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A Study of Cell-Mediated Immunity During a Primary Infection with Pseudorabies Virus

John George Chosay

A STUDY OF CELL-MEDIATED IMMUNITY DURING A PRIMARY INFECTION WITH PSEUDORABIES VIRUS

by

John George Chosay

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A STUDY OF CELL-MEDIATED IMMUNITY DURING A PRIMARY INFECTION WITH PSEUDORABIES VIRUS

John George Chosay, M.S.

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This research defines the role and sequence of cell-mediated immune events in an *in vivo* virus infection. Pseudorabies virus infected CF1 mice lungs, were assessed by histological or immunohistochemical means. Using fluorescein isothiocyanate labled anti-T lymphocyte (ATG) and lissamine rhodamine B200 labeled anti-macrophage globulins (AMG), the number of lymphocytes and macrophages were determined. An early increase in T cells (four hours post-innoculation) was followed by an increase in macrophages (16 hours post-innoculation). Thereafter tissue levels of T cells and macrophages remained constant. Thymus dependent lymphocytes failed to show increase macrophages clustering as pathology progressed. The first pathological changes in the bronchi and bronchiolar linings were the result of viral replication rather than the result of macrophage accumulation.

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John George Chosay

Chosay, John George

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INTRODUCTION

The study of cell-mediated immunity during a primary infection involves two complex interactions, immunology and virology. Immunology deals with the host protective system, while virology in part is the study of the virus and its host interactions.

Two principle host protective mechanisims come into play, humoral or antibody mediated, and cellular immunity or cell-mediated. Both systems are regulated by the interactions of three cell types: B lymphocytes, T lymphocytes, and macrophages. Humoral immunity implies the response of a specific serum antibody with a specific antigen. The host organism acquires these antibodies through either active or passive immunity. Active immunity requires the host to manufacture antibodies either through direct vaccination or infection. The predominant cell in the humoral response is the B lymphocyte (B cell).

B cells are thymus independent lymphocytes that arise and mature within the bone marrow. Mature B cells circulate within the vascular system and populate the spleen and medullary zones of peripheral lymph nodes. They can be differentiated from T cells by the presence of immunoglobulin molecules on their cell surfaces. When B cells encounter a recognizable antigen, they undergo cell differentiation to become plasma cells. Plasma cells are responsible for the high levels of antibodies produced. Each plasma cell produces antibodies that are specifically directed against the original B cell recognized antigen.

In vivo, antibodies may function in the direct removal of the antigen by activating complement, or they may label (opsonize) antigen to facilitate phagocytosis or T cell cytotoxicity.

Normally, antibody response is maintained by the host as long as a defense against the antigen or virus is required. A humoral response is usually efficient in combating infection before extensive cellular damage results. However, a humoral response takes time to develop with perhaps a week passing before titerable antibodies are detected. A viral infection can cause extensive cellular damage in 24 hours or less. Obviously, the humoral response is not the most effective mechanism in controlling an infecting agent during the early stages of infection (Bladen, 1970). This early role is relegated to a cell-mediated immune response.

In cell-mediated immunity T lymphocytes and macrophages play the major roles in the destruction of antigen or virus. It was early recognized that subjects with B cell deficiencies recovered normally from viral infections. By contrast, individuals with a T lymphocyte deficiency were extremely susceptible to diseases such as measles, varicella and cytomegalovirus (Fenner and White, 1976). The precursor T lymphocytes (or T cells) are located in the bone marrow. These precursor cells pass into the blood and migrate to the thymus where they mature into functional T cells (Weissman, 1967). On maturation, T cells displaying specific marker antigens circulate through the body and populate the central areas of periferal lymph nodes. Once stimulated by a specific antigen, they show either cytotoxic activity, lymphokine production, or cooperation with B cells for antibody response.

The cytotoxic activity of T cells is due to the response of one of three T cell sub types. Garovoy and Carpenter (1980) have stated that these three T cell types are either cytotoxic T lymphocytes (CTL), killer cells (K cells), or natural killer cells (NK cells). Cytotoxic T lymphocytes derive their specificity from prior immunization. Killer cells depend upon the binding of an antibody to direct its cytotoxic activity. Natural killer cells may recognize the target cell directly through the major histocompatibility complex.

T lymphocytes, in response to recognized antigen, produce specific soluble proteins (lymphokines) that act in a variety of protective fashions. Some of these lymphokines act as chemotactins to eosinophils, neutrophils, lymphocytes, or macrophages. Others prevent cellular migration, enhance macrophage motility and phagocytosis, or recruit uncommitted lymphocytes by stimulating cellular division of cells within the effected area. Interferon is also classed as a lymphokine and sensitizes the surrounding cells to viral particles and activates the natural killer cells (Trinchieri, Santoli, & Dee, 1978). All lymphokines are involved in either enhanced migration of protective cells into an infected area, or activation of protective cells (Cohen & McCluskey, 1973).

T lymphocytes also interact with macrophages and B cells in the production of antibodies. The T cells function by recognizing the macrophage processed antigen and presenting it to the B cells to initiate an antibody response.

Macrophage also functions in the cell-mediated immunity response. Its phagocytic activity is a factor in removing foreign materials. This phagocytic activity can be nonspecific or highly specific, as seen in its effector function of cell-mediated immunity. Macrophages may attach and phagocytize (ingest) an antibody-antigen complex. Macrophages identify immune reactions by specific cellular receptors for the Fc portion of antibodies and for C3b of complement. Additionally, they can recognize specific foreign or altered cells by their (macrophage) immune response antigens (Ia antigens). These Ia antigens are expressed on macrophage cell surfaces and serve as a direct binding site for other cells (Dausset, 1981).

A direct interaction between lymphocytes and macrophages has been shown by Lipsky & Rosenthal (1975). Lymphocytes from previously immunized animals have been shown to express an ability to form macrohage-lymphocyte clusters. Rosenthal showed that the clustering was greater in macrophages that were previously exposed to the antigen.

With viral infections the first several hours may be critical to the final outcome. There is little literature dedicated to these early events and to the role of humoral or cell-mediated immunity in its resolutions. Most of the available literature's data has been collected by tissue culture techniques (Zinkengel and Rosenthal, 1981) or passive cellular transfers (Bladen, 1971). These procedures create an artificial circumstance which may or may not represent the actual host in vivo cellular response. Therefore, one of the purposes of this study was to document the early response of T cell and macrophages at the site of an

in vivo virus infection, and to look for specific cellular relationships between these cells such as clustering.

To accomplish these aims specific cellular antibodies were developed and labeled with fluorescent dyes to differentiate the immune cells in vivo. Antibodies specific for each cell type were labeled with a different dye, facilitating identification and enumeration of cells in a single tissue preparation.

Another purpose of this study was to correlate pathological changes of a virus infection with cell-mediated events. Virus infected cells may lyse due to the intercellular events attributed to viral maturation, or cellular destruction may be through cell-mediated immune response to altered cellular antigens. In the first instance virus adsorbs to the cell membrane, penetrates and takes control of the cell's biochemical pathways and resources. Massive alteration of the cell membrane result in loss of integrity and release of virus particles. In the second instance cytotoxic T cells recognize infected cells through the antigenically altered membrane and cause lysis. This latter effect was shown by Doherty and Zinkernagel (1974). They compared the cytotoxic activity of lymphocytes with and without T cells against lymphocytic choriomeningitis virus infected cells. To show this, virus infected radioactive cells were split into three equal groups. Presensitized lymphocytes and complement were added to the first group. To the second group equal number of the above lymphocytes, complement and anti-T cell antibody were added. In this group the anti-T cell antibody and complement were added to lyse the T cells. The last group served as a

control. After 16 hours a sample of media was removed from each culture, and counted. The lymphocyte plus complement group showed a significant increase in the lysis of virus infected cells as compared to the control. The anti-T cell treated lymphocytes plus complement showed a significant decrease in lysis as compared to lymphocytes and complement alone. Therefore, one can see that some of the cellular damage was brought about by the cytotoxic effects of the T lymphocyte.

In the studies to be described, a herpes virus, pseudorabies, was used to infect mice. This virus infection is fatal in both pigs and rodents. In pigs and rodents the virus has been reported to affect the central nervous system and to produce pneumonia (Baskerville, 1973; Doliv, Beretta, Bonitas, and Foroglou, 1978). Pseudorabies is an enveloped double stranded DNA virus. The viral envelope contains cellular components of the host cell from which it was produced. In order to eliminate these foreign host antigens the virus was passed several times through cells of the chosen animal model.

This project was divided into three phases. The first phase was to establish the pseudorabies virus in CF1 mouse cells. The second phase was to establish the sequential histopathological changes in the lungs of infected mice. The third phase was to determine the interactions of macrophages and T lymphocytes in the maturation of the lesions.

METHODS AND MATERIALS

Animals

CF1 mice (Mus musculus) were obtained from either Charles River Laboratories, Portage, Michigan or from the colony maintained at Western Michigan University, Kalamazoo, Michigan. Male animals were used throughout the experiment. Two kilogram New Zealand White male rabbits, purchased from Norman Longshow's farm (Augusta, Michigan), were used for the preparation of anti-T cell and anti-macrophage sera.

Animal Cell Culture

Primary mouse kidney cultures were produced from 7 day old CF1 mice. The kidneys were aseptically removed and diced into .5 cubic centimeter pieces. The pieces were placed into a 125 ml erlynmeyer with a magnetic stirring bar and 15 ml of .25% trypsin (Gibco) solution in calcium and magnesium free physiological buffered saline. The trypsin was then allowed to act at 23 degrees Celsius while stirring for 10 minutes. The erythrocyte rich first solution was decanted and discarded. Fifteen ml of the trypsin solution was again added and allowed to digest for 20 minutes. This second solution was decanted into a 15 ml centrifuge tube and centrifuged for 15 minutes at 400 X g. The supernant was decanted and the cell pellet resuspended with 5 ml of culture medium. Culture medium consisted of Earle's MEM, 15% heat inactivated fetal calf serum, 50 units/ml penicillin, and 50 mcg/ml streptomycin. These cells were then counted with a hemacytometer and

3 million cells were added to 75 square centimeter flasks, with 15 ml of culture medium. The cells were cultured at 37 degrees Celsius with 5% CO_2 in air.

Virus Culture

To rid the pseudorabies virus of unwanted mouse envelope antigens, the virus was passed through mouse host cells. CF1 mouse kidney cultures were established as previously described. When these cultures became about 70% confluent, 2 million plaque forming units (PFU) of pseudorabies virus suspension, donated by Dr. Darwin Buthala of Western Michigan University, was added to each flask. After 3 days of culturing or when 70% of the cells showed viral destruction the virus was harvested. The total infected cellular mixture was then dispensed in 1 ml aliquots and frozen at -70 degrees Celsius until needed.

The virus was passed three times in mouse kidney cultures and then checked for infectivity by the plaque forming units assay. To determine the PFU, mouse kidney cell cultures were produced in 60 x 20 mm, plastic culture dishes, incubated at 37 degrees Celsius in 5% CO_2 atmosphere. Virus was then thawed and serially diluted (by 1:10 fold dilutions steps) with physiological buffered saline (PBS) until a 1 to 1 x 10^{-9} fold dilution was reached. Cell cultures, 70% sheeted, were rinsed once with 5 ml PBS. To each plate .5 ml of a viral dilution was added, and allowed to attach for 30 minutes at room temperature. The excess fluid was then poured off and the culture overlayed with a mixture of .9% Nobel agar (Difco) and culture medium 199 (Gibco) containing 10% heat

inactivated fetal calf serum (Flow). The cultures were then incubated at 37 degrees Celsius in a 5% CO_2 gas incubator for 24 to 48 hours with periodic checking for viral plaques. The number of plaques at each viral dilution was recorded and the number of PFU/ml determined.

Antibody Production

Antibody production was accomplished by subcutaneously injecting live mouse cells into New Zealand White rabbits. Both anti-T cell and anti-macrophage serums were made, using CF1 mouse cell types mixed with an adjuvant.

The anti-T cell serum was made against T cells from the thymus of a 7 week old mouse. The thymus tissue was aseptically removed, diced and vortexed for 2 minutes in 10 ml of PBS. The larger pieces were allowed to settle and the thymocyte rich supernatant solution decanted. The suspended cells were sedimented, washed twice with 5 ml PBS, and reconstituted with 15 ml of Earle's MEM culture medium. Cells were counted with a hemacytometer, and 25 square centimeter tissue culture flasks (Falcon) seeded with 5 ml of culture medium containing 3 million cells. The thymocytes were cultured for 2 days to remove unwanted macrophages, which adhered to the flask and left the T cells in suspension. A sample of these purified thymocytes was stained with trypan blue (Gibco) and counted by a hemacytometer to determine the number of viable cells per ml. One million viable cells in 2 ml of PBS were mixed with 2 ml of Freund's complete adjuvant (Gibco). This mixture was injected into a male New Zealand White rabbit. One ml was

injected subcutaneously into the ventral, proximal portion of each leg. After 3 weeks, the rabbit was again injected with live cells by the same procedure, except that Freund's incomplete adjuvant (Gibco) was used.

Anti-macrophage serum was made in a similar manner to the anti-T serum. To obtain a large concentration of macrophages, in vivo stimulation was required. For this, 5 ml of mineral oil was injected intraperitoneally (IP) into 10 to 12 week old CF1 mice. After 3 days mice were sacrificed by cervical dislocation and their peritoneal cavities flushed twice by injecting 10 ml of PBS. While massaging the abdominal walls, the macrophage rich fluid was slowly withdrawn into a syringe. The flushings were collected in a 50 ml silicone (Siliclad) coated centrifuge tube, and the cells were sedimentated at 800 x g for 15 minutes. The mineral oil surface layer and supernatant was removed by vacuum aspiration. The cellular pellet was washed twice with PBS and then again with 5 ml of medium. Three million cells were cultured in 25 cm squred culture flask. Culture were gassed with 5% CO_2 and incubated at 37 degrees Celsius. They were fed at 48, 72 and 96 hours with harvesting after 120 hours. for harvesting of macrophages, 5 ml of trypsin-versene solution (calcium and magnesium free PBS with .025% trypsin and .52 mM EDTA) were used to loosen adherent cells. Cells were then washed twice and resuspended in 5 ml of PBS. Macrophages were processed exactly like the T cells in that they were counted with trypan blue for viability, injected into a rabbit with Freund's complete adjuvant for the first course and injected into a rabbit with Freund's incomplete adjuvant in the second course.

To obtain anti-macrophage and anti-T cell serum at their highest titer, the rabbits were routinely bled and tested. Antibody levels were measured by finding the highest serum dilution capable of agglutinating specific cells. For routine testing, rabbits were bled through the lateral ear vein, withdrawing 1 ml of blood with a 1 ml syringe and a 25 gauge needle. The blood was clotted for 24 hours at 4 degrees Celsius, separating approximately .5 ml of serum. Serum was diluted 100 fold with .9% saline, and 1 ml of this dilute was placed into two, 13 x 75 mm glass tubes. Starting with the second tube, duplicate two fold dilutions were made with PBS to 1:12,800. A CF1 mouse speen was surgically removed and minced into pieces. These pieces, along with 15 ml of PBS were placed in a 25 ml centrifuge tube and vortexed for 2 minutes. the suspension was allowed to settle for 1 minute. The supernate was decanted, washed with 10 ml PBS, and counted with a hemacytometer. This cellular suspension was then diluted to 5×10^5 white blood cells per ml. One ml of the suspension was then added to each serial dilution of serum along with control tubes that contained 1 ml of saline. These tubes were vortexed and incubated at 4 degrees Celsius for 24 hours before being checked for agglutination. Each serum dilution was then checked and the highest dilution with agglutination of cells was recorded.

Two weeks after the rabbits received their second course of antigen, they were exsanguinated through the left femoral artery. The blood was collected, clotted, and the serum separated. Serum was then frozen at -70 degrees Celsius until needed.

Conjugation of Rabbit Globulin

For direct labeling of T cells and macrophages, anti-T cell and anti-macrophage antisera were labeled with fluorescein isothiacyanate, or lissamine rhodamine B200 (Calbiochem-Behring), respectively. The procedure for processing either sera was the same except for the quantities of serum and fluorescent dye added.

The first step in conjugation required separation of the immunoglobulins by precipitating with ammonium per sulfate. The serum was diluted with equal volumes of ammonium persulfate working solution (60% saturated ammonium persulfate), and incubated at 25 degrees Celsius for 2 hours with continuous mixing. The precipitated globulin mixture was centrifuged at 1,570 x g at 4 degrees Celsius for 30 minutes, and the supernatant fluid carefully removed and discarded. An equal volume of distilled water was added to the precipitate. the mixture was then allowed to sit until the precipitate dissolved. A third precipitation was then carried out exactly like the second.

The globulins were conjugated according to the procedure of Rinderknecht (1962). First, the protein content of the globulin mixture was determined using the BioRad protein assay kit (BioRad). A globulin solution and equal volume of .06 sodium carbonate-bicarbonate buffer (ph 8.5) was prepared and shaken for 5 minutes at room temperature with .5 mg of fluorescent dye per milligram of globulin protein. The mixture was then centrifuged for 5 minutes at 400 x g. The supernatant was passed through a 2.8 x 25 cm Sephadex G-25 (Pharmacia Uppsalo, Sweden)

column. The column was prepared by overnight degassing of the gel prior to pouring the slurry. The gel was allowed to settle and then flushed with 1 liter of .12 M sodium phosphate buffer at ph 6.5. Separation of labeled globulin from unreacted dye was easily followed because the labeled globulin moved through the column displaying a moving band of color. The effluent was collected in 5 ml fractions and each fraction was frozen and stored at -70 degrees Celsius.

Removal of Nonspecific Staining from Globulins

After conjugation, two additional processing steps were required before the globulins were satisfactory for direct staining of tissue sections. First the nonspecific staining characteristics were eliminated by absorbing the globulins with various antigens and second the globulin titers were adjusted to optimize their staining quality.

Cultures of mouse kidney cells, T cell, macrophage, and bone marrow B cells were grown as previously described. Each cell type was stained with each labeled globulin and checked for nonspecific staining.

Absorption with CF1 kidney cultures removed antibodies directed against normal mouse antigen, such as epithelial-like and fiberblastic-like cellular antigens. confluent cell cultures, of 25 square centimeter size, were rinsed with 10 ml of PBS, and 5 ml of conjugated globulin was added. The cultures were incubated at 37 degrees Celsius for 30 minutes, and the globulin poured from the flasks. This procedure was repeated until no staining was detected and then the globulin was refrozen in 5 ml. quantities.

Next cross-staining antibodies were removed from these sera so that anti-T cell serum did not stain macrophages or B cells, and anti-macrophage serum stained only macrophages. For the adsorption of anti-T cell globulin (ATG), mouse macrophage cultures were prepared as previously described. The ATG was first absorbed by macrophage cultures followed by bone marrow cells. Bone marrow cells were used because they are rich in both B cells and macrophages. Bone marrow cells were prepared by the following procedure. Mice were killed by cervical dislocation. The long bones, such as the tibia and femor, were carefully removed and both ends of the bones cut off to expose the marrow. A 5 ml syringe and 25 gage needle was filled with PBS and injected into the marrow. The PBS was slowly forced through the bone and collected at the other end. All four long bones were processed and marrow collected into a 50 ml centrifuge tube (Falcon). Bone marrow suspensions were then centrifuged at 400 x g for 10 minutes and washed twice with two changes of PBS (10 ml each). After a final centrifugation, the supernatant was discarded and 5 ml of anti-T cell globulin was added, mixed, and handled as before. Only one adsorption was necessary to remove unwanted anti-macrophage and anti-B cell antibodies form the conjugated anti-T cell globulin.

Anti-macrophage globulin (AMG) was absorbed with cultured white blood cells and thymocytes to remove any B cell and T cell antibodies respectively. The thymocyte cultures were developed as described in the anti-T cell antibody production procedure. Here, 1×10^7 thymocytes were washed twice with 5 ml of PBS. This suspension was again centrifuged

to produce a cellular pellet. The pellet was mixed with 5 ml of anti-macrophage serum, and incubated at 37 degrees Celsius for 30 minutes. After incubation it was centrifuged at 800 x g, and the supernatant collected and saved.

Next, a pool of 10 ml of heparanized blood was obtained from four CF1 mice via cardiac puncture. Ten ml of PBS was added to the blood to make a two fold dilution. Two centrifuge tubes were then set up with 5 ml of Ficoll-Hypaque gradient solution (Gibco). Ten ml of diluted blood was carefully overlayed on the gradient solution. Both tubes were sealed and centrifuged at 800 x g at 4 degrees Celsius for 30 minutes. After centrifugation, a buffy coat of white blood cells containing T cells, B cells, and macrophages, was aspirated from the gradient. The aspirate was washed twice with 5 ml of PBS. The cells were then cultured for 2 days in 25 square centimeter flasks with 10 ml of culture medium. This culturing removed macrophages by adherance and left mainly T and B cells in suspension. The cellular suspension was again centrifuged at 400 x g for 10 minutes and washed with 5 ml of PBS with another centrifugation to produce a cellular pellet. Anti-macrophage globulin was absorbed with this pellet, as previously described.

To remove sub cellular particulate, the absorbed globulins were centrifuged at 100,000 x g at 4 degrees Celsius for 30 minutes in a Beckman L2-65B ultracentrifuge. The globulins were then refrozen and stored at -70 degrees Celsius until needed.

The absorbed globulins were prepared in 5, 10, 20, and 30:1 dilutions with 5 ml of PBS. One ml of each dilution was placed on

cryostat sections of CF1 mouse thymus, as described in the antibody staining section. The sections were then evaluted. The antibody dilution that stained the desired cell with the least amount of background staining was used.

Testing of Conjugative Globulin

To define antibody labeling characteristics, labeled cells were identified by alternative methods. T cells have specific cellular receptors for sheep red blood cells and form rosettes, whereas macrophages do not. Macrophages, on the other hand, have a high level of phagocytic activity and rapidly ingest latex particles (Ross and Winchester, 1980).

Testing of each antibody was done by evaluating the fluorescent cells' ability to form rosettes or to phagocytise latex spheres. To do this, mouse leucocytes were prepared as previously described, except that silicone treated tubes were used to limit adherent loss of macrophages. The leucocytes were suspended in 5 ml of PBS, counted, and diluted to 1 x 10^6 cells per ml.

One ml of the previously mentioned leucocyte suspension was mixed with 1 ml of PBS containing 30 mM sodium azide to inhibit membrane motility. Cells were centrifuged and resuspended with 1 ml of the sodium azide solution. Next, .25 ml of anti-macrophage or anti-T cell globulin was mixed with .5 ml of sodium azide treated leucocytes. The cells were incubated at 37 degrees Celsius for 30 minutes, then centrifuged at 400 x g for 10 minutes and washed with 2 ml of PBS. One ml of

a 2% solution of sheep red blood cells (Gibco) was added to each tube. The tubes were centrifuged at 100 x g for 10 minutes, then incubated at 4 degrees Celsius for 3 hours. All but a small volume of supernatant was aspirated from the tubes. The tubes were then gently rolled at a vertical position until the pellet dispersed. A small drop of each suspension was placed on a Reich counting slide and covered with a coverslip. From the anti-T cell antibody stained slides, each green cell was counted and scored for its rosetting capabilities. A cell was considered to be a rosette if it had three or more red blood cells attached. Next, the slides that were stained with the red fluorescent anti-macrophage antibody were also evaluated for rosetting cells.

In the second phase of antibody testing, 1 million leucocytes were incubated for 1 hour at 37 degrees Celsius with 1 ml of a .5% LB-11 latex bead (Sigma) suspension. The cells were then washed twice with the 30 mM sodium azide solution and resuspended with 1 ml of the same solution. One half of a milliliter of the cellular suspension was then mixed with .25 ml of either anti-T cell or anti-macrophage globulin in 13 X 75 mm tubes. These mixtures were incubated for 30 minutes at 37 degrees Celsius, washed twice with PBS, and resuspended with 1 ml of PBS and observed for fluorescence and phagocytoses.

Viral Innoculation of Mice

To study the natural defense mechanisms of the lungs, mice were intranasally infected with low doses of pseudorabies virus. To facitate deep respiratory instillation, mice were anesthetized with ether.

Anestheziation was stopped at the point where the animal showed convulsive breathing. Alveolar exposure was insures when a drop of innoculum was placed into the nasal cavity at the time of inhalation. This method also reduced the probability of swallowing the nasally deposited droplet of virus.

Pseudorabies virus was diluted with PBS to contain 6,000 PFU/mL. Mice were infected with 50 microliters of this viral suspension, thus each mouse received approximately 300 PFU.

To study the cellular pathologic effects of pseudorabies virus, lungs from 16 virus infected mice were removed, embeded in wax, and stained with hemotoxylin and eosin. Groups of four mice were innoculated and killed at 0, 48, 72, and 96 hours post-innoculation.

For the immunohistochemical studies, 30 mice were infected and killed at specific times by overdosing with ether. Groups of 5 mice were killed at 0, 24, 48, and 72 hours post-infection. One group of 5 mice progressed until killed by the infection and a control group innoculated with PBS (placebo) was killed 24 hours post-innoculation. In order to look at the early events occurring during infection, a short term study was conducted in which mice were infected in the same way, but were sacrificed at 0, 2, 4, 8, 16, 32, and 48 hours post-innoculation.

Sectioning and Staining of Tissue

For histopathology, infected mouse lung tissues taken at 0, 48, 72, and 96 hours post infection were processed according to the following schedule.

Table 1

Paraffin Wax Embedding Schedule of Mouse Lungs

10% formalin	2 days
70% alcohol	1 hr.
95% alcohol	1 hr.
95% alcohol	1 hr.
absolute alcohol	1 hr.
absolute alcohol	1 hr.
xylene	1 hr.
xylene	1 hr.
paraffin	2 hr.
paraffin	2 hr.

After the last step, the lung tissue was placed in a metal mold and covered with hot paraffin. The cooled blocks were then serially sectioned at 6 micrometers. These sections were melted onto the slides and stained with hematoxylin and eosin according to the following schedule.

Table 2

H. & E. Staining Schedule of Lung Sections

xylene	2 minutes
xylene	2 minutes
absolute alcohol	1 minute
absolute alcohol	1 minute
90% alcohol	1 minute
tap water	4 dips
harris hematoxylin	15 minutes
tap water	4 dips
acid alcohol	3 dips
tap water	4 dips
ammonia water	6 dips or blue
distilled water	10 minutes
eosin	20 minutes
95% alcohol	1 minute
95% alcohol	1 minute
absolute alcohol	1 minute
absolute alcohol	1 minute
xylene	2 minutes
xylene	2 minutes
xylene	2 minutes

After the slides had been taken through the last xylene step, two drops of Permount (Fisher) were added and cloverslips positioned. These

stained sections were used for evaluation of pathological effects of pseudorabies virus infection.

For immunohistochemical staining, fresh whole lungs were placed on a metal mounting platform and covered with O.C.T. Compound (Lab-Tek Products). The specimens were frozed at -20 degrees Celsius and 6 micrometer sections taken. Sections were melted on a glass slide and refrozen at -20 degrees Celsius until stained. To reduce antigenic loss, section were stained less than a week after the first group of mice were sacrificed.

Before staining, the frozen sections were fixed in 4 degrees Celsius acetone for 10 minutes and then air dried. The dryed sections were enclosed within a circle of wax, and a mixture of a 1:10 dilution of anti-T cell globulin and a 1:20 dilution of anti-macrophage globulins was used to flood the sections. The slides incubated for 30 minutes at 37 degrees Celsius, then rinsed with two changes of PBS. The wax circle was wiped away and 2 drops of FA Mounting Fluid (Difco) was added before covering with a coverslip.

To visualize the cellular staining characteristics, a Leitz, Opthoplan microscope was equiped with a 3 mm BG 12 excitation and a K 510 suppression filter for the green fluorescence of the anti-T cell globulin, and a 2 mm G BG 36 excitation and K 580 suspression filter for the red fluorescence of anti-macrophage flobulin. Simultanous staining and observation of both T cells and macrophages allowed visualization of the spacial relationship of these two cells types. The best visualization was noticed with the first set of lens. With

these lens, one could see the T cells fluorescing green and macrophages fluorescing orange. To better indentify the macrophages the second set of lens were used to remove the green fluorescence of T cells.

Evaluations were made on one transverse midsection from the lung of each animal. The slide was stained as mentioned previously and examined with a phase contrast setting at 400 X magnification. An area was pinpointed by phase contrast so that the entire field of vision was filled with alveolar tissue. The microscope was then changed to a fluorescent setting and each cell type was counted and recorded. the microscope was then readjusted for the phase contrast setting and moved to a new area. The movement was from left to right and from top to bottom. This procedure was repeated seven times on each slide. The results were averaged for each group of mice at that time period. This experiment was repeated twice: once with time intervals of 0, 24, 48, and 72 hours and again at 0, 2, 4, 8, 16, 32, and 48 hours.

RESULTS

Antibody Production Response

Shown in Figure 1 are the average titers of rabbits immunized with either T cells or macrophages. Clearly, macrophages (titer of 1/2,000) stimulated a greater response than did T cells (titer of 1/720). The primary antibody response to the first antigen injection of both antigens peaked after two weeks. After the second injection of either antigen, the rabbits responded in approximately one week with almost a doubling of the titer, macrophages gave a titer of 1/2,800 and T cells elicitated a final titer of 1/1,800.

Antibody Specificity

The specificities of the heterologous anti-macrophage and anti-T cell globulins, were tested using their conjugated forms. Each labeled globulin was observed to nonspecificaly stain normal mouse kidney cell antigens. This nonspecific staining characteristic both anti-T cell and anti-macrophage globulins were removed by repeated adsorption with mouse kidney cell cultures. These adsorbed globulins were checked for cross staining between macrophages and T cells, and both globulins showed slight cross reactivity. The cross reactivity was lowered by dilution, however this decreased specific staining. To retain fluorescent intensity both globulins were adsorbed with their opposing purified cell types, anti-macrophage globulin by a purified culture of T lymphocytes and bone marrow cells, and the anti-T cell globulin by

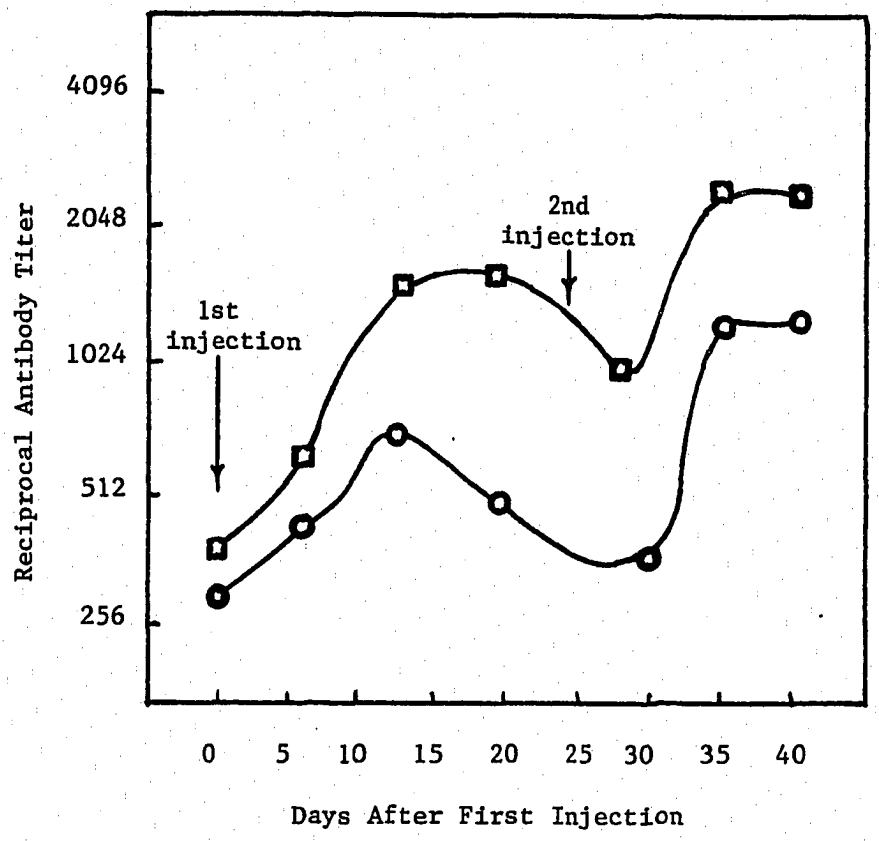

Figure 1. Mean agglutination titers of anti-T cell and anti-macrophage sera. Each line represents the average of two rabbits. Open squares display anti-macrophage sera data and open circles show anti-T lymphocyte sera titer.

a purified culture of T lymphocytes and bone marrow cells, and the Anti-T cell globulin by purified macrophages and bone marrow cultures. These globulins were found to stain specific cells most intensely at dilutions of 20:1 for anti-macrophage globulin and 10:1 for the anti-T cell globulin.

The specificity of cellular labelling of these globulins was confirmed by established cellular differentiation techniques (see Table 3). T cells were identified by rosetting and macrophages by the phagocytosis of latex spheres. Ninety-five percent of the anti-T cell globulin stained cells were positive for sheep red blood cell rosetting and negative for latex ingestion (less than 5%), while greater than 95% of the anti-macrohage globulin stained cells were positive for latex particle ingestation and negative for sheep red blood cell rosetting (less than 2%) (see Figures 2 and 3).

Viral Pathology

By 24 to 48 hours post viral innoculation, one could see animal behavioral changes. Mice began to scratch and rub their noses. On Days 2 and 3, some mice were showing a loss of the lateral facial hair. By Days 3 and 4, breathing rhythm began to show loss of smoothness and developed a shallowness. When pseudorabies virus was allowed to run its couse, all mice died within 4 to 5 days.

Histopathological effects were observed as early as 24 hours. the first sign was leucocytosis of the alveolar spaces (Figure 4). By 48 hours, spotty cellular damage of the bronchial and bronchiolar epithelium

Table 3

Immunohistochemical Staining vs. Alternate Enumeration Technique

Stain	Alternate Methods	
	Sheep RBC Rosette Forming	Phagocytocis of Latex Beads
Anti-Macrophage Globulin	< 5%	>95%
Anti-T Cell Globulin	>95%	< 2%

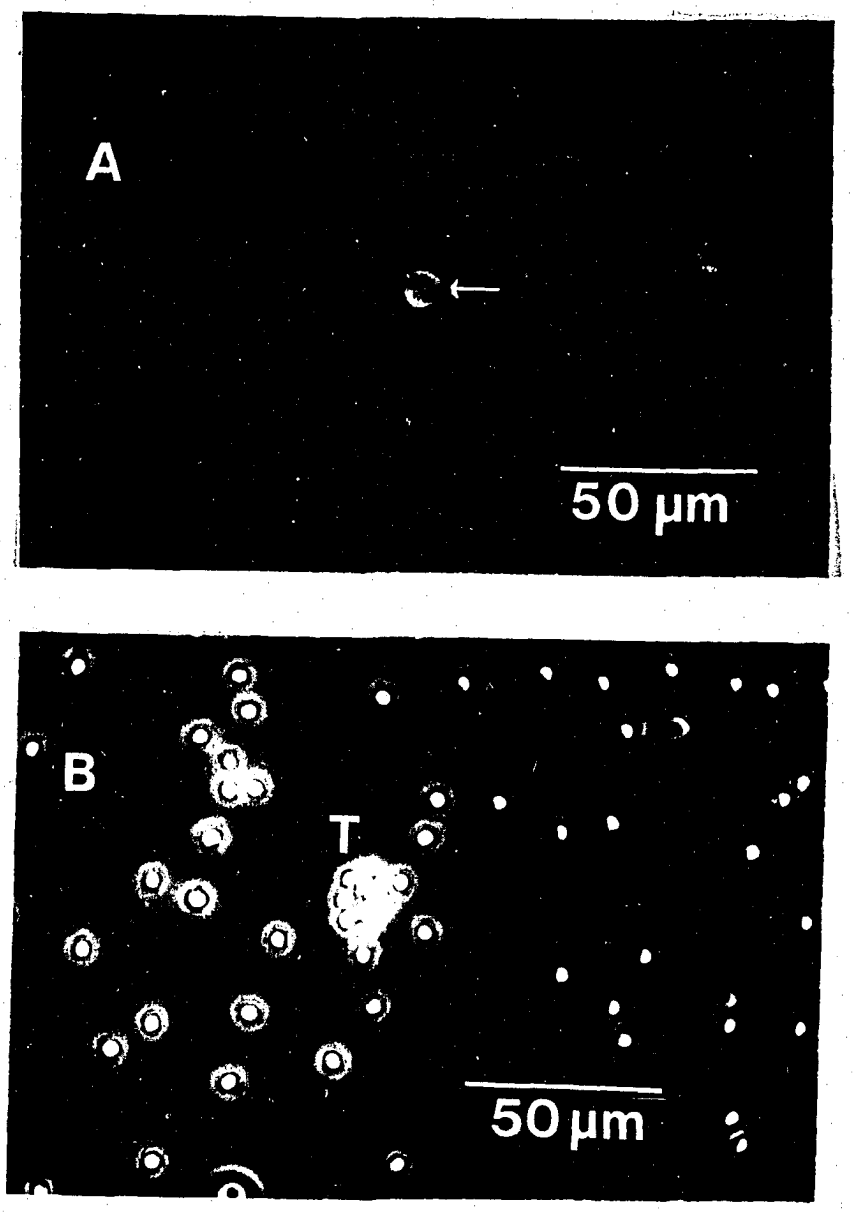

Figure 2. Testing of conjugated anti-T cell globulin. Mouse leucocytes were stained with the FITC conjugated anti-T cell globulin in the presence of sodium azide at 4 degrees Celsius. These cells were then washed with PBS and allowed to rosette with sheep red blood cells. Photograph A shows the fluorescent emission of a labeled T cell. Photograph B displays the rosetting capabilities of the same cell in A, except that phase contrast lighting was used to show the sheep red blood cells.

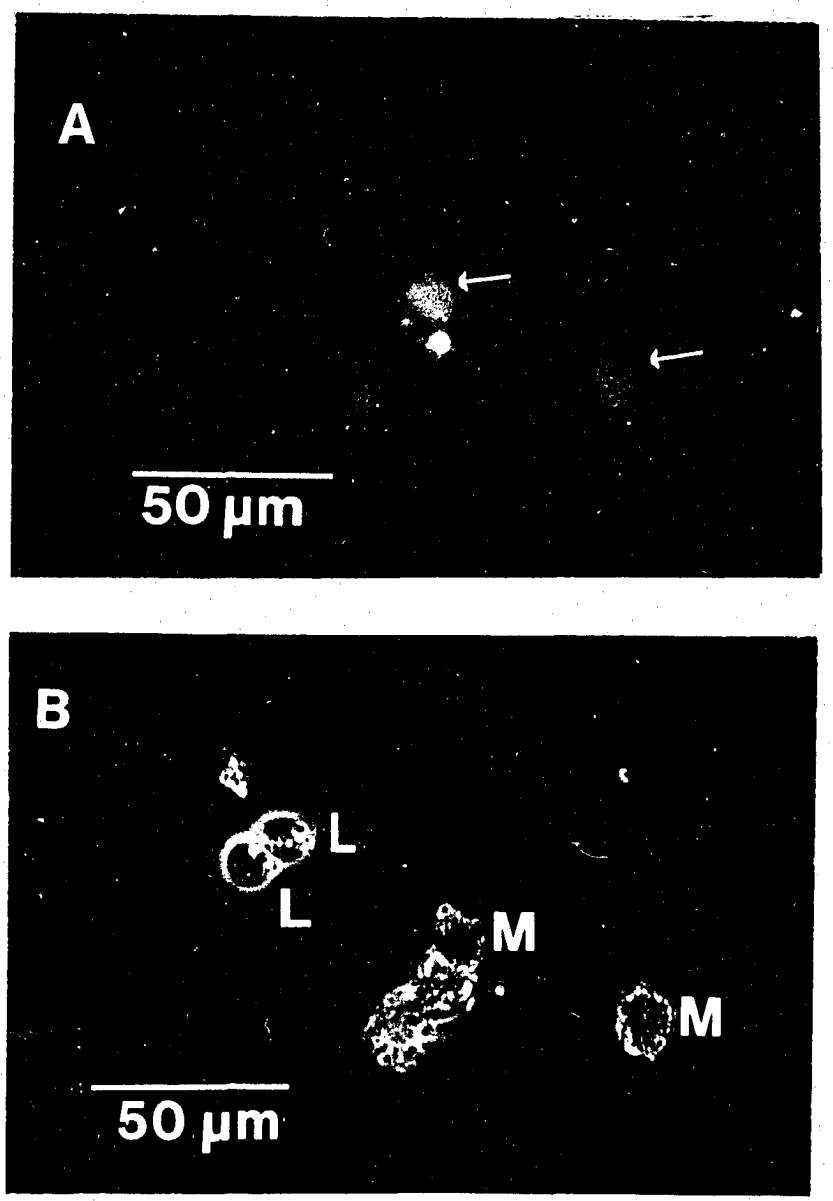

Figure 3. Testing of conjugated anti-macrohage globulin. Mouse leucocytes were incubated at 37 degrees Celsius in the presence of .5 percent LB-11 latex beads. These cells were then washed in PBS and stained with lissamine rhodamine conjugated anti-macrophage globulin at 4 degrees Celsius in the presence of sodium azide. Photograph A shows the red fluorescence of two macrophages (depicted with arrows). Photograph B shows the exact same leukocytes, but with phase contrast lighting. Notice the macrophages labeled with the letter M are heavily filled with latex beads and that the two lymphocytes labeled with the letter L only show external bead attachment.

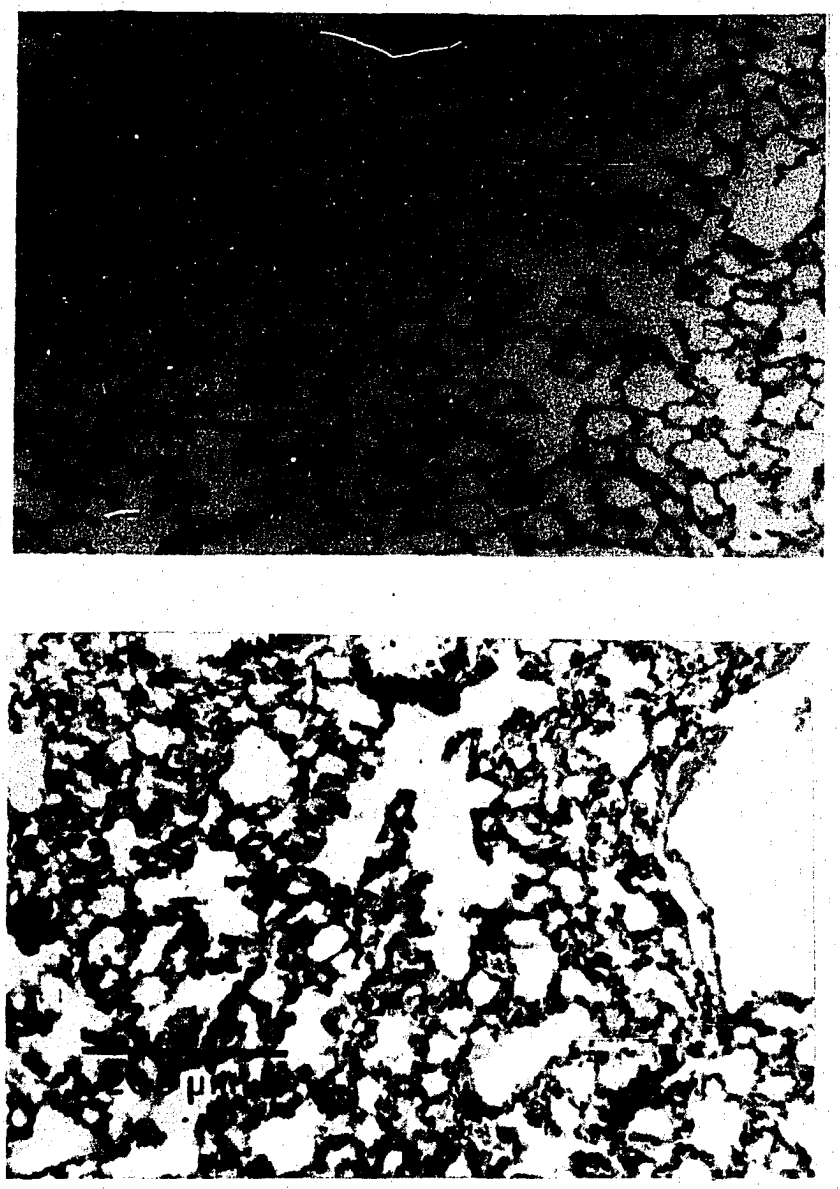

Figure 4. Low power observation of infected and control mouse lungs. Photograph A illustrates the clean alveolar area of control mouse lungs. Photograph B shows heavy leucocytic infiltration of the alveolar area at 48 hours post-innoculation. Both photographs were of mouse lung stained with hematoxylin and eosin.

was observed. Tissue sections showed epithelial decilliation and nuclear changes (Figure 5). Some nuclei tended to round up and locate closer to the basal membrane. By Day 3, nuclear and cytoplasmic inclusion bodies were observed. Leucocytic infiltration extended throughout the lung, and the infected bronchial and bronchiolar lesions had then increased in size and depth. Some of these lesions reached the basal membrane.

From Day 3 to Day 4, an exudate of cellular debris was noticed within some airways as well as occasional hemorrhage. The most prominent and consistent observation was a perialveolar mononuclear cell infiltration. To assess the contribution of T cells and macrophages to this mononucler cell response, immunohistochemical stains were used.

T Cell and Macrophage Involvement

To determine the T cell and macrophage involvement in the inflammatory process, a time sequence study was performed at 9, 24, 48, and 72 hours post infection. Slides were prepared from each group of five animals. The zero hour group and the placebo group showed about equal numbers of cells per field for both T cells and macrophages. T cells averaged 6 cells per field where macrophages averaged 2 cells per field. Twenty-four hours post-infection both cell types reached peak numbers. T cells showed a peak value of 18 and macrophage a peak value of 26 cells per field. These values along with the values found at 48 and 72 hours were significantly higher ($p < .05$) than the placebo control and the zero hour values. There was no significant difference in the 24, 48, and

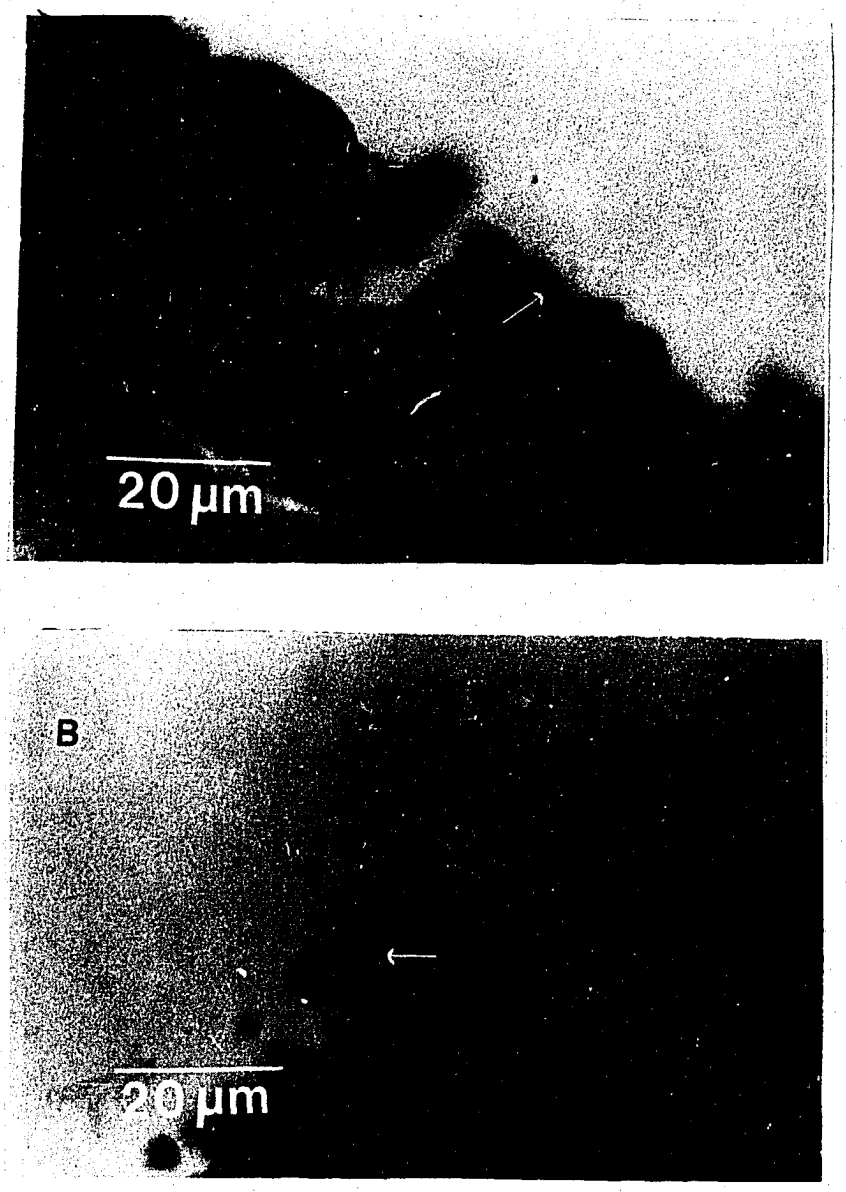

Figure 5. High power observation of infected and control mouse bronchial linings. Photograph A depicts normal bronchial linings (notice tall columnar cells and arrow showing normal cillia). Photograph B shows pseudorabies infected mouse bronchial linings at 72 hours post innoculation (notice destroyed cillia and shortness of cells). Arrow points to numerous vacuoles.

72 hour readings as determined by the student T test. The T cell and macrophage involvement appeared at 24 hours in the alveolar area (Figure 6 and 7). At 48 hours, some macrophages and T cells were noticed in the bronchial and bronchiolar linings. By 72 hours, infiltration of both cell types were equally distributed throughout the lungs. Thus, both cell types responded to the virus within 24 hours.

Observations at 0, 2, 4, 8, 16, 32, and 48 hours post-infection were then done to more closely depict the actual response time for these cell types. At zero hours, both cell types displayed results consistent with the previous study. A difference between infiltration by the two cell types began at 2 hours (Figure 8). The T cell number at 4 hours showed a significant increase ($p < .05$) over the placebo and the zero hour, but the macrophage value remained relatively unchanged. At 4 hours, the number of T cells per field had already peaked, with a value of almost three times the control value. The number of macrophages remained relatively unchanged until 16 hours (Figure 9). However, by 16 hours the macrophage number suddenly peaked and leveled off. The value was also almost three times the original value and had a significant difference between the 8 and 16 hour readings ($p < .05$). By comparing Figures 8 and 9, one can see that there was an approximate 12 hour lag between the T cell and the macrophage response.

Through the use of a direct simultaneous labeling technique, cellular contact between T cells and macrophages was then checked (Figure 10). The average number of macrophage-T cell contacts were divided by the average number of macrophages to give the percentage of

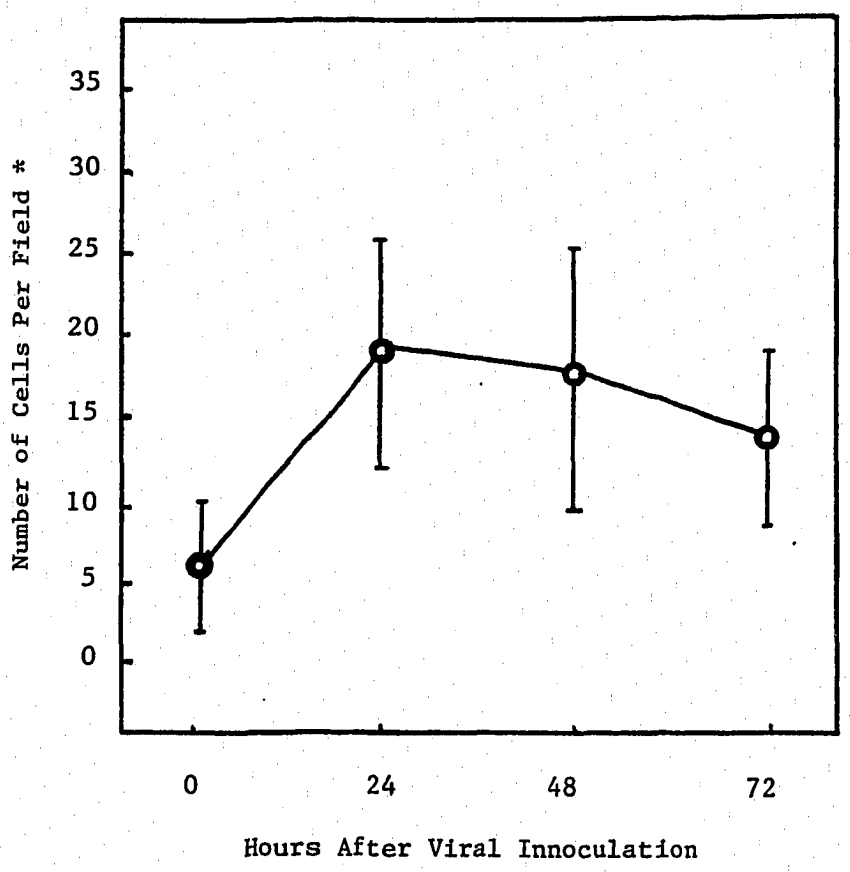

Figure 6. Three day study of T cell influx within virus infected mouse lungs. Each point represents the average of five mice. Vertical bars display standard deviations. *Area of field is 1.73×10^{-1} mm^2.

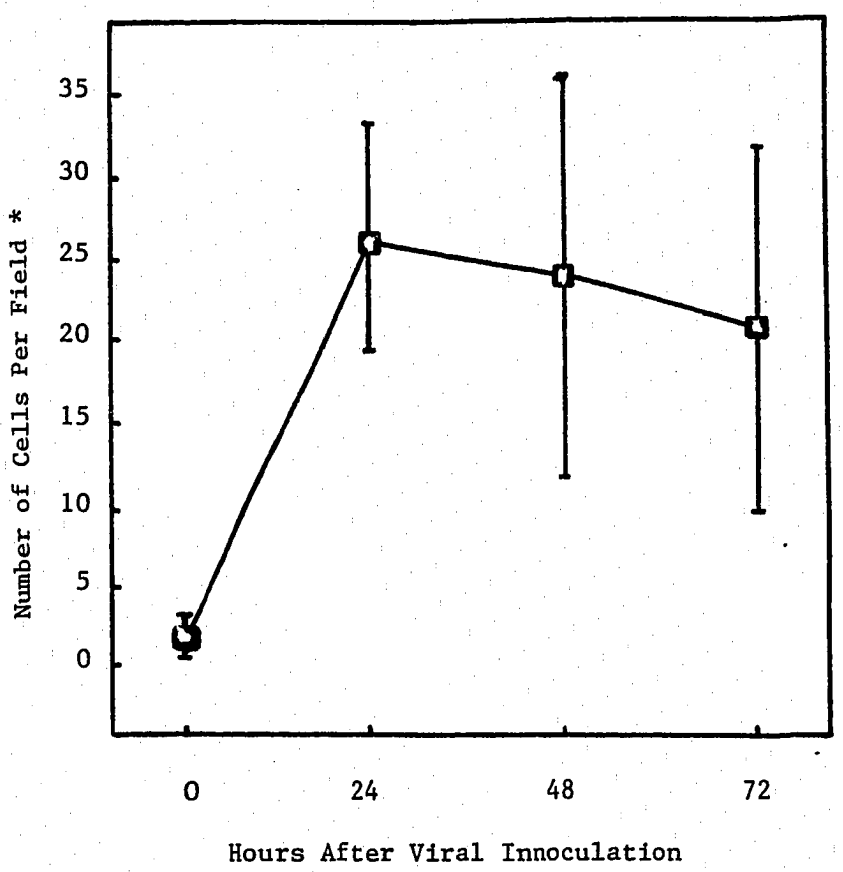

Figure 7. Three day study of macrophage influx within infected mouse lungs. Each point represents the average of five mice. Vertical bars display standard deviations. *Area of field is 1.73×10^{-1} mm^2.

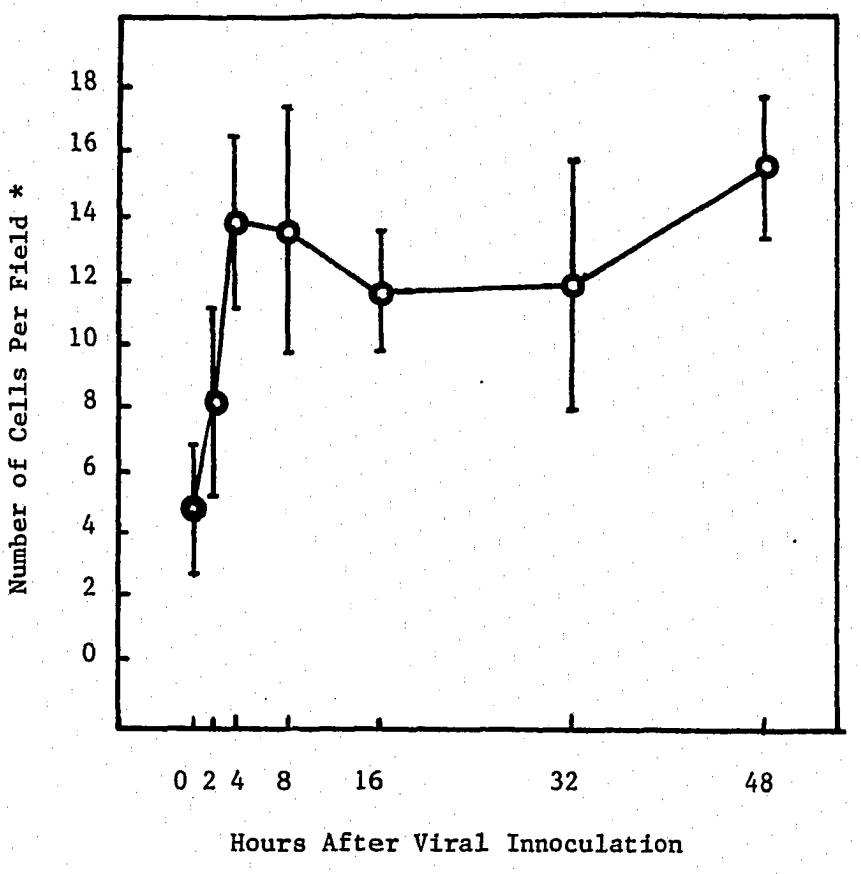

Figure 8. Short term study of T cell influx within infected mouse lungs. Each point represents the average of five mice. Vertical bars display standard deviations. Notice how T cells peak by as early as 4 hours. *Area of field is 1.26×10^{-1} mm^2.

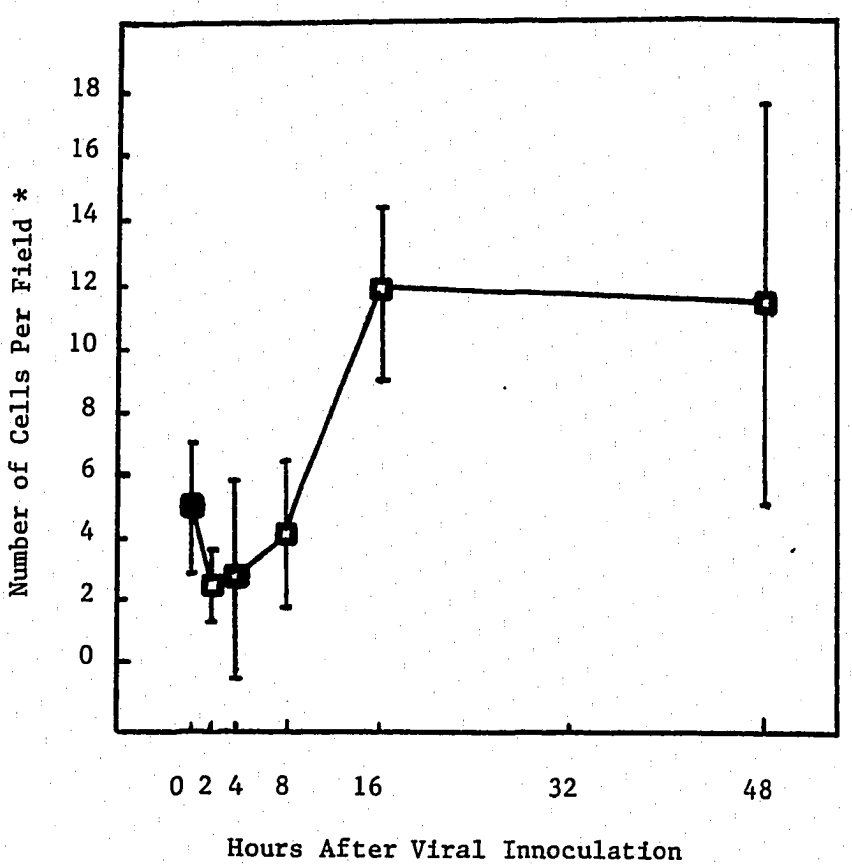

Figure 9. Short term study of macrophage influx within infected mouse lungs. Each point represents the average of five mice. Vertical bars display standard deviations. Notice that macrophage influx does not peak until 16 hours. *Area of field is 1.26×10^{-1} mm^2.

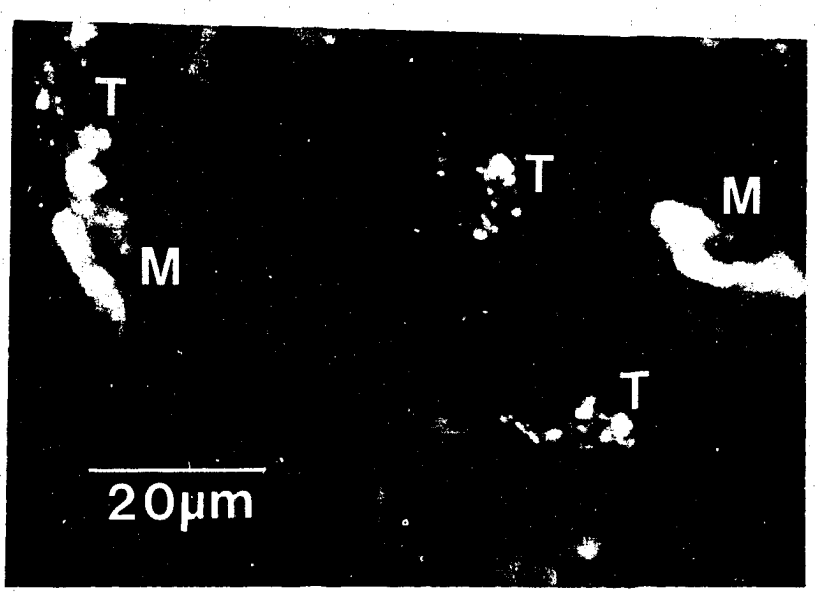

Figure 10. T cells and macrophages in pseudorabies infected mouse lung. T cells and macrophages were simultaneously observed using both anti-T cell and anti-macrophage globulin on cryostat sections of infected lungs. M & T represent the red fluorescent macrophage and the green fluorescent T cell respectively. Also on this slide was a rare shot of a possible macrophage-T cell interaction.

macrophage clusterings per visual field. No significant increase in the T lymphocyte and macrophage association was noticed during the course of infection. However, the ratio of T cells to macrophages at their peaks (4 and 16 hours respectively) seem to be a 1:1 ratio.

DISCUSSION

Antibodies

During the production of anti-macrophage and anti-T cell globulin different immulogical characteristics were noted in titer and specificity. Both antibody titers displayed the classical primary and secondary response (Roitt, 1974, pp. 70), with a slow increase in titers during the primary response and a sharp increase after the second antigen injection. This secondary response developed the high concentration of antibody needed for direct antibody conjugation. Anti-macrophage agglutination titers were consistently twice as high as the anti-T cell serum titers. These agglutination titers paralleled the staining capacities of conjugated anti-macrophage globulin and anti-T cell globulin, allowing the use of the former at a dilution of 20:1 and the latter at 10:1. Since the numbers of cells and animal innoculation conditions were identical for macrophages and T cells, the response difference may have been due to 1) trypsinization exposing more antigenic sites on macrophages, 2) macrophage cultures being more homogenous, 3) macrophage cultures having more natural antigenic sites, or 4) macrophage cells were already activated to express other antigenic sites.

Trypsin, a pancreatic protolytic enzyme, digests peptide bonds between arginine or lysine, affecting cell surface membrances. This potentially exposes protein chains for immune recognition (Arnal-Monreal, Goltzman, Knaack, Wung, and Huang, 1977). Therefore, removing attached

macrophages from the culture flask by trypsin could expose hidden antigenic sites. Another possible explanation for the increased antigenicity of macrophages over T cells pertains to the purity of the antigen employed. By using only adherent cells, a considerable increase in the concentration of macrophages was obtained (Rocklin, 1980). Because of macrophage surface morphology and size, they possibly have a greater number of natural antigenic sites than T cells. Macrophage diameter exceeds that of T cell by about 2 microns, and have a higher density of major histocompatibility antigens than T cells (Biddison, 1982; Benacerraf, 1981). To stimulate macrophage migration into the peritoneal cavity, mineral oil was used. This procedure activates macrophages causing considerable physiological changes and probably membrane antigens expression. It has been shown by Ezekowitz, Austyn, Stahl, and Gordon (1981) that stimulated macrophages increase in size, membrane activity, and surface markers. All of these factors may have contributed to macrophage antigenicity resulting in a higher anti-macrophage titer than anti-T cell titer.

Staining

The results of this study documents that pseudorabies virus infection of CF1 mice lungs cause a rapid cell mediated immune response. This response was observed through histochemical and immunochemical staining. Hematoxylin and Eosin staining showed alveolar build up of infiltrating leucocytes and located pathological changes. The immunochemical stains facilitated localization and quantitation of the T cells and macrophages.

Using these two methods of observation, a time sequence of host response and viral pathological changes were noted. Following inhalation of the virus a latent period of approximately 12 to 24 hours was usually observed, during which no virus could be recovered (Levine, Buthala, and Hamilton, 1971). During this latent period many cellular and tissue changes occurred. The first observable tissue alteration occurred 4 hours post-innoculation, when the number of T cells per area increased to three times the normal. After another 12 hours, the number of macrophages in the area increased to three times their normal value. Both cell types first appeared diffusely throughout the alveolar area, but did not reach the surface of the bronchial linings.

The 4 hour change in T cell concentration, as observed by anti-T cell globulin staining was the first statistically significant event. By 2 hours, even though not having a P value of less than .05, there was still a doubling of the control value. This 2 hour reading and its linear relationship with the 4 hour value shows that the T cell response to the virus was almost immediate. There are several possible explanations for this response.

Following cellular infection by a herpes virus such as pseudorabies, a number of immediate changes take place. First the virus envelope fuses with the target cell membrane, thus inserting into the cell new antigens. Then the virus nucleic acid is released into the nucleous of the cell, and within 1-2 hours directs the production of several enzymes and nonstructural proteins. These nonstructural proteins can be found in the cell nuclear membrane and the cytoplasmic membrane well before

mature virus is produced (Warshawski, 1978). A population of circulating T cells have membrane receptors that recognize these viral antigens, or the neoantigens instilled into the cellular membrane similar to the observation of Valdimarsson, Agnarsdottir, and Lachmann (1975) for measles virus. Once the T cell receptors interact with the target antigen a number of lymphokines are produced such as Interleukin II (IL II) and lymphocyte chemotactic factor. The latter would attract more lymphocytes to the areas and the former would trigger differentiation and cellular division.

Another reaction that would be taking place simultaneously to the T cell response would be the interaction of macrophages and the neoantigens. As shown by Mizel (1982), macrophages on contact with antigen produce monokines such as Interleuken I (IL I) which cause T cells to activate and proliferate, thus increasing the number of antigen reactive cells in the area of antigen concentration.

Another possibility is that virus infected fibroblasts or other interstitial cells could have produced interferon(s) that cause the activation in the T cells at 4 hours post-innoculation. Horoszewicz, Leong, Berardino, and Carter (1978) have shown how fibroblasts can be used for large scale production of human fibroblastic interferon. A release of interferon would cause activation of a specific subset of T cells called NK cells. Zarling, Eskra, Borden, Horoszewicz, and Carter (1979) showed that fiberblastic interferon could activate NK cells. This activation was expressed by an increase in target cell lysis. The increased lysis of infected cells would then cause an increase of accessible viral antigens.

The T cells observed early during infection may have been natural killer cells (NK). Herberman and Orthaldo (1981) observed that natural killer cells can respond to a viral infection in 4 hours or less. Also, natural killer cells display some T cell antigens (Cantor, Kasai, Shen, LeClerc, and Glimcher, 1979), and could have been stained by the anti-T cell globulin used in this experiment.

The next cellular change occurred by 16 hours post-innoculation with the accumulation of macrophages into the infected area (Figure 8). Again, the mechanism of action is hard to define. Macrophages may have been drawn into the area by soluble chemotactic lymphokines, that were produced by the early arriving T or NK cells. Many independent lines of evidence have suggested that T cell recognition of virus (or other antigents) triggers macrophage involvement (W.H.O., 1972). The data presented by this in vivo study agree with the in vitro systems of Bloom and David (1976), which illustrate that T cell lymphokines affect macrophage activity and involvement. The three lymphokines that have been directly related to macrophage involvement are migration inhibitory factor (George and Vaughan, 1964), macrophage chemotactin (Nelson, Quie, and Simmons, 1975), and macrophage activation factor (Nelson, 1976). Two of these factors, macrophage inhibitory factor and macrophage chemotactin, may have been related to accumulation of macrophages into T cell recognized areas. A 12 hour lag period between T cell and macrophage peak involvement shows that there was time for the T cells to produce these celular factors, and possibly go through a cell replication cycle. A replication cycle may be needed to produce an effector T cell

population. This could be very similar to pokeweed mitagen stimulation of T cells *in vitro*, for after T cells in culture are stimulated they divide and then produce lymphokines (Kite, 1973, pp. 75-76).

Another possible explanation for the macrophage accumulation was that after 12 hours the virus may have been shed, and the increased number of T cells picked up the virus and expressed then to the macrophage. This expression may be done through direct cell contact. It was believed that T cells and macrophages transmit information or specificities by cell to cell clustering (Lipsky and Rosenthal, 1975; Braendstrup, Werdelin, Schevach, and Rosenthal, 1979; Lyons, Tucker, and Uhr, 1979). Using the simultaneous double cell staining technique, there was no observed increase in cell clustering. It could be that clustering is a later developed response, or that very few clusters are required to initiate a large response. Earlier studies were done using primed animal lymphocytes, where the animals were allowed to develop an increased population of sensitive T cells to the chosen antigens. So cell clustering may be a later event in the host's protection mechanism. Therefore, with no increased cellular clustering one could assume that the T cell's influence on macrophages is mediated *in vivo* by diffuse factors such as lymphokines and that the T cell macrophage clustering involves a small percentage of the total population.

As shown by my data, the increase in T cells and macrophages must be recruited directly from blood and not the result of local cell replication. With a greater than three-fold increase in T cell and macrophage numbers in a 4 or 8 hour period (data of Figures 4 and 5),

there was hardly time for replication. Even fast dividing cultured tumor cells can only double in number after 12 hours (Gray, Dean, and Mendelsohn, 1979, pp. 383-407). This theory of cellular attraction agrees also with other papers in the roles of monocytes and interstitial cells in generating alveolar macrophages (Adamson and Bowden, 1980; Bowden and Adamson, 1980; Velo and Spector, 1973). My study also agrees with the paper of Wyde, Couch, Mackler, Cate, and Levy (1977) by showing that a cell-mediated immune response can occur in one or two days to a primary antigen. They showed that there was a difference in a low and a high passage influenza virus infection. They demonstrated histologically that mice intranasally innoculated with the high passage virus (virus that was passed through cell culture more than three times) developed an early cell-mediated response in one to two days. Their in vivo data on high passage virus showed that a large percent of these cells probably came from the thymus, for by Day 3 there was already a 49% decrease in thymus size.

Another study that pertains to the influx of immune cells into an infection was the study of Williams and Waksman (1969). However, their study dealt with a delayed type hypersensitivity reaction to a second encounter of purified protein derivative (PPD), a bacterial antigen. Using fluorescein conjugated Lewis rat anti-DA rat antibody with hybrid thymus graph implants (Lewis X DA) in thymectomized irridated Lewis rats, William and Walksman displayed an increase in the number of graphed thymus cells at skin test sites to PPD. At 8 hours they showed that 15% of the cells at the test sites were thymus derived. At 16 hours, 20% of

of the cells were thymus derived. The 24 hour data showed that only 5% of the cells at skin test sites were of thymus origin. The sudden decrease was reportedly caused by the increase in the number of nonspecific cell over the specific cells. They only showed data reading for 0, 8, 16, and 24 hours post-innoculation. Therefore, their response may be quite similar to this paper, for by summing the T cell and macrophage response together and only looking at the 8 and 16 hour readings, one gets a similar curve.

Following the influx of mononuclear cells into the area, one could begin to see the destruction of mouse lung cells. This did not occur until 24 to 48 hours. Cellular pathology, observed by light microscope, displayed itself first by decilliating the bronchial and bronchiolar linings, and gradually increased to loss of cells. This loss started at isolated spots and spread profusely from there. Knowing where the first signs of cell destruction occurred, and the location of both the macrophage and T cells, we can now speculate on how cellular destruction occurs. The first signs of destruction are probably caused by the direct lytic effects of the virus, because cell destruction was first observed on the bronchial linings where few macrophages and T cells were found. Later cell destruction may be caused by a cell mediated mechanism, because after a few days the entire lung was infused with these immune cells. This agrees with Wells, Albrecht, and Ennis (1981) where influenza infected mice lungs substantially increased in weight and size by Days 3 and 7. They also showed that the spleen cells of these infected animals displayed an increase in specific target cell

lysis against influenza virus tainted mouse cells. This increase in their lytic ability began to rise by Day 3 and peaked by Day 7. This showed that it takes a few days to develop a strong cytotoxic cellular response.

SUMMARY

During the course of a controlled pseudorabies virus infection of mice, four major events were noted. First, T cells and macrophages accumulate in the infected area probably through the effect of soluble humoral factors, such as lymphokines, and are not increased by self replication of indigenous macrophages and T cells. The initial triggering mechanism is still not well defined. On possibility is that infected interstitial cells possibly release interferon, which activates the NK cells or T cells already in the area. Another possibility is that macrophages or T cells, which are already present in a low number, release interleukins or chemotactins. These soluble factors attract NK cells or T cells. These cells then attract macrophages by producing lymphokines that draw on the macrophages' reserves within the blood. The macrophages (or monocytes) would respond to the antigens by producing monokines that further activate these cells and other immune cell types to develop a cytotoxic cellular response. Second, T cells precede macrophages and possibly go through a cell cycle to produce effector cells. Thirdly, the increase in clustering of macrophage and T cells was not noticed, which indicates that clustering is a later occurence or that it is done only by a small percentage of cells. Finally, the first signs of cellular destruction are not from the immediate cell mediated response, but are from the direct effects of virus lytic destruction during replication. This is shown by having very few T cells and macrophages in the early infected areas.

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