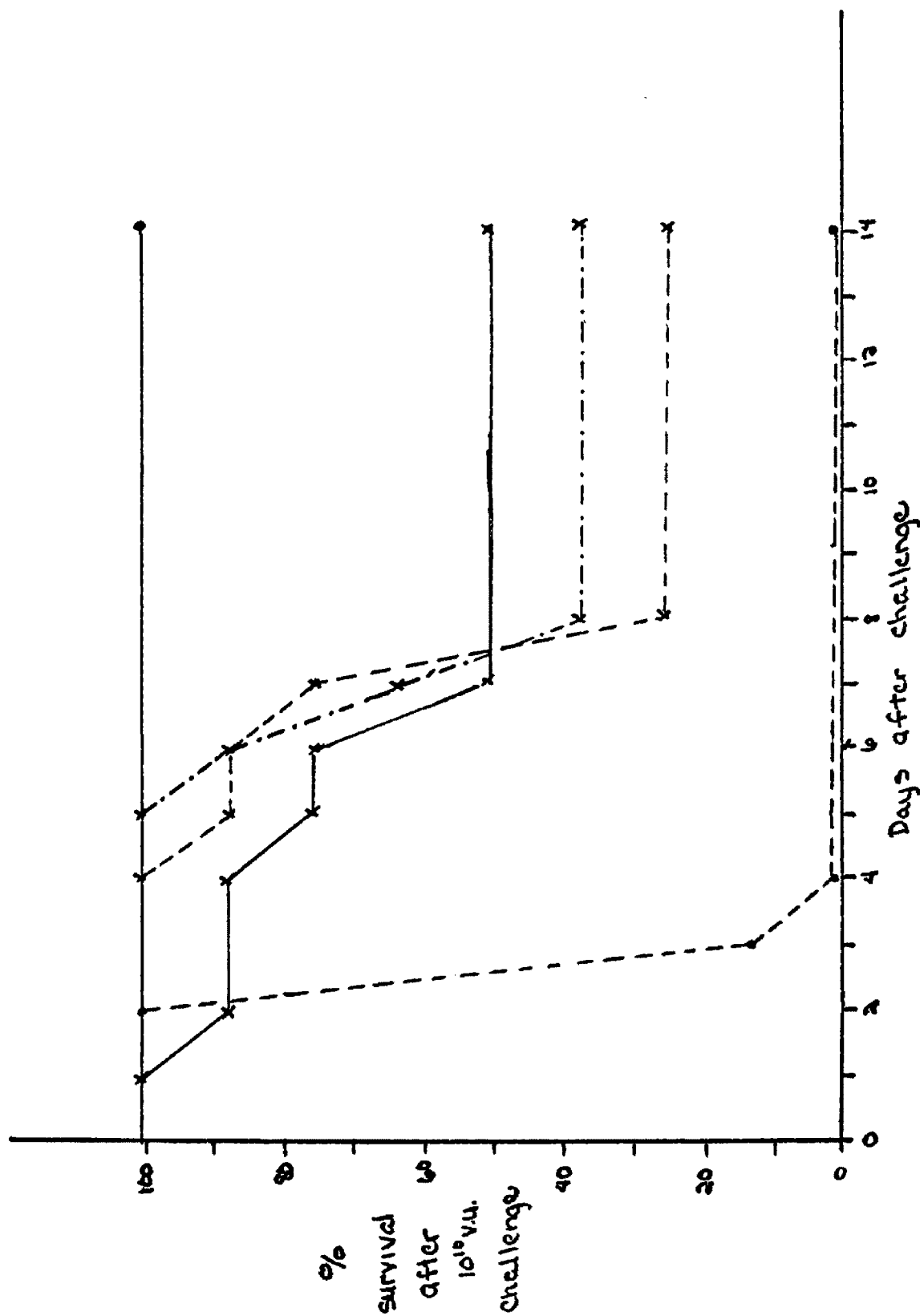
Fig. 22 - Effects of treatment with 0.25 ml ATS iv in mice immune to challenge with F. tularensis 425 F_4G. BCG-EEA immune, not ATS ——— ; BCG-EEA immune, ATS treatment 24 hours prior to challenge x—·—x, with the challenge x—··—x, 24 hours after challenge x———x; non-immune, no ATS ·——·.

and EEA and to evaluate the degree to which antibody and B-cells are involved. Groups of normal and BCG-EEA immunized mice were treated i.p. 7 and 14 days after immunization with 150 mg/kg of cyclophosphamide. Twenty-one days after immunization mice were challenged i.p. with F. tularensis strain 425 F_4G. Results are shown in Table XII and Figure 23. Treatment with cyclophosphamide reduced the LD_{50} to less than 10^4 organisms, this demonstrates the importance of specific antibody and the participation of the B-cell in immunity induced by BCG and EEA.

Table XII. Effects of treatment with two 150 mg/kg doses of cyclophosphamide (CP) on mice immune to F. tularensis strain 425 F_4C.

Immunization with BCG-EEA[a]	CP Treatment	Challenge dose of F. tularensis (v.u.)	d/t[b]	MTD[c]
+	+	10^8	8/8	3.6
+	+	10^6	8/8	4.9
+	+	10^4	8/8	4.3
+	+	-	3/8	4.0
+	-	10^8	1/8	6.0
-	+	10^8	8/8	2.8
-	+	10^6	8/8	2.5
-	+	10^4	8/8	4.8
-	+	-	3/8	6.0
-	-	10^4	8/8	3.3

[a] 0.2 ml Klett 100 i.v.

[b] Deaths/total challenged.

[c] Mean time of death.
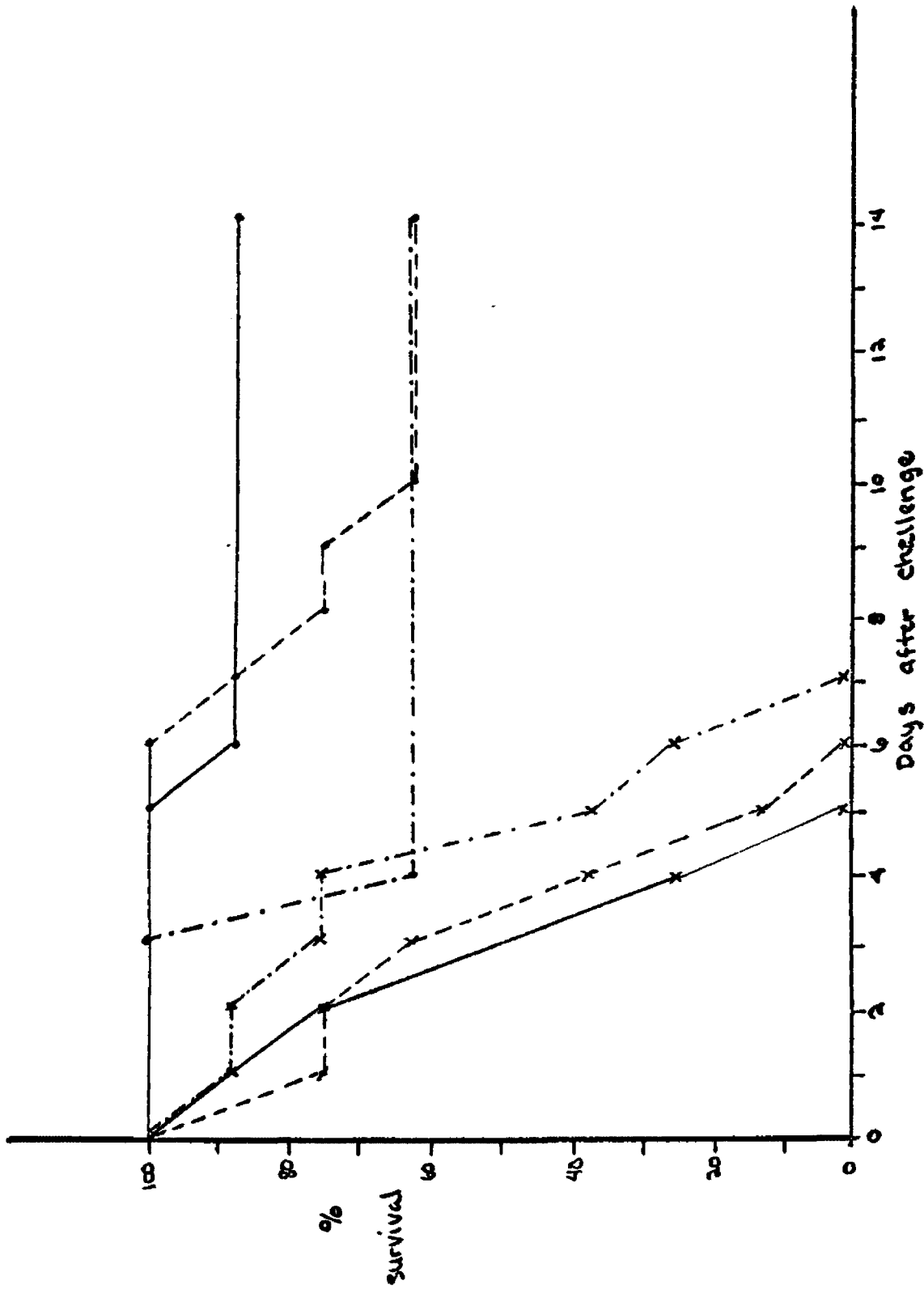
Fig. 23 - Effects of treatment with two 150 mg/kg doses of cyclophosphamide in mice immune to challenge with F. tularensis strain 425 F.C. CP treated, challenge 10^8 x——x, 10^6 x-·-x, 10^4 x--x v.u., none ·——·, (all BCG-FBA immune); BCG-FBA immune, no CP ————; non-immune, CP treatment ·——·.

Experiment 3 - Immunosuppressive effects of anti-macrophage serum and silica.

Allen (1) and Thorpe and Marcus (72, 73,74) maintain that immunity to tularemia results from stimulation of the reticuloendothelial system. Anti-macrophage serum and silica are effective in eliminating macrophages, The primary effector cell of the RES. Experiments were performed to determine the immunosuppressive effects of these immunosuppressive agents.

a. Groups of normal and BCG-EEA immunized mice were treated i.p. with AMS 24 hours prior to, simultaneously with, and 24 hours after i.p. challenge with F. tularensis strain 425 $F_4 G$. Table XIII and Figure 24 show AMS treatment prior to challenge reduced the LD_{50} by greater than 4 logs. Treatment after challenge reduced the LD_{50} by 8 logs, which demonstrates a requirement for the macrophage in production of immunity to F. tularensis strain 425 $F_4 C$.

b. Groups of mice were treated i.p. with 50 mg of Min-U-Sil 4 hours prior to challenge, simultaneously with challenge, and 4 hours after challenge with F. tularensis strain 425 $F_4 C$. Results shown in Table XIV and Figure 25 agree with the results obtained with AMS. Min-U-Sil decreases the LD_{50} by greater than 8 logs, demonstrating the importance of the macrophage in immunity produced by BCG and EEA. No difference exists in data obtained from groups treated at different times in relation to challenge.

Table XIII. Effects of AMS injected i.p. on mice immunized with BCG-EEA and challenged i.p. with F. tularensis strain 425 F_4C.

Immunizing Antigen	Time of AMS Treatment (0.25 ml - i.p.)	Challenge dose	d/t[a]	MTD[b]	LD$_{50}$
BCG-EEA[c]	24 hours prior to challenge	10^{10}	6/8	4.00	
		10^8	6/8	5.00	10^6
		10^6	4/8	5.25	
BCG-EEA	with challenge	10^{10}	7/8	3.6	
		10^8	5/8	5.0	$10^{7.4}$
		10^6	1/8	7.0	
BCG-EEA	24 hours post challenge	10^{10}	7/8	4.2	
		10^8	7/8	4.0	10^6
		10^6	4/8	3.6	
BCG-EEA	-	10^{10}	0/8	-	$>10^{10}$
None	24 hours prior to challenge	10^6	8/8	2.0	$<10^2$
		10^2	8/8	3.7	
None	with challenge	10^6	8/8	1.0	$<10^6$
		10^2	8/8	3.3	
None	24 hours post challenge	10^6	8/8	1.1	$<10^6$
		10^2	8/8	3.0	
None	None	10^2	8/8	3.2	$<10^2$
None	0.25 ml	-	0/8	-	-

[a] Deaths/total challenged.

[b] Mean time of death.

[c] 0.2 ml Klett 100 i.v.
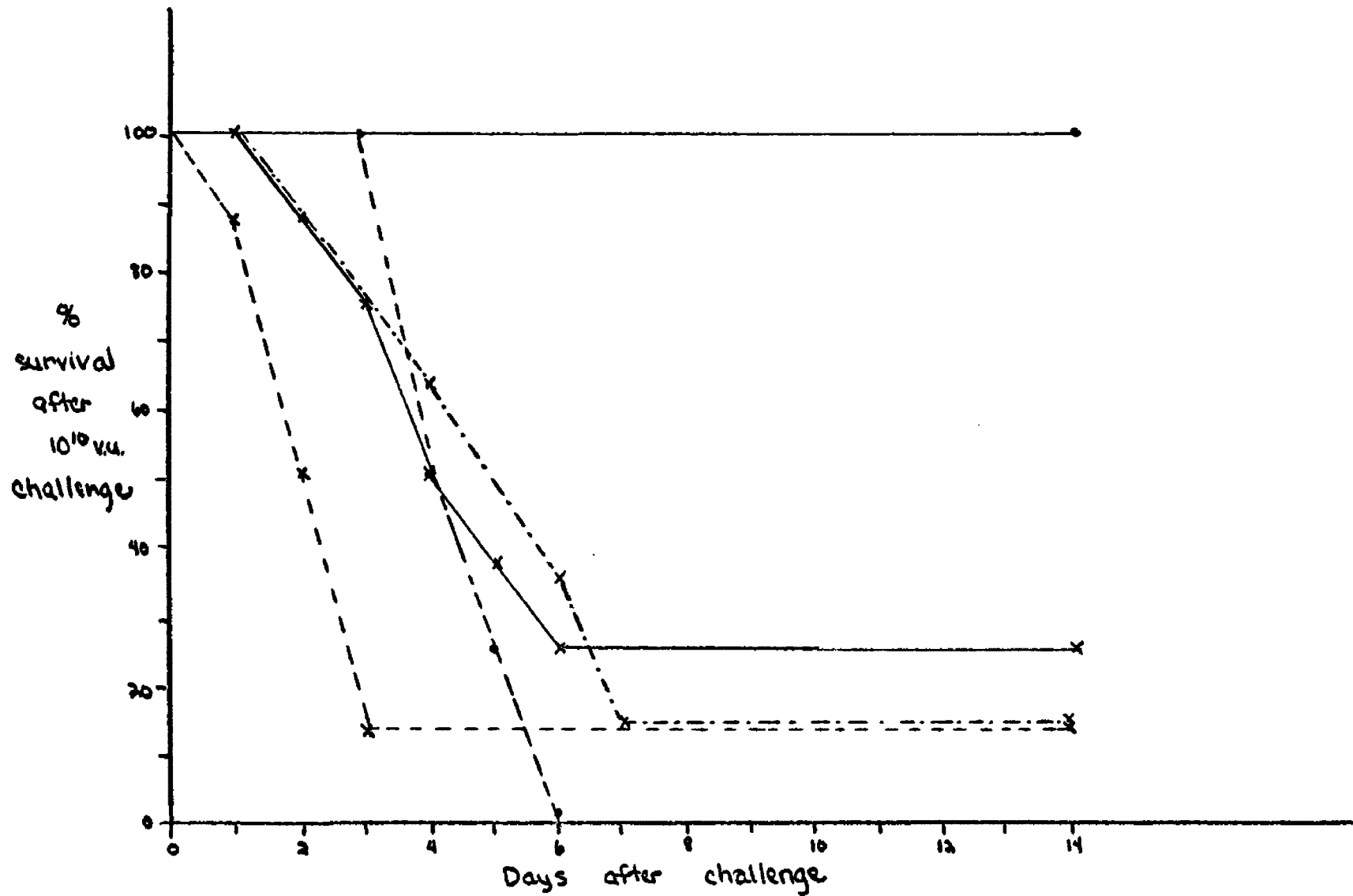
Fig. 24 - Effects of treatment with 0.25 ml of AMS ip in mice immune to ip challenge with $\underline{F}$. $\underline{tularensis}$ strain 425 F_4G. BCG-ERA immune, no AMS ————— ; AMS treatment 24 hours prior to challenge x---x, with challenge x-··-x, 24 hours after challenge x———x.

Table XIV. Effects of i.p. treatment of Min-U-Sil on mice immunized with BCG and EEA and challenged i.p. with F. tularensis strain 425 F_4G.

Immunizing Antigen	Time of Treatment (50 mg/ml i.p.)	Challenge Dose	d/t[a]	MTD[b]	LD$_{50}$
BCG-EEA[c]	4 hours prior to challenge	10^8	8/8	3.5	$<10^2$
		10^6	8/8	4.6	
		10^4	8/8	4.6	
		10^2	5/8	4.6	
BCG-EEA	with challenge	10^8	8/8	3.8	$<10^2$
		10^6	8/8	3.9	
		10^4	8/8	3.7	
		10^2	5/8	4.2	
BCG-EEA	4 hours post challenge	10^8	8/8	3.2	$<10^2$
		10^6	8/8	5.6	
		10^4	8/8	5.6	
		10^2	8/8	5.5	
BCG-EEA		10^8	0/8	-	$>10^8$
None	4 hours prior to challenge	10^4	8/8	2.3	$<10^2$
		10^2	8/8	2.3	
None	with challenge	10^4	8/8	1.6	$<10^2$
		10^2	8/8	2.3	
None	4 hours post challenge	10^4	8/8	2.1	$<10^2$
		10^2	8/8	1.8	
None	None	10^2	8/8	2.0	10^2
None	50 mg/ml	-	0/8	-	-

[a] Deaths/total challenged.

[b] Mean time of death.

[c] 0.2 ml Klett 100 i.v.
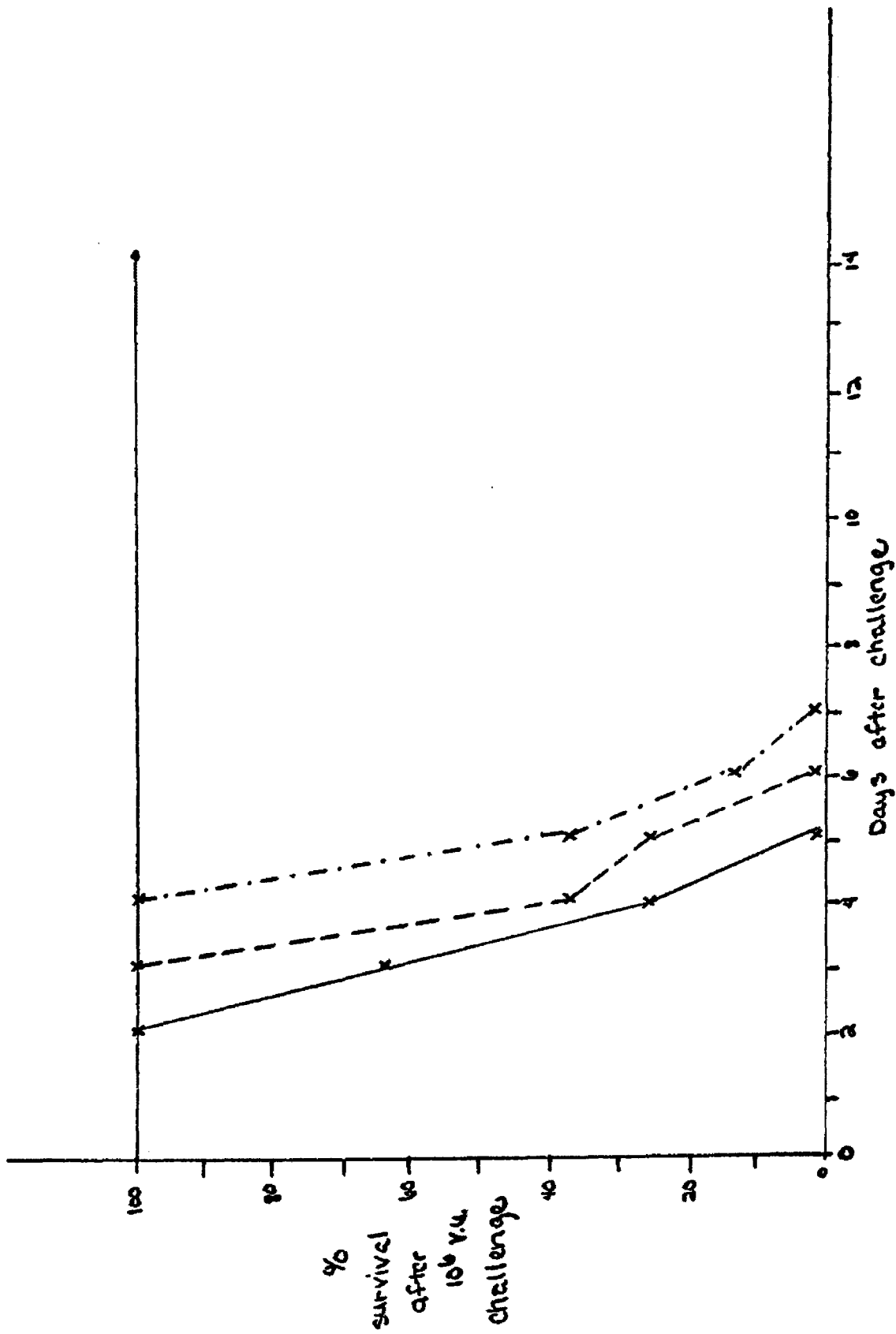
Fig. 25 - Effects of treatment with 50 mg/mouse of Min-U-Sil (ip) in mice challegned ip with F. tularensis 425 F.G. BCI-SEA immune, no treatment ————; BCI-EFA immune, Min-U-Sil 4 hours prior to challenge x---x; with challenge x——x; 4 hours after challenge x--x.

Effects of immunosuppressive treatment on hypersensitivity reactions.

Elimination of immediate or delayed hypersensitivity occurs with some immunosuppressive treatments. Figure 26 shows the effect of various immunosuppressive treatments on the hypersensitivity skin reactions to EEA in mice immunized with BCG and EEA. All immunosuppressive agents were administered i.p. Anti-thymocyte serum decreased by 50% the immediate hypersensitivity response and reduced the delayed reaction by 75%. The immediate hypersensitivity response was reduced by 87.5% with cyclophosphamide; however, the delayed reaction was decreased only 37.5%. Min-U-Sil and AMS eliminated both hypersensitivity responses.

Immunity to F. tularensis strain Schu.

Experiment 1 -

Claflin and Larson (13) showed immunity induced by attenuated F. tularensis to F. tularensis strain Schu is accompanied by delayed hypersensitivity to ether-extracted antigen. A requirement for specificity was also detected. Experiments were performed to determine if the combined BCG-EEA vaccine which also induced delayed hypersensitivity to EEA, induced immunity to F. tularensis strain Schu. Groups of normal mice and mice immunized with BCG, EEA from F.tularensis strain 425 F_4C or strain Schu or both were challenged with F. tularensis strain Schu. Tables XV and XVI show extension in MTD in BCG-EEA mice but LD_{50} and mortality ratios remain unchanged. No significant difference is observed in data obtained from those experiments comparing EEA from the Schu strain or the 425 F_4C strain.
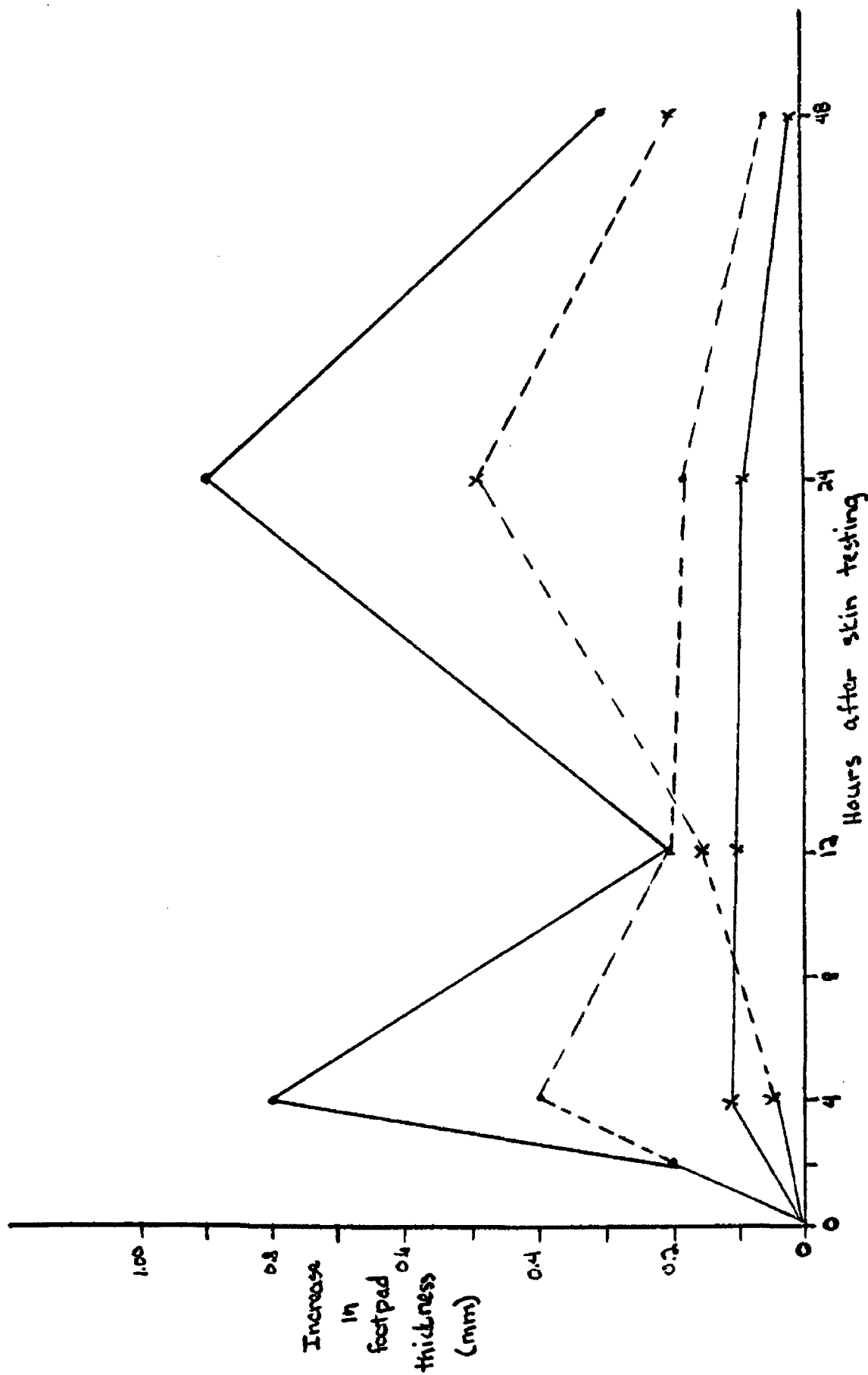
Fig. 26 - Effects of immunosuppressive agents of the immediate and the delayed hypersensitivity reactions in mice immunized with 0.2 ml of a Klett 100 BCG-BEA suspension. BCG-BEA immune, no treatment ——, 0.25 ml ATS ---, 0.25 ml ALS + 50 mg Min-U-Sil x——x, 150 mg/kg CP x--x.

Table XV. The effects of Immunization with BCG and ether-treated cells of F. tularensis strain 425 F_4G in mice infected with serial dilutions of F. tularensis strain Schu.

Challenge dose of F. tularensis (v.u.)	BCG and EEA		BCG		EEA		Control	
	d/t[a]	MTD[b]	d/t	MTD	d/t	MTD	d/t	MTD
10^3	10/10	6.6	8/8	4.875	8/8	5.25	8/8	4.3
10^2	10/10	7.5	8/8	4.400	8/8	5.60	8/8	4.0
10	10/10	8.6	8/8	4.400	8/8	5.25	8/8	3.7
LD_{50}	<10 cells		<10 cells		<10 cells		<10 cells	

[a] Death/total challenged.

[b] Mean time of death.

Table XVI. Effects of immunization with viable BCG and ether-treated cells of F. tularensis strain Schu in mice challenged with serial dilutions of F. tularensis strain Schu.

Challenge dose of F. tularensis (v.u.)	BCG and EEA		BCG		EEA		Control	
	d/t[a]	MTD[b]	d/t	MTD	d/t	MTD	d/t	MTD
10^3	8/8	6.6	8/8	4.1	8/8	4.9	8/8	3.75
10^2	8/8	6.6	8/8	4.2	8/8	5.9	8/8	4.00
10	8/8	6.3	8/8	3.9	8/8	4.9	8/8	3.75
LD_{50}	<10 cells		<10 cells		<10 cells		<10 cells	

[a] Death/total challenged.

[b] Mean time of death.

Experiment 2 -

BCG immune animals given a booster dose of purified protein deriva-
tive (PPD) a few hours before or simultaneous to challenge have been
shown to protect animals against growth of malignant melanoma (Larson
unpublished data). Boosting with PPD is believed to stimulate production
of lymphocyte products of importance in delayed hypersensitivity reaction.
Experiments were performed to determine if BCG-EEA immune mice boosted
4 hours prior to challenge with PPD have increased immunity to F.
tularensis strain Schu. Table XVII shows the results observed. PPD
boosting of BCG-EEA immune mice does not induce protection against
challenge with F. tularensis strain Schu.

Table XVII. Effects of boosting BCG-EEA immune mice with PPD (i.p.) 4
hours prior to (i.p.) challenge with F. tularensis strain Schu.

Immunizing Antigen	PPD Treatment	Challenge dose (v.u.)	d/t[a]	MTD[b]
BCG-EEA[c]	300 ug	10^3	8/8	5.1
BCG-EEA	300 ug	10^2	8/8	5.4
BCG-EEA	300 ug	10	8/8	5.0
BCG-EEA	-	10^3	8/8	5.1
BCG-EEA	-	10^2	8/8	5.8
BCG-EEA	-	10	8/8	5.9
EEA[c]	300 ug	10^2	8/8	3.8
EEA	-	10^2	8/8	3.8
BCG[c]	300 ug	10	8/8	3.3
BCG	-	10	8/8	3.4
None	300 ug	10	8/8	3.0
None	-	10	8/8	3.3

[a] Death/total challenged.

[b] Mean time of death.

[c] 0.2 ml Klett 100 i.v.

CHAPTER IV

DISCUSSION

Francisella _tularensis_ is a facultative intracellular organism which infects cells of the reticuloendothelial system of susceptible animals. As in brucellosis and tuberculosis, resistance to tularemia generally has been considered to be directly related to an increase in the functional capacity of phagocytic cells. Stefanye, et al. (69) observed that intracellular destruction by normal guinea pig peritoneal exudate cells of various strains of F. _tularensis_ correlated with the virulence of the organisms _in_ _vivo_. Huziwara, et al., as cited by Claflin and Larson (13), demonstrated that virulent and avirulent strains of F. _tularensis_ were phagocytosed at the same rate. However, virulent organisms were capable of intracellular proliferation whereas avirulent strains were digested. Phagocytes from tularemia immune guinea pigs, rabbits and mice possessed increased ingestive and digestive capacities as compared to cells from normal animals. Thorpe and Marcus (72, 73) maintained that the degree of intracellular destruction of the organisms in phagocytes taken from both normal and immune animals was independent of antibody. These studies implied that increased resistance in acquired immunity to tularemia was dependent on enhanced capabilities of the phagocytes to destroy the organisms and that specific antibody is of secondary importance. Further work by Thorpe and Marcus (74) and Allen (1), with mice, and Woodward, et al. (79), with rats, demonstrated that peritoneal exudate cells or spleen cells from immune animals could protect adoptively normal recipients which were challenged with fully

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virulent F. tularensis. Although these studies implicated a cellular immune mechanism, the heterogeneous population of cells utilized should be considered. Splenic cells from mice are histologically highly heterogeneous, containing lymphocytes of both thymus (T-cell) and bone marrow or bursal-equivalent (B-cell) origins and also phagocytes of the reticuloendothelial system (24). Peritoneal exudate cells, including those stimulated with special irritants, contain, in addition to macrophages, up to 30% lymphocytes and 10-15% PMNs. The heterogeneity of the cell population in transfer studies results in difficulty of evaluation the role of individual cellular elements as mediators of immunity. Even though resistance may rest with the phagocytic cell, the induction and expression of their activity may depend upon interaction with other lymphoid cells or their products (24).

Larson (43) showed that other factors may determine the final outcome in resistance of rats to tularemia. He observed a degree of protection in animals challenged with fully virulent F. tularensis when ether-extracted antigen was employed as a vaccine. Bell, et al. ([4]) demonstrated a similar phenomena in mice challenged with F. tularensis strain 425 F_4G.

From data presented in this thesis, it is apparent that in mice challenged with high doses of F. tularensis strain 425 F_4G, immunity was dependent upon a combination of specific immunization plus non-specific stimulation of the reticuloendothelial system. Several lines of evidence support this theory. Viable Mycobacterium bovis strain BCG or heat-killed Bordetella pertussis are both excellent stimulants of non-specific immunity. Mice stimulated with these vaccines were not protected against challenge with F. tularensis strain 425 F_4G or F. tularensis strain Schu. This is

in contrast to results obtained with other facultative intracellular
parasites. Mackaness (50,51) has shown that BCG immunization is
protective in animals challenged with _Listeria monocytogenes_. Elberg
(22) and Elberg, _et al._ (23) could stimulate non-specific resistance to
brucellosis. However, these authors also demonstrated an immune serum
requirement _in vitro_, which demonstrates a possible requirement for
specific humoral factors. Immunity to F. _tularensis_ strain 425 F_4C was
not dependent solely on the activation of the RES but required other
specific components. Active immunization with ether-extracted antigen
(EEA) simultaneously with viable BCG protected mice against a challenge
of greater than 10^{12} organisms of F. _tularensis_ strain 425 F_4C. Ether-
extracted antigen alone protected mice to a 10^6 challenge dose of the
same organism. Specific antibody was shown to be present, but the titer
did not correlate with the high degree of protection afforded with the
combined antigen. Similar results were obtained when heat-killed B.
pertussis was employed with EEA. Passive transfer of immune serum into
BCG immune mice resulted in an increase of at least two logs of protection
when compared to animals given immune serum alone. These results imply
a mechanism involving the macrophage as an ultimate effector or killer
cell aided by opsonization of antibody.

To define more clearly the immune mechanism responsible for protection
of animals against tularemia and other facultative intracellular parasites,
the cooperation between cellular components was investigated. Immuno-
suppressive agents carefully administered can be effective in elimination
of one of the cellular components involved in the induction of an immune
response. Turk, _et al._ (75) demonstrated, histologically, a depletion

of the B-cell areas of the spleen and lymph nodes with relatively little

effect on the T-cell areas in animals treated with cyclophosphamide.

Functionally, a normal T-cell response was associated with reduced B-cell

activity. Mice which had been immunized with BCG-EEA and then treated

with CP gave an LD_{50} of less than 10^4 cell of F. tularensis strain 425

F_4G, whereas the LD_{50} was greater than 10^3 for mice which had been only

immunized and not treated with CP. Cyclophosphamide treatment decreased

the immediate hypersensitivity response to EEA but had little effect on

the delayed hypersensitivity response. These results indicated the

participation of the B-cell in immunity to F. tularensis strain 425 F_4G.

Elimination of B-cells responsible for antibody production and of

immediate hypersensitivity, implied the elimination of a specific antibody

component in the response, in question. This data further substantiated

a requirement for specific factors, in addition to enhancement of the

RES, in immunity to tularemia. These results concur with those of

Elberg, et al. (23) and Whitby and Rowley (78). These authors noted an

augmentation of phagocytosis and intracellular killing when macrophages

from animals immune to brucellosis and salmonellosis (respectively) were

incubated with immune serum. The antibody involved in the immune response

to tularemia seems to be an opsonin which aides the ingestion and digestion

of the invading organisms. There is, however, a possibility that cyto-

philic antibody with high avidity for macrophages was also involved in

production of the immune response to F. tularensis. This antibody has

been associated with immunity in salmonellosis (65), and is a possible

explanation for a lack of correlation in the antibody titer and the degree

of resistance obtained with BCG-EEA immunization. The increase in

protective effects of active immunization in comparison to passive transfer of immune serum into BCG immune animals could also be explained by a cytophilic component not found in the immune serum.

Intravenous treatment with anti-thymocyte serum of BCG-EEA immune mice reduced the LD_{50} by 1-2 logs in mice challenged subcutaneously with F. tularensis strain 425 F_4G. Although a decrease in the LD_{50} was observed, the effects of ATS in this system were far less detrimental than those observed by Claflin and Larson (13) in mice immunized with the live vaccine strain of F. tularensis. Mackaness (50, 51) observed complete abolition of immunity to Listeria monocytogenes in immune mice treated with anti-lymphocyte globulin. These results also contrast with the data obtained in the present experiments. It has been observed that the route of challenge is important in ATS treatment. The immunosuppressive effect is most apparent when animals are challenged intravenously and least apparent in subcutaneous challenges, however, mice do not survive i.v. challenge with F. tularensis. This may be explained by the inaccessibility of extravascular lymphocytes to intravenous administration of ATS. Partial abolition with subcutaneous challenge is perhaps indicative of a T-cell function in immunity to tularemia. T-cells are known to be necessary for the induction of cell-mediated immunity and in the production of a delayed hypersensitivity response. Treatment with ATS reduced the delayed hypersensitivity response to EEA but had little effect on the immediate hypersensitivity reaction. The effects of ATS on the delayed response shows an interaction of the vaccine or a component of the vaccine with the T-cell. This interaction of antigen with T-cells could stimulate production of lymphocyte products which are postulated by several

investigators to activate the cell-mediated immune apparatus. The absence of the T-cell and the functions associated with this cell may be an explanation for the decrease in protective effects of the BCG-EEA vaccine in animals treated with ATS.

Anti-macrophage serum (AMS) and silica (Min-U-Sil) are known to be effective in elimination of the major phagocytic cells of the RES. BCG-EEA immune mice, treated intraperitoneally with a 50 mg dose of Min-U-Sil are no longer resistant to a challenge of greater than 10^2 cells of $\underline{F}$. $\underline{tularensis}$ strain 425 F_4C, compared to untreated controls which are resistant to a 10^{10} challenge. Treatment with AMS was less effective but reduced the LD_{50} by greater than three logs. This difference in immunosuppressive effects of the two anti-macrophage agents was probably due to a difference in the mechanism of action on the macrophage. Min-U-Sil is a faster acting agent that is engulfed rapidly by the macrophage. This mechanism is more direct and probably effects more macrophages. Anti-macrophage serum requires absorption onto the macrophage and the action of the immune mechanisms of the animal to destroy the macrophage-antibody complex. This process is more indirect and requires a greater length of time for destruction of the macrophage. Min-U-Sil would be engulfed also by the other phagocytes (granulocytes) of the RES, eliminating any phagocytosis of the infecting organism. The effects of AMS is more specifically directed at the macrophage and would not involve the other cells mentioned, (see materials and methods). Reduction of resistance by AMS and Min-U-Sil demonstrates the importance of the macrophage, and phagocytic cells in general, in manifesting immunity to $\underline{F}$. $\underline{tularensis}$ strain 425 F_4C. Absence of digestion and

inactivation of the invading organisms by phagocytic cells can allow growth of massive numbers of organisms; this can overpower the immune apparatus. These results support hypothesis made by Allen (1), Thorpe and Marcus (72,73,74),and Stefanye, et al. (69) who maintain that immunity to tularemia is due to enhanced phagocytic activity of the RES. Another explanation for the effect of these immunosuppressive agents could be the ability of macrophages to process the antigens required for the stimulation of a sufficient number of antibody producing cells. Insufficient processing of antigen could affect the activity of the T-cell resulting in the absence of the soluble mediators which may play a role in the immune response in question. Abolition of the immediate and the delayed hypersensitivity responses is observed with both AMS and Min-U-Sil, if treatment occurs prior to skin testing. This demonstrates a requirement for the macrophage in both of these responses. It has been observed by several workers (8, 10) that macrophages are required for a delayed hypersensitivity response. However, the role of the macrophage in immediate hypersensitivity is less well defined. In sufficient processing of the antigen with the resultant absence of antibody production could explain the effect of these immunosuppressive agents on immediate hypersensitivity (39).

Immunization with BCG-EEA induces delayed hypersensitivity to EEA in mice and guinea pigs, a condition not observed in animals immunized with EEA or BCG alone or with other preparations of F. tularensis killed vaccines. Claflin and Larson (13) have shown delayed hypersensitivity to EEA in animals immunized with the live vaccine strain of F. tularensis. The relationship between delayed hypersensitivity and acquired cellular

resistance to infection remains a classical problem, especially in relation to facultative intracellular organisms. Techniques performed in these studies have demonstrated a separation of these phenomena. Delayed hypersensitivity to EEA and resistance to tularemia are independent phenomena. This is demonstrated by several lines of evidence. First, resistance of mice to challenge with high doses of F. tularensis strain 425 F_4G is afforded by immunization with EEA in combination with viable BCG or heat-killed B. pertussis. However, delayed hypersensitivity to EEA was induced by the BCG-EEA complex but not by the B. pertussis-EEA complex. Second, even though the BCG-EEA combined vaccine and the live vaccine strain (LVS) both induced delayed hypersensitivity to EEA, only LVS induce protection to challenge with F. tularensis strain Schu (13). Immunization with the BCG-EEA complex extended the mean time of death; which demonstrates some degree of protection, but the vaccine was ineffective in preventing eventual death from tularemia. Third, decrease of the delayed hypersensitivity response to EEA with ATS is not comparable to the slight reduction of the protective effect of the combined vaccine. Fourth, in vitro study of delayed hypersensitivity reactions induced by BCG-EEA demonstrated production of macrophage inhibition factor (MIF). However, in vivo techniques for the production of MIF and other lymphocyte products resulted in no increased immunity to F. tularensis strain Schu. This is in opposition to observations and hypotheses made by Waksman (77) and Mackaness and Blanden (52).

These four preceding points represent a study that is in opposition to those by Collins and Mackaness (15) with salmonellosis and Mackaness and Blanden (52) with tuberculosis. However, observations made by Gordon

(31) using different methods are similar and demonstrate a lack of
correlation between delayed hypersensitivity and immunity to tularemia.
These results indicate that these manifestations are independent
immunologic phenomena. Delayed hypersensitivity does not reflect the
status of resistance to infection in a host.

CHAPTER VI

SUMMARY

Immunity to F. tularensis strain 425 F_4C results from a combination of specific antibody and increased bacteriocidal capacities of the reticuloendothelial system in cooperation with the T-cell or its products. Several lines of evidence support this hypothesis. 1) Immunization with ether-extracted antigen in combination with BCG or heat-killed Bordetella pertussis protect mice from challenges of greater than 10^{12} cells of F. tularensis strain 425 F_4C. This is an increase of greater than 6 logs of protection over EEA immunized mice. 2) Passive transfer of immune serum into BCG stimulated animals protects mice from challenge with 10^8 (LD_{50}) cells of F. tularensis strain 425 F_4C, whereas, immune serum alone protected mice from a challenge of only 10^4 (LD_{50}) cells of the same organism. 3) The protective effects of the combined BCG-EEA antigen are decreased by Min-U-Sil and anti-macrophage serum and immunosuppressive agents which are effective in elimination of macrophages. 4) Immunity induced by the combined BCG-EEA antigens is abolished by treatment with cyclophosphamide, an immunosuppressive agent effective in elimination of the antibody producing cell. 5) Treatment with anti-thymocyte serum partially eliminated the immunity induced by the combined vaccine, which possibly indicates participation of the T lymphocyte and the lymphocyte products associated with these cells.

Immunosuppressive techniques demonstrate a two or perhaps three cell interaction in immunity to tularemia. Cooperation of antibody from B-cells and phagocytic macrophages are primarily involved in protection of mice against F. tularensis strain 425 F_4C. However, production of

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lymphocyte products by the T-cell could be of secondary importance and might be considered as a secondary cell for the induction of resistance.

Delayed hypersensitivity to EEA and acquired resistance to tularemia are thought to be independent phenomena. Supportive evidence for this is as follows: 1) Resistance to high doses of F. tularensis strain 425 F_4G is induced with both BCG-EEA and B. pertussis-EEA; however, only the BCG-EEA complex induces delayed hypersensitivity. 2) Both BCG-EEA and LVS induce delayed hypersensitivity to EEA, but only the live vaccine strain protects animals from challenge with the highly virulent Schu strain. 3) Reduction of the delayed hypersensitivity response with ATS reduces resistance only slightly (1-2 logs). 4) In vivo MIF induction does not increase the protective effect of the BCG-EEA complex in mice to challenge with F. tularensis strain Schu.

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