UNIFORMITY AND DIVERSITY OF MACROPHAGES IN CELLULAR IMMUNITY

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BRIAN CHIH-TZU WU

This is to certify that the

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Major professor

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UNIFORMITY AND DIVERSITY

OF MACROPHAGES IN CELLULAR IMMUNITY

By

Brian Chih-Tzu Wu

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ABSTRACT

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OF MACROPHAGES IN CELLULAR IMMUNITY

By

Brian Chih-tzu Wu

This study was undertaken to determine whether differences in immunologic behavior, in the cellular immunity system, exist among common macrophage types found in different anatomical locations.

Peritoneal macrophages, Kupffer cells from the liver, macrophages from the spleen, and alveolar macrophages from the lung of the mouse were maintained in tissue culture. Both normal and immune cells were challenged with Brucella suis and the fate of the resulting intracellular bacteria determined.

B. suis multiplied unrestrained in all four types of normal macrophages. In the immune cells, there was marked suppression of growth, and killing of the intracellular bacteria by macrophages from the peritoneal cavity, liver and spleen; the alveolar macrophages from the lung displayed no such increased resistance; the intracellular growth curves were identical with those obtained with normal cells.

INTRODUCTION

The effector cell role of the macrophage in cellular immunity in bacterial diseases has been well established. However, the term macrophage has been used to describe many cells of differing morphology and anatomic distribution. Indeed, metabolic and enzymatic differences have been reported for phagocytic cells of quite similar morphology. In studying cellular immunity, the question has arisen as to whether differences in immunologic behavior may also exist among this group of cells. Accordingly, this study was undertaken to determine whether any difference in immunologic behavior could be demonstrated among four common varieties of macrophages; peritoneal macrophages, Kupffer cells from the liver, macrophages from the spleen, and alveolar macrophages from the lung.

Methods for maintenance and cultivation of macrophages *in vitro* offered a convenient and direct means of measuring immunologic parameters of isolated cell types. Further, methods for isolation and purification of macrophages from tissues have been developed. *Brucella suis* was selected as the nearest to ideal facultative intracellular parasite for these studies; thus, the *in vitro* culture of macrophages derived from different sources in the same animal, followed by challenge *in vitro* with *B. suis* formed the system used in the following study.

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Anatomic Distribution and Structure of Mouse Macrophages

There is no generally accepted definition of a macrophage. Nelson(51) suggested a loose definition, that macrophages are large mononuclear cells with a special and highly developed capacity for endocytosis and especially phagocytosis, whether this capacity has already been demonstrated or is still only potential. Macrophages also possess large numbers of lysosomes containing acid hydrolases, notably acid phosphatase, with the presence of microvilli (visualized electron microscopically) and ruffles or hyaloplasmic veils on at least part of the cell membrane. Macrophages are found in all body organs and fluids with a wide range of diversity in structure and morphology. A few of the more prominent ones are discussed as follows:

Peritoneal Macrophages

Peritoneal macrophages are easily obtained from experimental animals and hence have been studied more extensively than other macrophages. When examined by light microscopy, peritoneal macrophages measure 10μ to 30μ in diameter with an oval or kidney shaped nucleus of 6μ to 12μ in diameter. The abundant cytoplasm is slightly basophilic and may contain many vacuoles and granules. The cells are ameboid(41, 42). By phase contrast microscopy peritoneal macrophages are seen to contain abundant light gray diffuse cytoplasm with dark gray rod-shaped mitochondria usually grouped near the centrosphere(62). The pronounced tendency of macrophages to adhere to and spread on glass surfaces creates hyaloplasmic flow which gives rise to expansions of the characteristic ruffles or hyaloplasmic veils on the cell membrane(62). Examination with the electron microscope(52) shows three layers in the membrane which is approximately 80Å thick, with

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protuberances and invaginations. Peripheral cytoplasm in the numerous cellular processes is finely granular and usually lacks the structure seen in the rest of the cytoplasm. Three main types of cytoplasmic vesicles are found: small pinocytic vesicles; organelles with fine granular material and; large dense vacuoles. North and Mackaness(52) compared the ultrastructure of peritoneal macrophages from mice immunized with Listeria monocytogenes with macrophages from normal mice. The cytoplasmic membrane of immune macrophages is smoother, and has fewer protuberances and invaginations than the membrane of nonimmune macrophages. The mitochondria are smaller but more numerous. Compared with nonimmune peritoneal macrophages the cytoplasm of immune macrophages contains fewer vesicles and a very extensive Golgi apparatus.

Spleen Macrophages

Palade(58) describes the ultrastructure of splenic macrophages as being similar to that of peritoneal macrophages. In addition Swartzendruber(72, 73) reported on EM observations of mouse spleen tingible-body macrophages which had phagocytized cellular debris, possibly derived from small lymphocytes or erythrocytes which had given them their tingible property. He then suggested that these structural specificities were associated with the specific activities of splenic macrophages, i.e. removal of effete cells.

Liver Macrophages (van Kupffer Cells)

Various methods have been described for separating Kupffer cells from liver parenchymal cells(4, 5, 47, 61). Beard and Rous(4) in 1934, allowed the macrophages to ingest particles of iron prior to perfusing the liver. The macrophages were subsequently removed from suspension with a magnet. They cultivated the isolated Kupffer cells in vitro and characterized them. They recognized the morphological similarities between liver macrophages and

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spleen or peritoneal macrophages. Later deMan(40) studied the morphology of Kupffer cells with the electron microscope and he reported that these irregularly shaped cells project into the lumen of the sinuses that they line and have fine cytoplasmic extensions inserted between adjacent parenchymal cells. The surface area of the cells is large because of the many projections and invaginations of these cells. The intensive phagocytic activities of kupffer cells are reflected in the EM observations made by Rouiller(66). He found that the cytoplasm contains many vesicles, lysosomes, phagosomes, and erythrocytes in various stages of digestion.

Blood Monocytes

Bloom in 1938(6) described the morphology of peripheral blood cells. His classic description is still generally accepted. He reported that blood monocytes are approximately 10 to 11 u in diameter. The rounded nucleus is usually indented, there is a more abundant cytoplasm than in lymphocytes, and the cytoplasm is slightly basophlic and is often finely reticulated. Supravital staining of living macrophages with Janus green reveals the presence of a large number of short rod-like mitochondria. Low and Freeman(33) examined the monocytes with the electron microscope and found that they contain numerous small circular or rod-shaped mitochondria, many small reticular structures, and some ribosomes in an abundant cytoplasm. Monocytes of the mouse also have a characteristic dark, irregular band of chromatin adjacent to the inner margin of the nuclear membrane. All these characteristics have been used to distinguish between lymphocytes and monocytes.

Alveolar Macrophages

Leake and Heise, in their extensive review(31), have summarized evidence obtained from electron and light microscopy which supports the concept that

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alveolar and peritoneal macrophages represent two morphologically distinct cell types. They submitted six major morphological differences between alveolar and peritoneal macrophages of germ-free rats: 1) The nuclei of alveolar macrophages are round or slightly oval and the peritoneal macrophages have elongated, deeply idented nuclei; 2) Rough ER is seen frequently in peritoneal macrophages and rarely seen in alveolar macrophages; 3) Peritoneal macrophages have a more extensive Golgi apparatus than do alveolar macrophages; 4) Alveolar macrophages have many dense granules which are not observed in peritoneal macrophages; 5) The mitochondria in peritoneal macrophages are larger and more elongated than mitochondria of alveolar macrophages; 6) The size of alveolar macrophages is approximately 1.5 times that of the peritoneal macrophages. Karrer(26, 27) confirmed this report.

Other Organ and Tissue Macrophages

Macrophages present in lymph nodes have tingible body characteristics but are not actively phagocytic; Ada(1) suggested that they may represent inactive macrophages. Kotowiecki(30) concluded in his recent review that the macrophages in the thymus resemble those of lymph nodes in structure. Macroglia, neuroglia, and microglia cells have long been considered to be macrophages because in some specific conditions they function as free macrophages [i.e. trauma(74)]. In addition macrophages are present in virtually every other organ and tissue. They are ultrastructurally similar, except for alveolar macrophages, although the environment and state of activity of the cell may be reflected by alterations in morphology(31).

Giant Cells and Epithelioid Cells

In certain diseases, including brucellosis, tuberculosis, sarcoidosis, and silicosis, giant cells are found in lymph nodes as well as in granulomatous lesions(65). At least two types of giant cells are found in granulomas. Langhan's giant cells have multiple nuclei located at their periphery, while the foreign-body giant cells contain numerous centrally located nuclei; both lack lysosomes and intact phagocytic vacuoles(71).

Epithelioid cells have a similar structure, and they contain lysosomes but not intact phagosomes. Both epithelioid and giant cells apparently are terminal forms of mocrophages since they are inert but give morphological evidence of having been extremely active(71).

Cellular Kinetics of Cellular Immunity

Because of the wide range of morphological diversity, the origin of peripheral blood monocytes and the tissue macrophages of the reticuloendothelial system have been the subject of conjecture for many years. Recently, improved research techniques, such as radioactive labeling and tracing of cell division and migration, have given some insight into these problems.

The myelogenous origin of the mononuclear phagocytes was first demonstrated in radiation chimeras given allogenic bone marrow cells(3), later by the transfusion of H^3-thymidine labeled bone marrow cells into syngenic animals(77), and by labeling studies of X-irradiated animals with partial shielding of the bone marrow(20). Still other evidence has been found which supports the concept that bone marrow cells serve as principle progenitors of alveolar macrophages(60) and liver Kupffer cells(7), as well as the macrophages found in the thymus and lymph nodes(76).

It has been conclusively shown that blood monocytes are immature circulating macrophages. Evidence in support of this conclusion comes from their short half life in blood and evidence showing their constant migration through capillaries into tissues(20). It is thus suggested by Van Furth(75) in his recent extensive review, that the mononuclear phagocytes originating in the bone marrow are transplanted by the peripheral blood and eventually become tissue macrophages.

Labeling experiments(68) have shown that the macrophages in a sterile imflammatory exudate are recruited from peripheral blood monocytes because of the rapid rate of accumulation of labeled macrophages at an inflammatory site. When different types of stimuli are used, the picture becomes slightly modified. North(53) infected mice with either BCG or L. monocytogenes. He found that 10-14% of the peritoneal macrophages synthesized DNA, and he attributed the acquired ability to divide as due to acquisition of a state of delayed hypersensitivity. Similar experiments had been conducted by Spector et. al.(69), who showed that upon injection of complete or incomplete Freunds' adjuvant into the skin of mice, a chronic inflammatory lesion developed. This coincided with a continuous proliferation of macrophages. The chronicity of the inflammatory lesion appeared to be due to this proliferation, and the influx of monocytes from blood accounted for only one percent or less of the macrophages present. North(54) studied the cell kinetics of liver during the process of development of cellular immunity. He found that in mice infected with L. monocytogenes, the labeling index of the Kupffer cells 30 minutes after a H^3-thymidine pulse rose to as much as 18% above normal. The peak of macrophage proliferation always preceeded the acquisition by the host of an effective antimicrobial immunity and the

emergence of delayed-hypersensitivity. He emphasized that the time of macrophage proliferation in the liver coincides with that in the peritoneal cavity during L. monocytogenes and BCG infection. He then suggested that the simultaneous, localized, mitosis of macrophages in the liver and peritoneal cavity of Listeria infected mice represents a special feature of the host's immune response to infection with facultative intracellular organisms. Mackaness has further investigated this problem(34). After exposing a group of mice to a sublethal dose of whole body X-irradiation, these mice and a group of normal mice received immune cells from Listeria-infected donors. All the mice were challenged with Listeria intravenously. After 24 hours, each mouse was pulse-labeled with tritiated thymidine. The mice were killed 30 minutes, 24 hours and 48 hours after pulsing, and the amount of labeled DNA in the liver was measured. The corresponding viable Listeria in the same tissue was counted. He found that in irradiated mice the viable Listeria count increased three logs with no increase of labeling in 48 hours. In the unirradiated mice, the viable Listeria decreased, but the amount of radioactivity continued to rise over a period of 48 hours. Since only one pulse of thymidine was given, the increase in radioactivity between 24 and 48 hours in the unirradiated animals must have indicated immigration of labeled cells from an extrahepatic source. Mackaness reasoned that division of tissue macrophages is limited and cannot match the numerical potential of the migratory phagocytes.

In 1964 Khoo and Mackaness(28) studied the proliferative activity of peritoneal macrophages in mice systemically infected with either L. monocytogenes or Brucella abortus. They found a gross difference in the time-course of the infections caused by these organisms. In each infection the

DNA synthesizing cells rose progressively to a peak on the third day after the bacterial population in the spleen had reached a maximum. The DNA synthesis fell abruptly in both primary and secondary Listeria infections, in which the bacterial population was quickly eliminated. However, DNA synthesis persisted at a high level in Brucella-infected mice, in which bacterial inactivation occured much more slowly. This tends to support the idea that the release of antigen and cell products, particularly from inactivated bacteria is one of the factors responsible for proliferative activity of macrophages.

The Involvement of Lymphocytes in Cellular Immunity

Frenkel(17) transferred cells from the spleen and lymph nodes of hamsters infected with intracellular parasites Besnoitia jellisoni, or Toxoplasma gondii, to isogenic normal hamsters. Cellular immunity was demonstrated in the normal recipients by challenge in vivo with the homologous parasite; transferring serum showed only a slight protective effect. Therefore, antibody apparently did not account for the transfer of immunity. Mackaness(35) similarly investigated the transfer of adaptive immunity. Spleen cells from mice previously infected with L. monocytogenes or B. abortus were used. He found the recipient had been conferred with acquired resistance to challenge and also a proportionate level of delayed hypersensitivity. He then challenged animals which had previously received BCG immune spleen cells with Listeria. He found no cross resistance (same as non-specific resistance, see cellular immunity section) developed unless the recipients were also injected with an eliciting dose of BCG. He suggested the activation of host macrophages was mediated through a product resulting from specific interaction between sensitized lymph cells and the infecting organism. McGregor et. al.(46) studied the cell kinetics of lymph cells involved in cross transfer experiments. They injected tritiated thymidine into Listeria-infected and adaptively

immunized rats (recipient of immune cells). Spleen and thoracic duct cells were periodically collected for radioautographic study. Their results suggested that immunologically committed cells appearing at the sites of bacterial implantation were generated in stimulated regional lymph nodes. These cells entered the blood via the efferent lymphatics and main lymphatic ducts. Unlike long-lived small lymphocytes these cells did not reenter the lymph. The tendency was toward localization in the inflammatory area. In their recent studies(29), they confirmed this speculation. They demonstrated that these "no return" lymphocytes which were responsible for mediating cellular immunity were members of a line of rapidly proliferating lymphocytes.

Cellular Immunity

The importance of the host defense mechanism at the cellular level has been recognized only in recent years(36). Most chronic diseases caused by facultative intracellular parasites, e.g. listeriosis, brucellosis, tuberculosis, leishmaniasis and histoplasmosis, share some common characteristics during pathogenesis. Namely: 1) there is a consistent association of the chronic disease with delayed hypersensitivity; 2) this form of acquired resistance cannot be passively transferred with serum; 3) changes in the antibacterial activity of the host macrophages occur; 4) non-specific resistance toward diverse challenge organisms develops; 5) the acquired resistance progressively decreases during the convalescence from primary infection and under certain circumstances resistance can be reestablished at an accelerated rate with a small inoculum of live or killed organisms; 6) this recall of resistance is immunologically specific for homologous antigen and; 7) antibody is often found with high titer during infection but its contribution in eliminating the disease agent is often low or no effect(19, 37, 43, 67, 82). The implications of these phenomona are intimately related with the concept of local immunity.

In tuberculosis, Dannenberg(12) examined the dermal lesions (granuloma) and the development of cellular immunity and hypersensitivity in the BCG immunized rabbit. He found that macrophages developed into mature epithelioid cells under the stimulus of tubercle bacilli and their products. This development was accompanied by a decrease in the number of bacilli present in the lesion. The activated macrophages, as determined by the criteria of marked increase in lysosomal enzyme activities, were found only in localized lesions (granulomatous tissue). He suggested that cellular immunity is a local rather than systemic phenomenon. Later Dannenberg(13) argued that the products of local lesions possibly released periodically into the general circulation cause the activation of macrophages systemically. This would render the host sensitive to tuberculin (generalized cellular hypersensitivity). Mackaness(38) concluded that the persistent nature of tuberculosis immunity was contributed by the constant stimulation of living organisms in localized areas.

The concept of local immunity was further expanded by cross resistance experiments and supported by the time-course studies of cellular immunity. Pullinger(64) in 1936, showed that guinea pigs simultaneously inoculated with mixed tubercle bacilli and B. abortus failed to develop a Brucella infection. Nyka(55) later confirmed this finding by reversing the testing organisms. He found that the doubly infected animal, when compared with M. tuberculosis-infected mice, had milder clinical symptoms, less severe lesions in the lungs, and much longer survival time. Collins et. al.(11) infected mice with different infecting doses of Salmonella montevideo and subsequently challenged then with virulent S. enteritidis. The results showed that resistance to challenge infection varied inversely with the

size of the residual population of S. montevideo in the tissue from the primary infection. S. montevideo and S. enteritidis are antigenically unrelated. Based upon the above observations, the resistance thus developed by the animal during infection is not specifically directed against the infecting organism. This type of resistance apparently depends upon an altered state of the host macrophages.

Concerning the time-course of cellular immunity, Mackaness(38) showed that Listeria infected animals within 12 weeks of convalescence can regain full resistance if reinforced with homologous bacteria. However, the time needed to establish this recall-resistance progressively increases as convalescence occurs. By the 12th week the animals had reverted to normal and lost the resistance recall mechanism. This localized persistance of bacteria is essential for the establishment of cellular immunity. It has long been observed, but neglected. In 1958, McCune et. al.(44) reported that in isoniazid-treated BCG-infected mice, once the "sterile-state" is established, the tuberculin test is no longer positive.

Brucellosis

Brucella are known to be facultative intracellular parasites(8, 12). Braude(8, 9) studied the histopathologic changes during Brucella infection. He showed that within 24 hours after infection of guinea pigs with B. abortus, nearly all Brucella detectable in the peripheral blood were within neutrophils. These phagocytes were subsequently removed from the circulatory system by the spleen, liver and other organs. Focal aggregation of polymorphonuclear leukocytes and mononuclear phagocytes in these organs led to development of granulomas. Phagocytized Brucella were not destroyed but rather multiplied intracellularly until onset of the mature granuloma. This was marked by the appearance of epitheloid cells and the gradual disappearance

of bacteria from the tissue. Holland and Pickett(23) studied the role of cellular immunity in Brucella infection by using an in vitro testing system of peritoneal cavity exudate cell culture. Their findings are summarized as follows: 1) B. suis, B. abortus, and B. melitensis multiplied extensively within normal mouse, rat and guinea pig peritoneal cell cultures; 2) cells cultured from Brucella-infected animals greatly restricted the intracellular growth of smooth and non-smooth Brucella; 3) antiserum to Brucella did not have any influence on the pattern of smooth Brucella behavior in either normal or immune cell cultures and; 4) cellular resistance did not develop when animals were vaccinated with heat-killed Brucella, though these animals did produce agglutinating antibodies. The similarity of brucellosis with other chronic diseases caused by facultative intracellular organisms thus has been recognized.

It has been repeatedly observed that the individual who recovers from a previous infection of brucellosis obtains a state of hypersensitivity to Brucella antigen(70). Following injection intradermally of Brucella antigen, a local necrotic reaction develops as well as a systemic reaction as characterized by the appearance of granulomatous lesions(15). In experimental brucellosis(39) it was found that delayed-type hypersensitivity appears by the fourth day of infection as detected by skin reaction. More recently, Darlington and Scherago(14) using in vitro migration inhibition demonstrated that B. abortus infected animals developed delayed-type hypersensitivity at the fourth week with an onset of orchitis at the same time.

Mackaness(39) studied the cellular events occuring during Brucella infection in mice. He found that delayed-type hypersensitivity was detected at the fourth day of infection and that the bacterial population continued to rise for eight days. Thereafter, resistant macrophages appeared in the

peritoneal cavity and the number of localized bacteria correspondingly decreased. When the bacterial population had been reduced to small proportions, host resistance also decreased, but the level of hypersensitivity continued to rise. The explanation of this observation is still a subject of conjecture.

A proposed model of pathogenesis of brucellosis by McCullough(43) deserves more than just mention. After Brucella pass through the primary barrier, they are collected by the lymphatics and transported to the regional lymph nodes. Bacterial multiplication occurs intracellularly in fixed as well as wandering mononuclear phagocytes. If bacteria are released due to the death of phagocytes, the released bacterial antigens and cell products stimulate an intense local mononuclear cell proliferation and activiation. Meanwhile, the released antigens also activate the antibody-forming mechanism. Because of the dissemination of antigen, a generalized hypersensitivity state also develops. If the infection has been restricted to the regional area, granulomatous tissue will develop due to local inflammatory responses. If the infection progresses, release of bacteria from necrotic cells occurs, and extracellular growth of Brucella becomes a prominent feature. Now circulating antibody may play an important role in eliminating the spread of bacteria. If humoral and cellular immune mechanisms effectively bring the infection under control, the disease is progressively eliminated and eventually becomes terminated. In some instances infection is not completely eliminated. The possibility of developing chronic or relapsing disease depends upon the result of a constant combat between intracellular multiplication and the effectiveness of humoral and cellular immunity.

MATERIALS AND METHODS

Mice

Albino CF-1 female adult mice (Carworth Farms, Portage, Michigan), weighing from 25 to 30 grams were used in all experiments.

Strain of Brucella

Since mice are generally resistant to infection with Brucella, a highly virulent strain of B. suis, strain 1776, with ID_{50} for the guinea pig of two bacteria (Dr. McCullough unpublished data) was used. Stock cultures were grown on Trypticase Soy agar (Baltimore Biological Laboratories) slants, and 48 hour cultures were stored at -78 C. Cultures for regular use were maintained on Trypticase Soy agar slants and stored at 5 C. The culture was periodically checked for maintenance of smooth characteristics by the oblique lighting method of Henry(22). Forty-eight hour agar slant cultures were harvested by washing the slants with 0.1% peptone-saline solution, and then diluting to the desired concentration as determined by optical density reading at 650 nm on a Spectronic 20 (Bausch and Lomb).

Immunization

In order to find the proper bacterial dose for immunization, comparative studies were performed with different bacterial doses, from 10^3 to 10^7 organisms. Body weight loss, spleen, liver and lung enlargement and the killing ability of peritoneal cavity macrophages were compared. It was found that 10^6 organisms gave good stimulation of immunity as determined by the above criteria. Intravenous injection of 10^6 organisms was the route and dose of choice. Twenty-one days after immunization, the animals were

killed and macrophages from various sources were harvested.

Macrophage Collections

In order to eliminate contamination of macrophages by red blood cells, animals were bled to death by rupture of the post-orbital sinus before proceeding with collection.

Peritoneal Cavity - This technique was modified from the method described by Padawar(57). After disinfecting the skin of the whole animal with Roccal and alcohol, the skin was reflected away from a midline ventral incision extending from the mandible to the lower abdomen. A 10 ml syringe, with 22g needle was used and 6 ml tissue culture medium (see Standard Cell Culture Conditions section) was injected through the peritoneal wall. For aspiration to avoid injury and hemorrhage, the needle was cautiously reinserted into the left side of the peritoneum between the spleen and the abdominal wall. Four to five ml of cell suspension, containing 2×10^6 to 5×10^6 macrophages were usually obtained.

Spleen - The technique of Bennet(5) was used with minor modifications. Immediately after the collection of peritoneal cavity fluid as described above, the spleen was removed and placed in a sterile Petri-dish. Using a 1 ml tuberculin syringe with a 27g needle, 1 ml of GNK glucose saline solution (see Chemical Reagents section) was injected forcibly into the spleen. The spleen was removed aseptically and transferred to a sterile, siliconized Petri-dish containing 10 ml of cold tissue culture medium. The spleen was then cut in half. A rough stainless steel screen (60 mesh) was placed on top of the halves and used to squash the spleen. The shell of the spleen capsule was discarded. The fragments of splenic pulp were vigorously withdrawn and expelled with a pipette several times. The remaining clumps of gross debris were removed by filtration through cheese cloth. The cells

were washed twice with tissue culture medium by centrifuging at 1,000 rpm for five minutes (International Clinical Centrifuge); the cells were resuspended in tissue culture medium containing 25 units penicillin-G per ml. The number of cells obtained by this method differed between normal and immune mice. Generally, a normal mouse spleen yielded 6 X 10^6 to 9 X 10^6 macrophages, and an immune mouse spleen yielded 15 X 10^6 to 20 X 10^6 macrophages.

Lung - Two methods were used for routine cell harvest. Method A - A technique described by Myrvik(49) was adapted with some modifications. After removal of the spleen and liver, the upper trachea was grasped with a fine pair of artery forceps, the midline incision extended upward around the trachea, and the entire trachea, lungs and heart then dissected out en bloc, taking care to avoid puncturing the lungs. The excised thoracic organs were washed free from blood in cold HBSS (Hanks Balanced Salt Solution). Using a 2 ml syringe fitted with a dull point 20g needle, 1 ml of GNK was injected into the trachea and aspirated three times, then withdrawn to a collecting tube. This was repeated ten to twelve times. The cells were washed twice by centrifugation at 1,000 rpm for eight minutes. Cell yield by this method ranged from 1.5 X 10^5 to 2 X 10^5 macrophages per mouse. Method B - This method was described by Appel(2). The lungs were removed in sections from the opened pleural cavity. After washing off the contaminating RBC's in HBSS, the lungs were cut into small pieces of 0.5 mm^3 in size. Using a wide mouth Pasteur pipet, the pieces were vigorously withdrawn and expelled for five to seven minutes. The fluid was filtered through cheese cloth, and the filtrate washed twice with cold HBSS by centrifugation. The cell yield in general ranged from 2 X 10^5 to 3 X 10^5 by this method.

Liver - The method used was described by Garvery(18) and modified by Howard (7, 24). After the spleed was removed, the inferior vena cava was severed and 2 ml of GNK was perfused through the portal vein. The liver was then excised and cut into small pieces (1-2mm^3). These liver fragments were transferred and mixed with 2.5 ml of 0.02% collagenase (Worthington Biochemical Corporation, Freehold, N.J., enzymatic activity 194 u/mg). The mixture was then incubated at 10 C with constant but slow stirring for two and one-half hours. The fragments were then washed by centrifugation as above, 2.5 ml of EDTA-trypsin (see Chemical Reagents section) were added, and suspension was incubated at 37 C with stirring for 20 to 35 minutes until a paste-like suspension developed. Ten ml of GNK was added, the mixture was shaken and allowed to stand at room temperature for three minutes. The upper layer was then removed and filtered through cheese cloth. Ten ml of GNK were added to the filtrate, then the suspension was slowly centrifuged (300 rpm for three minutes). The large hepatic (parenchyma) cells with higher density than the Kupffer cells settled to the bottom. The supernatant was carefully transferred and washed three times at 1,000 rpm for five minutes. This method yielded 5 X 10^6 to 10 X 10^6 Kupffer cells with reasonable purity.

Standard Cell Culture Conditions

Many biological activities of macrophages are known to be influenced by the culture environment. This experiment was designed to compare macrophages from various sources in their ability to suppress or kill Brucella in vitro. Therefore, in order to eliminate possible nonspecific stimulation of the macrophages, the macrophages were cultured in low serum concentration.

Culture medium consisted of: Medium 199 supplemented with 1% of 200 mm L-glutamine, 1% vitamin mixture, and 10% fetal bovine serum (screened

for cytopathic and non-cytopathic bovine virus and gamma-globulins), made up to final volume with amine-free double distilled water. The adjustment of final pH to 7.0 - 7.3 was made with either 1N HCl or 1N NaOH. All of the medium ingredients were supplied by Microbiological Associates, Bethesda, Md. Fresh medium was prepared at one week intervals.

One ml volumes of cell suspension were dispensed into Leighton tubes with the following cell concentrations: liver, spleen, and peritoneal cavity macrophages, 5 X 10^5 to 7 X 10^5 cells; and lung macrophages, 1 X 10^5 to 2 X 10^5 cells. Cells were maintained in 5% CO_2 and 95% air atmosphere at 37 C. Cells were incubated for 72 hours before assay, and every 24 hours the medium was removed and replaced with 1 ml fresh medium without antibiotics.

Chemical Reagents

GNK was prepared as follows: NaCl, 8 gm; KCl, 0.4 gm; Dextrose, 1.0 gm; Penicillin-G, 25,000 units; Dihydrostreptomycin, 5,000 ug; distilled water, 1,000 ml. EDTA-trypsin solution was prepared as follows: NaCl, 8 gm; KCl, 0.4 gm; Dextrose 1.0 gm; $NaHCO_3$, 0.35 gm; EDTA, 0.2 gm; Phenol Red, 5.0 mg; trypsin, 0.5 gm (Worthington Biochemical Corporation, Freehold, N.J., enzymatic activity 194 u/mg); distilled water, 1,000 ml. Staphylococcus delta hemolysin was donated by Mrs. Donna Muirhead, Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan, and was supplied by Dr. Frank Kapral, Department of Medical Microbiology, Ohio State University, Columbus, Ohio.

Assays

Macrophage Lysis by Delta Hemolysin of Staphylococcus

Delta Hemolysin produced by Staphylococcus aureus is specific for animal leukocytes and will not effect B. suis. This was considered to be the method

of choice for breaking macrophages to release intracellular bacteria. Using cell suspensions, Muirhead(48) found 50 HD_{50} units to be adequate, however, in these experiments, 100 HD_{50} units and 15 minutes incubation at ambient temperature was found to be most satisfactory, resulting in 99% of macrophages lysed.

Determination of Intracellular Growth and Survival Curves

A 48 hour slant culture of B. suis 1776 was washed and suspended in 0.1% peptone-saline solution. After two consecutive washings the bacteria were resuspended to the desired concentration (bacteria to macrophage ratio was 10 to 1) in maintenance medium. The intracellular growth curves of B. suis were obtained by the following methods. One ml of the B. suis suspension was added to each Leighton tube. The mixture was incubated for two hours at 37 C to allow phagocytosis. The supernatant fluid was then removed, and the cells were washed free of extracellular bacteria with HBSS before addition of fresh maintenance medium (control experiments showed that with two washes nearly 93% of the extracellular bacteria were washed off). Unphagocytized bacteria were removed by periodic washing (at three and six hours) to control extracellular growth(45). After three hours and six hours of incubation at 37 C, one set of Leighton tubes of each kind of macrophages was removed, the medium was withdrawn, and the cells were washed once with HBSS. The volume was restored with 1 ml of 100 HD_{50} units/ml Delta-Hemolysin and the suspension incubated at room temperature for 15 minutes. The released bacteria were then diluted in peptone-saline solution, and differential plate counts were performed by using pour plates of Trypticase Soy agar.

In other experiments bacteria were preincubated with 0.5 ml of inactivated immune serum (50 C for 30 minutes) in the cold prior to suspending the medium for cell infection(45).

In each experiment for comparison of the behavior of macrophages from different sources, the various types of cells were collected from the same animal. Cell sets were run in duplicate for lung cells, and in triplicate for all others. All graphs were constructed by plotting averages of determinations derived from at least nine mice.

Bacterial Enumeration in Spleen, Liver, Lung, Blood and Peritoneal Exudates

Mice, five in a group, were infected with 1.5×10^5 viable *B. suis* intravenously. At one day, three days, seven days, and at seven day intervals thereafter, the number of bacteria in the spleen, liver, and lung homogenates, peritoneal exudate, and blood was determined.

Blood - Using aseptic technique, 1 ml of blood was obtained by severing the branchial artery. The blood was mixed with four ml of saline-peptone-heparin (10 units/ml) solution and plated out for bacterial count.

Spleen, Liver and Lung - Mouse spleen, liver and lung were removed aseptically, homogenized, and suspended in a final volume of 2 ml saline-peptone-heparin solution. The organs were homogenized with a motorized homogenizer (Tri-R Instruments, Inc., N.Y.) at 7,000 rpm. After serial dilution, aliquots were plated on agar medium. Slide agglutination with known antiserum was used to identify typical *Brucella* colonies. Very occasionaly a few contaminating bacteria were encountered, but offered no significant problem.

Histological Examination

Mouse lung, liver, and spleen were collected and fixed in 10% formalin. The specimens were then trimmed, dehydrated and embedded using conventional techniques(63). Sections of each tissue were stained with hematoxylin and eosin and examined to assess the histopathologic changes.

RESULTS

Cultivation of Macrophages in vitro

Monolayers of macrophages were produced from cultures of cells from the peritoneal cavity, spleen, lung, and liver. Macrophages from the peritoneal cavity attached to the cover slips within an hour; macrophages from lung, spleen, and liver required 12 to 24 hours for attachment. These differences were also reflected in the morphology of the macrophages. Under examination with a phase-contrast microscope, the peritoneal macrophages possessed relatively abundant cytoplasmic processes and granules, whereas lung, spleen, and liver cells had scant cytoplasm after 24 hours of culture. After a short period of cultivation the macrophages slowly spread and became phagocytic, whereas the lymphocytes were still floating and remained suspended in the medium. The majority of lymphocytes and other contaminating cellular elements together with the agglutinated erythrocytes were readily removed with the change of medium at 24 hours.

The peritoneal population of cells in the mouse consists of approximately equal numbers of macrophages and lymphocytes. The lymphocytes in culture soon died or were phagocytized by macrophages. By 48 hours the peritoneal macrophages were fully spread and the culture consisted nearly entirely of macrophages.

The predominant extraneous cell type in the culture of lung macrophages was the erythrocyte. Erythrocytes, however, became agglutinated and were readily removed with the changes in medium at 24 hours. Lung macrophages in general required 48 to 72 hours to become fully spread. Morphologically, lung macrophages were distinct from macrophages of other sources. They had round or slightly oval nuclei, were frequently binucleated, and had few extended cytoplasmic processes.

Macrophages made up a small proportion of cells from the spleen, the majority of cells being lymphocytes. In the experimental culture most lymphocytes died within 48 hours and were either removed from the cultures with the change of medium or were phagocytized by the macrophages. At 72 hours the culture consisted entirely of macrophages.

Since Kupffer cells had been treated with enzymes and repeatedly washed and centrifuged prior to cultivation, it was expected that the Kupffer culture would take a longer period of time before resuming its' normal morphology and function. After 24 hours, considerable differentiation of the cells was observed and conspicuous morphological change and growth had occured at 72 hours. The spreading of pseudopodia and appearance of highly dense optically reflective granules were observed at this time. Garvey(18) stated that his Kupffer cell preparation contained a maximal contamination with parenchymal cells of 1%. However, in this experiment, the contamination was estimated close to 5% even with two additional washings. However, since the parenchymal cells are serum sensitive(18), they rapidly degenerated and completely disappeared after 48 hours cultivation and changing of medium.

In all the cells cultured in vitro, with the exception of the alveolar macrophages, we observed a progressive increase in cell size and cell differentiation (activation). Associated with cell growth there was the development of phase-dense granules which accumulated in the prenuclear region. These eventually occupied the majority of the cytoplasmic area if the cells were allowed to grow over three to five days. Lung cells, however, failed to develop as fully as the others. After three days of culture, the alveolar macrophages began to show a relatively small quantity of phase-dense granules which were randomly distributed within the cytoplasm. The cell morphology was also maintained and not stretched out so readily as other cell types.

Extracellular Growth of B. suis After Repeated Washing of Parasitized Macrophage Cultures

To determine the degree of extracellular growth a preliminary experiment was done. Both normal and immune macrophage cultures were challenged with B. suis (see Materials and Methods). Two hours were allowed for parasitization. The culture medium was removed and the cells washed twice with 1 ml HBSS. The three specimens were pooled and plated for bacterial count. New culture medium was added to the washed cells. The removal of medium, washing of cells, and plating for bacterial count were repeated at hours three and six. The comparative bacterial counts appear in Table I.

The results show that with an inoculum of 10^6 organisms, that after parasitization, washing of the cells and thereafter, there were less than 100 bacteria existing extracellularly in a culture tube. Since phagocytosis theoretically could have been continuous, extensive multiplication of extracellular bacteria had to be avoided. To be sure, antibiotics can serve the purpose of reducing extracellular bacterial growth. However, Patterson and Youmans(59) had shown that some antibiotics can be readily taken up by the cells and hence increase the intracellular killing ability, this also had to be avoided. Accordingly, no antibiotics were used in these experiments as it seemed possible to obtain dependable data in their absence, due to the small number of extracellular bacteria and the slow growth rate of Brucella.

In the preparation of lung, liver, and spleen macrophages (see methods), to reduce the incidence of contamination, medium containing penicillin and streptomycin was used for the collection and processing of the cells. After washing and placing the cells in Leighton tubes, antibiotic

Table I

Extracellular Population of B. suis at 0, 3 and 6 Hours

Cultivation After Initial Repeated Washing

	Normal Animal					Immune Animal			
	Cell Source				Medium	Cell Source			
Time	Perito-neum	Spleen	Lung	Liver		Perito-neum	Spleen	Lung	Liver
0 hr	8.3×10^5	8.2×10^5	10×10^5	9.3×10^5	5.6×10^6	9.7×10^5	9.6×10^5	10×10^5	10×10^5
3 hr	43	37	88	11	1.5×10^7	102	41	38	89
6 hr	72	80	44	98	2.8×10^7	71	66	27	73

a. Bacterial inoculum was 10^6 organisms/ml.

b. The figures shown were the average data of 12 mice.

free medium was used, except for spleen cells. In this case, 25 micrograms of penicillin was used for the first 24 hours, thus, in all cases, the cells, after washing, had been cultured in the absence of antibiotic for 48 or 72 hours prior to actual use in an experiment. In the case of peritoneal cells, they were never exposed to antibiotics.

Intracellular Growth of B. suis in Cultures of Normal Macrophages from Various Sources

Lung alveolar macrophages, peritoneal cavity cells, spleen cells and liver Kupffer cells were obtained from normal mice, and cultured *in vitro* for 72 hours. The culture fluid was replaced by one ml of *B. suis* suspension (in complete medium with a concentration of bacteria to cell ratio of 10 to 1). Two hours were allowed for parasitization. After washing off the extracellular bacteria, the intracellular concentration of *B. suis* was determined by applying delta-hemolysin to lyse the cells, and plating the lysate. Similar determinations were made at three and six hours. The results are shown in Figure 1.

In spite of the fact that the peritoneal macrophages showed a slight tendency of slowing down the intracellular growth of *Brucella*, among all the cell types the overall tendency of intracellular multiplication was comparable with the control. The variation among the various species of macrophages was slight.

Intracellular Growth of B. suis in Cultures of Immune Macrophages from Various Sources

Three weeks after mice had received 10^6 organisms by the i.v. route, the various cells were collected and cultured *in vitro*. After 72 hours the cultures then were challenged with *B. suis*. The number of intracellular

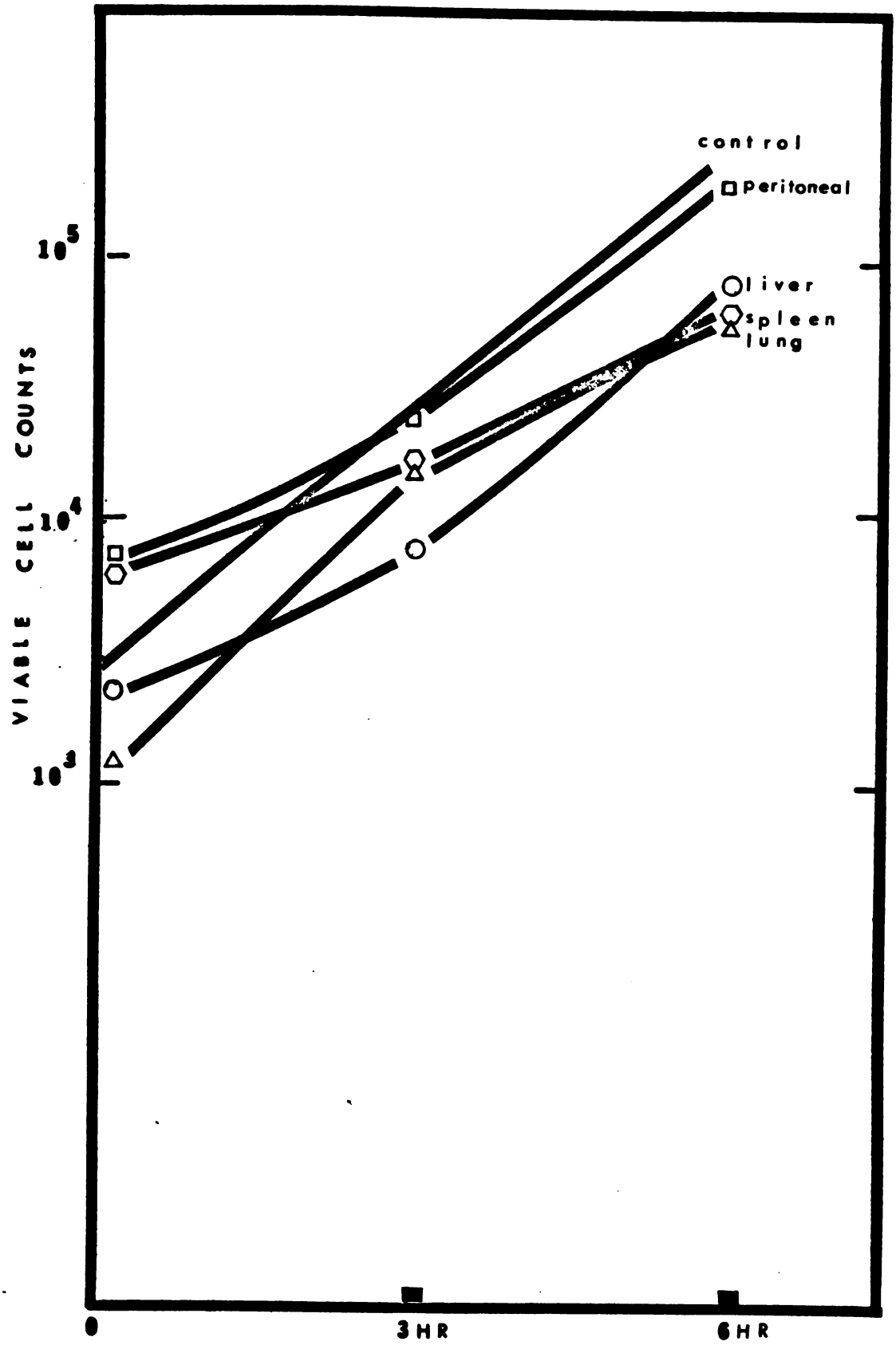

Figure 1 Intracellular Growth of B. suis in Cultures of Normal Macrophages from Various Sources.

* Control, the actual number of bacteria was higher, it is shown here for the purpose of comparison of the slopes of the curves.

bacteria were determined at zero, three, and six hours, and compared. The results are shown in Figure 2.

The results showed that there were some variations among the peritoneal, spleen, and liver cells in their ability to suppress the growth or kill intracellular B. suis. However, in all cases the overall effect was very comparable. The lung alveolar macrophages, however, showed no resistance to the multiplication of B. suis intracellularly. The intracellular growth curve was comparable to that obtained with normal cells.

Intracellular Growth of B. suis in Immune Macrophage Cultures from Various Sources with the Addition of Inactivated Immune Serum

In order to investigate the effects of humoral antibody on the cellular immune system the following experiment was done.

Heat inactivated immune mouse serum was incubated with brucellae prior to challenge of the cells, and the previous experiment was repeated. The intracellular growth curves are compared in Figure 3.

We observed that the immune cells from spleen, peritoneum and liver, with the addition of immune serum, exhibited similar patterns of killing (Figure 2) except that the drop of brucellae in liver cell cultures occured earlier. However, the lung cell culture did not demonstrate a drop of intracellular cell number, but instead maintained a steady level whereas the comparable curve in Figure 2 showed marked growth. However, due to the extreme difficulties of obtaining sufficient lung cells, the experimental results in Figure 2 and 3 were not derived from cells from the same animal and cannot be directly compared. Perhaps had the experiment have continued longer the general shape of the curves may have been similar.

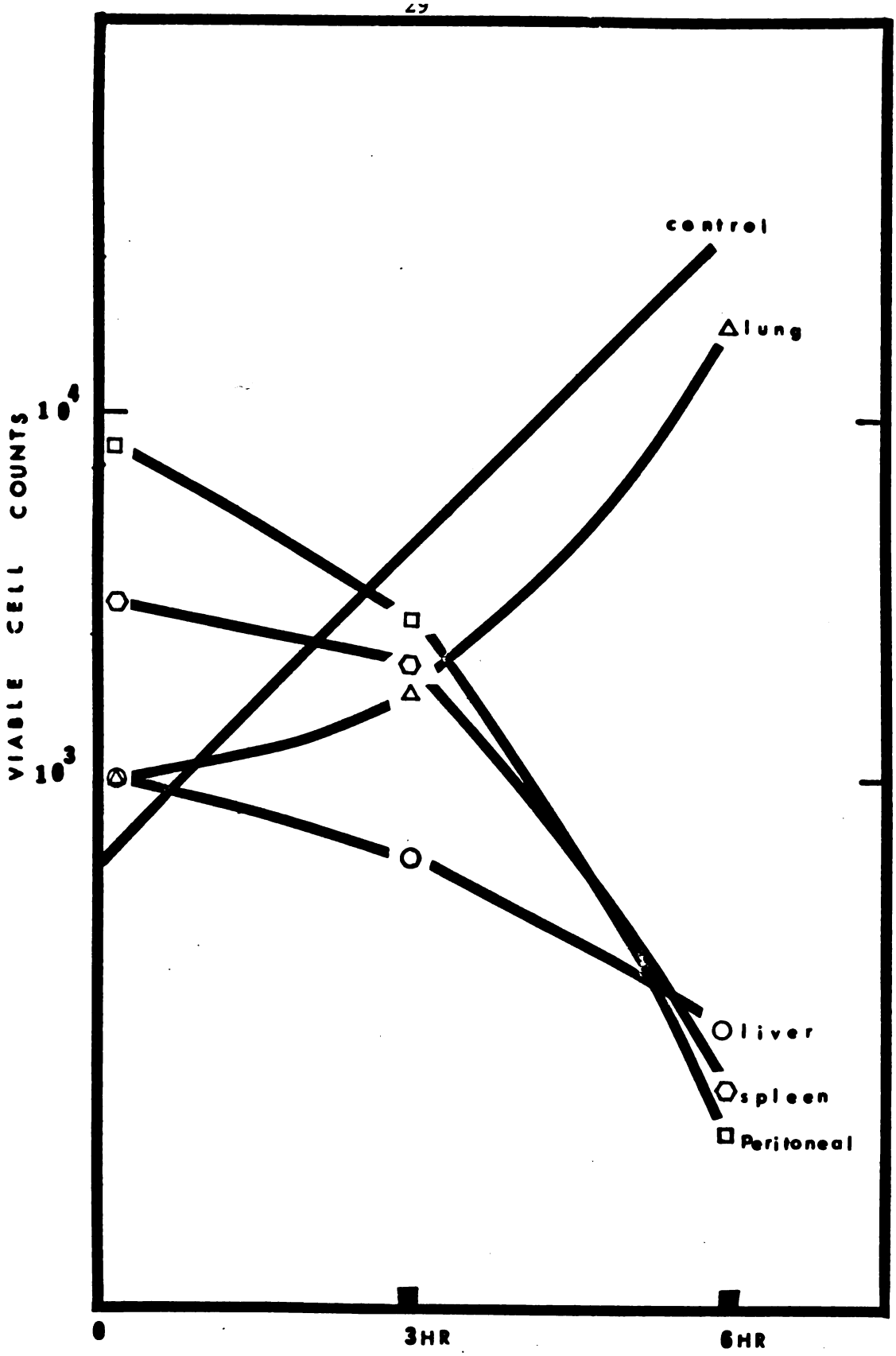

Figure 2 Intracellular Growth of B. suis in Cultures of Immune Macrophages from Various Sources.

* Control, the actual number of bacteria was higher, it is shown here for the purpose of comparison of the slopes of the curves.

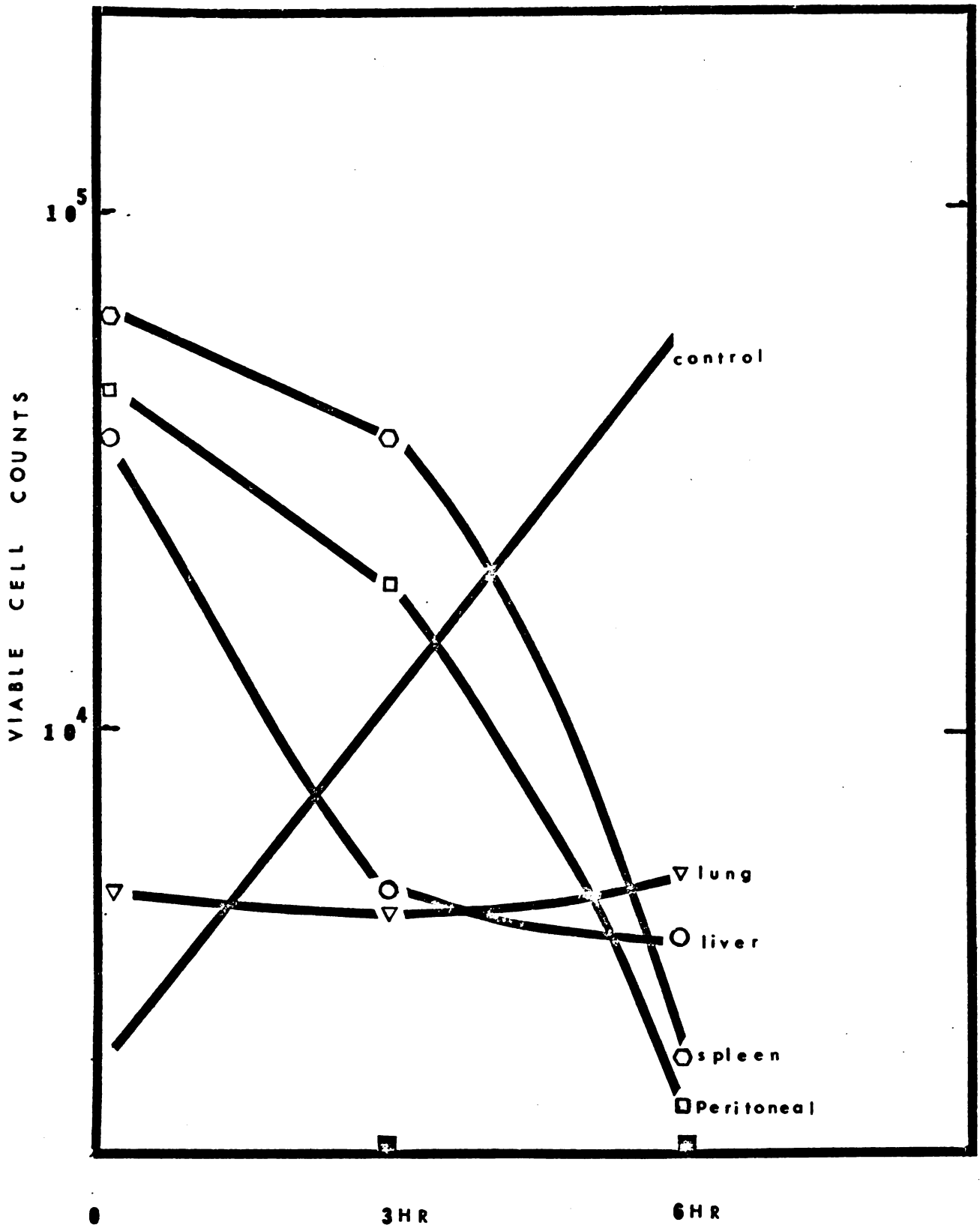

Figure 3 Intracellular Growth of B. suis in Immune Macrophage Cultures from various Sources with the Addition of Inactivated Immune Serum.

*Control, the actual number of bacteria was higher, it shown here for the purpose of comparison of the slopes of the curves.

Local Immunity as Measured by the Number of Bacteria in the Local Area

Since cellular immunity is mainly a local phenomenon, a constant stimulation by antigen(s) in a localized area is essential in maintaining the level of cellular immunity. To test this, 10^6 brucellae were inoculated into mice by the i.v. route. These animals were sacrificed and various organs and tissues were obtained at various time intervals during a nine week period. The number of viable brucellae in each was determined. The results of this experiment are shown at Figure 4 and Table 2.

The data show that the number of bacteria increased significantly in the liver and spleen initially. Subsequently, the number of bacteria declined slowly although a significant number ($10^4 \pm 10^2$) were still present in these organs at the end of nine weeks. The bacterial titer was sharply reduced in the lung and disappeared after the primary septicemia was over. The frequency of isolation and the titer fluctuated thereafter throughout the nine week period. The number of bacteria isolated from both peritoneal washings and blood were low and the incidence of isolation irregular, the possible significance of these data will be considered in the discussion.

Cellular Sensitization as Detected by Histopathologic Characteristics

It has been postulated that once an animal has been sensitized, the cellular immunity could be recalled only by the homologous antigen.(37). Since cellular hypersensitivity is also initiated by the localized antigen(s)(13), a sensitized animal when challenged with homologous antigen should react with cellular response. To test this hypothesis, the mice were infected by the i.v. route with 10^6 heat-killed B. suis antigen two months after the primary infection. The tissue sections of spleen, lung, and liver were

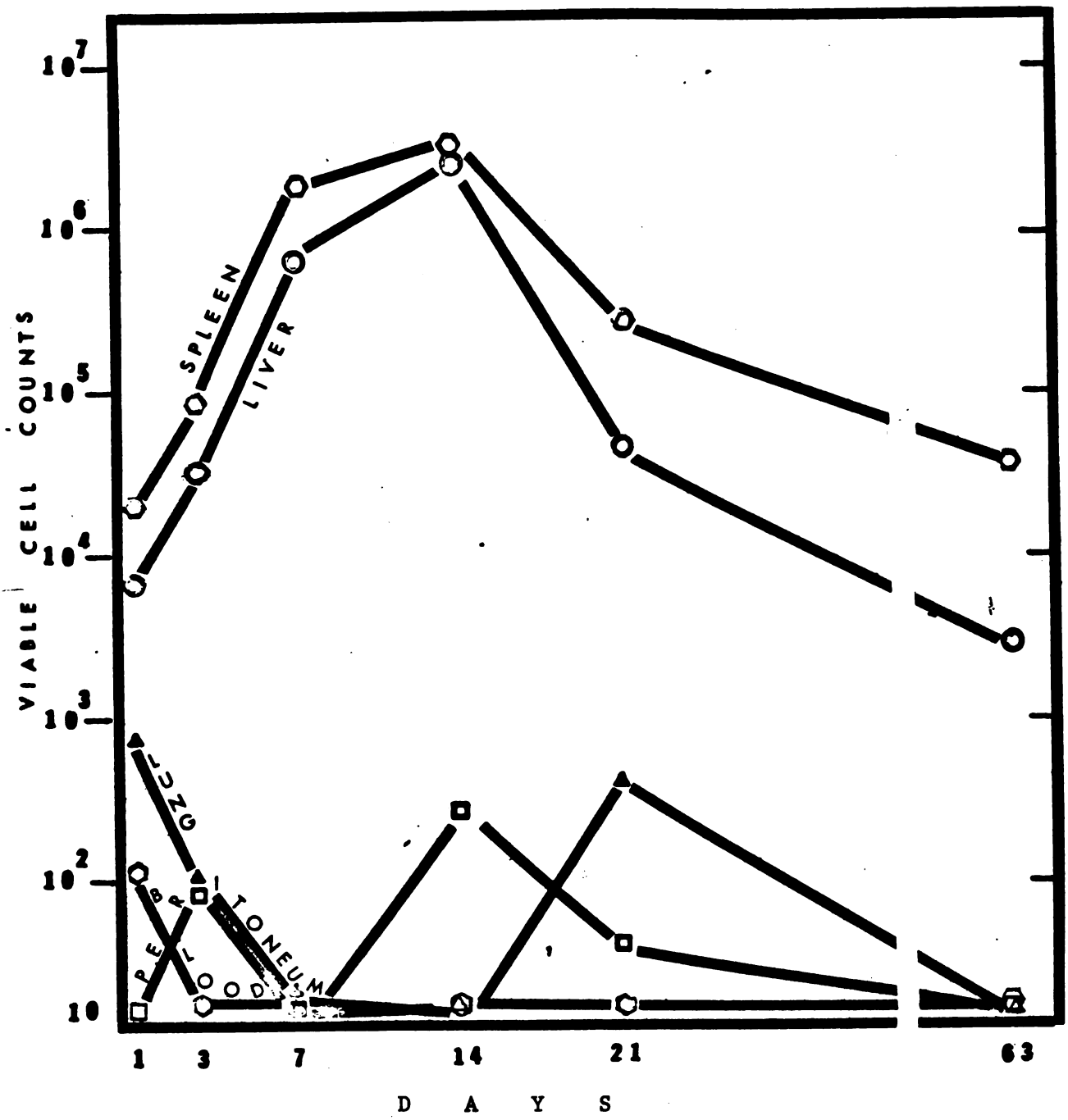

Figure 4 Viable B. suis Recovered from Various Organs in a Course of Nine Weeks Infection.

Table 2

Bacteria Recovered from Various Organs In a Course of 9 Weeks Infection

Day	Blood $\bar{Y}\pm$1S.D.[a,b]	Peritoneal fluid $\bar{Y}\pm$1S.D.[a,b]	Spleen $\bar{Y}\pm$1S.D.[a,b]	Liver $\bar{Y}\pm$1S.D.[a,b]	Lung $\bar{Y}\pm$1S.D.[a,b]
1	9.8 ± 158	<10	$2.1\times10^{4}\pm2.2\times10^{4}$	$5.3\times10^{3}\pm3.4\times10^{3}$	$5.8\times10^{2}\pm2.6\times10^{2}$
3	<10	84 ± 41	$8.3\times10^{4}\pm1.8\times10^{4}$	$4.2\times10^{4}\pm5.8\times10^{3}$	93 ± 33
7	<10	28 ± 52	$1.9\times10^{6}\pm6.7\times10^{5}$	$7.0\times10^{5}\pm5.0\times10^{5}$	0
14	<10	337 ± 567	$3.3\times10^{6}\pm3.0\times10^{6}$	$2.8\times10^{6}\pm3.7\times10^{5}$	<10
21	<10	46 ± 89	$3.5\times10^{5}\pm6.8\times10^{4}$	$5.0\times10^{4}\pm2.6\times10^{4}$	$5.1\times10^{2}\pm6.3\times10^{2}$
63	0	0	$4.7\times10^{4}\pm4.7\times10^{4}$	$3.8\times10^{3}\pm2.7\times10^{3}$	17 ± 105

a. $\bar{Y}$ is the mean of eight mice.

b. S.D. = Standard Deviation according to formula,

$$s = \sqrt{(\varepsilon Y^{2} - (\varepsilon Y)^{2}/n)/n-1}$$

obtained and processed for histological studies. A massive infiltration of granulocytes, and numerous granulomas were found in both the spleen and liver. However, pathological change in the lung was slight.

DISCUSSION

In evaluating the immunological comparability of the four types of cells used, it is necessary to consider the possible effects of the different methods used to obtain and process the cells. It is noted in this respect that after processing for 72 hours, the cells derived from the liver, spleen, and peritoneal cavity appeared comparable in terms of growth, development and state of activation. On the other hand the macrophages from the lung failed to develop as fully as the others, exhibiting fewer pseudopodia and mitochondria.

To sidestep the possibility of suppression of growth of intracellular bacteria by an antibiotic added to prevent extracellular growth, no antibiotic was used in these experiments (see Results section, page 22). It seemed unnecessary to add this variable to an already complex situation. The generation time of Brucella organisms is comparatively long. Further, the experiments were only allowed to run for six hours. Thus, relatively little increase in numbers of extracellular organisms was expected after the period of parasitization and repeated washing of the cells. This expectation was borne out by our experimental results. At all times after parasitization and washing, extracellular organisms did not exceed 100 per ml. This was true for all the cell types and for both normal and immune cells (Table I). The differences in numbers of intracellular organisms was marked in some instances, i.e. between normal and immune cells. Accordingly, it is quite clear that the small number of extracellular organisms did not significantly affect the determination of the behavior of the intracellular organisms.

It is apparent from the data presented in Figure 1, that B. suis was able to replicate in all the normal cell types without apparent inhibition.

In fact, the slope of the growth curves of intracellular growth in all cells approximated the slope of the growth curve determined in nutrient medium without cells.

As shown in Figure 2, immune cells derived from the liver, spleen and peritoneal cavity not only markly suppressed the intracellular growth but destroyed large numbers of the organisms. Cells derived from the lung of infected animals exhibited no such activity, the intracellular growth of B. suisbeing approximately the same as that in cells from a normal animal. These results show an apparent immunological difference between the behavior of lung macrophages and those derived from other sources.

Other works suggest that lung macrophages may be biologically different from other macrophages. Leake and Heise(31) have summarized evidence, obtained by electron and light microscopy, which supports the concept that alveolar and peritoneal macrophages represent two morphologically distinct cell types. Our morphological findings discussed earlier are likewise compatible with this hypothesis. Further, Myrvik et. al.(50) showed that peritoneal macrophages contain little lysozyme as compared to the alveolar macrophages of the same animal, but the destruction of bacteria was found to be greater in peritoneal cells than alveolar cells. These findings support the concept that the alveolar macrophages of the same animal may differ in their cytoplasmic composition of antibacterial substances as compared with macrophages from another source. Detailed biochemical studies have generated more information about the metabolic pattern of the alveolar macrophages and the peritoneal cavity cells. Using a radioisotope, Karnovsky(25) showed that during phagocytosis the alveolar macrophages depend to a considerable degree on oxidative phosphorylation to provide energy. The peritoneal macrophages depend only on glycolysis as the source of metabolic energy. Onchi et. al.(56) studied the

anaerobic and aerobic behavior of rabbit alveolar macrophages, and found that anaerobiosis inhibited phagocytosis. Leake et. al.(32) compared the content of lysosomal acid phosphatase and beta-glucuronidase in normal rabbit alveolar macrophage extracts to that obtained from extracts of peritoneal macrophages. Their data suggested that alveolar macrophages and oil induced peritoneal macrophages are constitutively different in their enzymatic activities.

Although such differences in physiological and functional properties of alveolar macrophages have been studied, we have found no reference to experiments dealing with differences in immunological behavior. Our results indicate that such a difference may exist.

Since specific cellular hypersensitivity is a concomitant of the cellular immune response, is there a difference in the degree of hypersensitivity among macrophages harvested from different organs of the same animal infected with B. suis? Do these differences reflect the same difference measured by the parameter of intracellular killing? In attempting to answer these questions, heat-killed Brucella antigen was inoculated into immunized mice. The histopathological characteristics of lung, spleen, and liver were studied. The results indicated that the lung tissue did not show any sign of granulomatous response and in fact little inflammatory response, while spleen and liver both showed massive granuloma formation in response to the Brucella antigen.

It has long been presumed that the granuloma may be a manifestation of delayed hypersensitivity. A review article appeared in 1967(16).

More recently, Warren(78, 79) has performed a series of experiments concerning the relationship between granuloma formation and delayed hypersensitivity. Using Schistosoma mansoni eggs or their soluble extracts, he

elicited a specific granulomatous reaction in the sensitized animal. He observed the following: 1) mice previously exposed to the schistosome eggs developed large granulomas more rapidly than control unsensitized animals(80); 2) and this reaction was specific in relation to other worm genera, and to other schistosome species(81; 3) this sensitization could be transferred only by cells, and not by serum, to other unsensitized animals(80); 4) also the granulomatous response was strongly inhibited by immuno-suppressive agents(82).

Based on the above, it is reasonable to assume that granuloma formation correlates in some degree with cellular sensitization. In this experiment, our results indicated that the lung tissue of an infected animal showed no sign of sensitization. In addition, as shown in Figure 5, the lung tissue was not expected to have as constant a stimulus as the other organs, judging from the data on bacterial isolation. Perhaps the local stimulus was not sufficient to allow a high degree of cellular immunity to develop.

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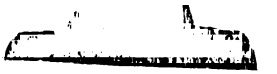

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