Histol Histopath (1987) 2: 217-222

Histology and Histopathology

An ultrastructural examination of murine alveolar macrophages following intranasal administration of *Propionibacterium acnes*

James A. Hightower[1], Marcia G. Welsh[1], R.A. Jackson[2] and J. David Gangemi[2]

Departments of [1] Anatomy and [2] Microbiology and Immunology, School of Medicine, University of South Carolina, Columbia, SC USA

Summary. Light and electron microscopic analysis of murine lungs or isolated pulmonary cells was performed three days after intranasal administration of the bacterial immunostimulant, *Propionibacterium acnes (P. acnes).* Our observations indicated that pulmonary alveolar and airway macrophages (PAMs) were the only cells with *P. acnes* bacilli in their cytoplasm. Bacilli were not observed in pulmonary interstitial macrophages, granulocytes, lymphocytes or pulmonary parenchymal cells such as type I and type II pneumocytes. Because of the morphological heterogeneity of PAMs observed in control and experimental animals, it was not possible from these studies to be certain about the relative abundance or complexity of lysosomes, endoplasmic reticulum, Golgi and other organelles in the two groups. However, we noted that it was not uncommon to observe in the same PAM, profiles of *P. acnes* and a well developed Golgi complex and endoplasmic reticulum. These *P. acnes* — associated morphological alterations occurred at a time when functional activities (e.g., phagocytosis, cytostasis) of PAMs were enhanced.

Key words: Electron microscopy — Alveolar macrophages — *Propionibacterium acnes*

Introduction

The critical role of alveolar macrophages in host defense against inhaled particles such as microorganisms and environmental toxins is well established (Hocking and Golde, 1979). Adjuvants such as *P. acnes* (Gangemi et al., 1983) and muramyl dipeptide (Fidler, 1981) have been used to stimulate the functional activities (e.g., phagocytic, microbicidal, cytotoxic) of these cells in several animal models. Moreover, prophylactic use of these immunostimulants has proved effective in enhancing nonspecific resistance to viral infection and tumor metastasis. The direct activation of peritoneal macrophages following ingestion of *P. acnes* has been well documented (Christie and Bomford, 1975). In contrast, the mechanisms (s) by which *P. acnes* activates alveolar macrophages is unclear. Previous observations in our laboratory (Gangemi et al., 1983) suggested that the route of *P.acnes* inoculation was an important factor in the ability of this immunostimulant to activate alveolar macrophages. Intranasal inoculation was highly effective whereas intraperitoneal inoculation was not. As a correlate to these functional studies, we were interested in morphologically characterizing the interaction of *P. acnes* with various cellular components of the lung. Our purpose in this study was to assess changes in normal structure of murine respiratory cells and tissues resulting from the intranasal instillation of *P. acnes.* This was accomplished using a variety of morphological techniques including light microscopy of paraffin and plastic embedded tissues, transmission electron microscopy, and histochemistry of cytocentrifuge preparations.

Materials and methods

Mice. — Male 6-8 week-old C3H/HeN mice from Charles River Breeding Laboratories, Inc. (Wilmington, MS.), and C3H/HeJ mice from Jackson Laboratories (Bar Harbor, MA) were routinely screened for Sendai virus prior to use in all of the experiments described in this study.

Treatment of mice with P. acnes. — *P. acnes* (CN 6134, 7mg/ml) was obtained from Wellcome Research Laboratories (Beckenham, England). Mice were lightly anesthetized with ether and then given i.n. inoculations of *P. acnes* (0.025 ml per nostril, 14 mg/kg). Control mice

Offprint requests to: Dr. James A. Hightower, Department of Anatomy, School of Medicine, University of South Carolina, Columbia, South Carolina 29208, USA

Grant 1N107D from the American Cancer Society awarded to JDG

were treated in a similar manner and given i.n. inoculations of sterile saline.

Macrophage tumor cytostasis assay. — The ability of alveolar macrophages to inhibit growth of methylcholantherene-induced C3H fibrosarcoma (line 1023) cells at effector-to-target cell ratios of 40:1, 20:1, and 5:1 was examined. Cells were pulsed with [^{3}H] thymidine 48 h after the addition of macrophages. Calculation of the cytostatic index was performed by using the following formula: cytostatic index = [1 − (T/N] × 100, where N represents the mean counts in cultures containing cells from untreated (control) mice, and T represents the mean counts in cultures containing similar cells from mice inoculated with *P. acnes* (Gangemi et al., 1983).

Light microscopy. — Mice were anesthetized with sodium pentabarbitol (50 mg/kg i.p.). The lungs were fixed by an intratracheal injection of 10% neutral buffered formalin. They were then removed and placed in fresh fixative for 24 hrs. Tissue was dehydrated, embedded in paraffin, sectioned at 7 μm, mounted and stained with either H&E to evaluate inflammatory responses generated by *P. acnes* or Taylor's method for demonstrating the presence of internalized gram-positive or gram-negative bacteria (Taylor, 1966).
Transmission electron microscopy. — Mice were anesthetized with sodium pentabarbitol (50 mg/kg i.p.). The lungs were fixed by an intratracheal injection of a fixative containing one part of 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) and two parts of 1% $0s0_4$ solution in the same buffer at 4°C (Collet, 1979). Small pieces of lung, as well as pellets of *P. acnes*, were immersed in this fixative for 1 hr. All tissue was stained *en bloc* in 0.25% aqueous uranyl acetate, dehydrated in a graded ethanol/propylene oxide series, and embedded in Spurr's low-viscosity embedding medium (Spurr, 1969). Thick sections (0.5 μm) were stained with toluidine blue. Thin sections (gray-silver) were stained with lead citrate and examined on a JEOL JEM-100 B electron microscope.

Cytocentrifuge preparations. -Appropriate concentrations of murine lung lavage cells (Gangemi et al., 1983) were cytocentrifuged for 10 min at 600 rpm (Shandon-Southern Cytospin, Sewickly, PA). Slides were then air-dried and stained with either Jenner-Giemsa, Taylor's method or for the cytologic demonstration of acid prosphatase (described below).

Jenner-Giemsa . — Cytocentrifuge preparations were stained for 2 min in filtered 0.3% Jenner in acetone-free methanol followed directly by 4 min in Giemsa: 10 cc of tris-maleate (pH 6.3). 30 cc of distilled water and 4.5 cc of filtered stock Giemsa (1 g Giemsa, 66 ml of glycerine and 66 ml of acetone-free absolute methanol). Blood cells were then identified and examined for the presence of bacteria which are known to stain magenta with Giemsa dyes (Preece, 1972).

Taylor's method . — Both paraffin embedded tissue sections and cytocentrifuge preparations were stained with Taylor's method (Taylor, 1966) which is a modification of the Brown and Brenn gram stain (Brown and Brenn, 1931) for the differential staining of gram-positive and gram-negative bacteria in tissue sections.

Acid phosphatase . — Cytocentrifuge preparations were incubated for 1 hr at 37°C in a solution containing AS-Bl phosphoric acid and fast garnet GBC. Naphtol AS-Bl, released by enzymatic hydrolysis, coupled immediately with fast garnet GBC forming insoluble maroon dye deposits at sites of enzyme activity (Li et al., 1970; Yam et al., 1971).

Results

Stimulation of Alveolar Macrophage Function Following Intranasal Administration of P. acnes

The route by which *P.acnes* is administered is an important factor in the ability of this bacterial adjuvant to enhance PAM function (Fig. 1). As illustrated, the intranasal route was highly effective in stimulating PAM phagocytosis and cytostasis while the intraperitoneal route was ineffective. The data given in Figure 1 were recorded from PAMs isolated three days after *P. acnes* treatment; however, kinetic studies (data not presented) indicate that elevated phagocytic and cytostatic functions persist for at least 15 days. PAM activation was never observed at any time following i.p. *P. acnes* treatment.

Light microscopy

Although lung lavages reveal that *P. acnes* causes an approximate 5-fold increase in inflammatory cells 3-4 days after i.n. inoculation (Gangemi et al., 1983), this was not detected by inspection of paraffin embedded sections thus demonstrating that the inflammatory response is a histologically subtle event. In contrast to i.n -treated macrophages which are typically situated in intraalveolar sites immediately adjacent to alveolar septa (Fig. 2A), most of the neutrophils (PMNs) and lymphocytes reside interstitially within alveolar septa. The control pulmonary alveolar macrophages (PAMs) are large ovoid-shaped cells (20-25 μm dia.) which possess eccentrically-positioned, euchromatic, oval (ovoid) nuclei with prominent nucleoli. The abundant lightly basophilic vacuolated cytoplasm contains many small, rod-shaped or ovoid granules which stain lightly with toluidine blue. In i.n.-treated mice, *P. acnes* bacilli (Fig. 2A) were observed in the cytoplasm of many PAMs. They were not, however, seen in PMNs of i.n. -treated animals or in PAMS or PMNs of control or i.p.-treated animals. A single section of a *P. acnes*- treated PAM may possess from 0 to approximately 30 of these large (1.5-2.09 μm dia.), intensely staining, ovoid bacilli (Fig. 2A). Cytocentrifuge preparations of lung lavage cells stained with Jenner-Giemsa (Fig. 2B) and Taylor's method for gram-positive and/or gram-negative bacteria

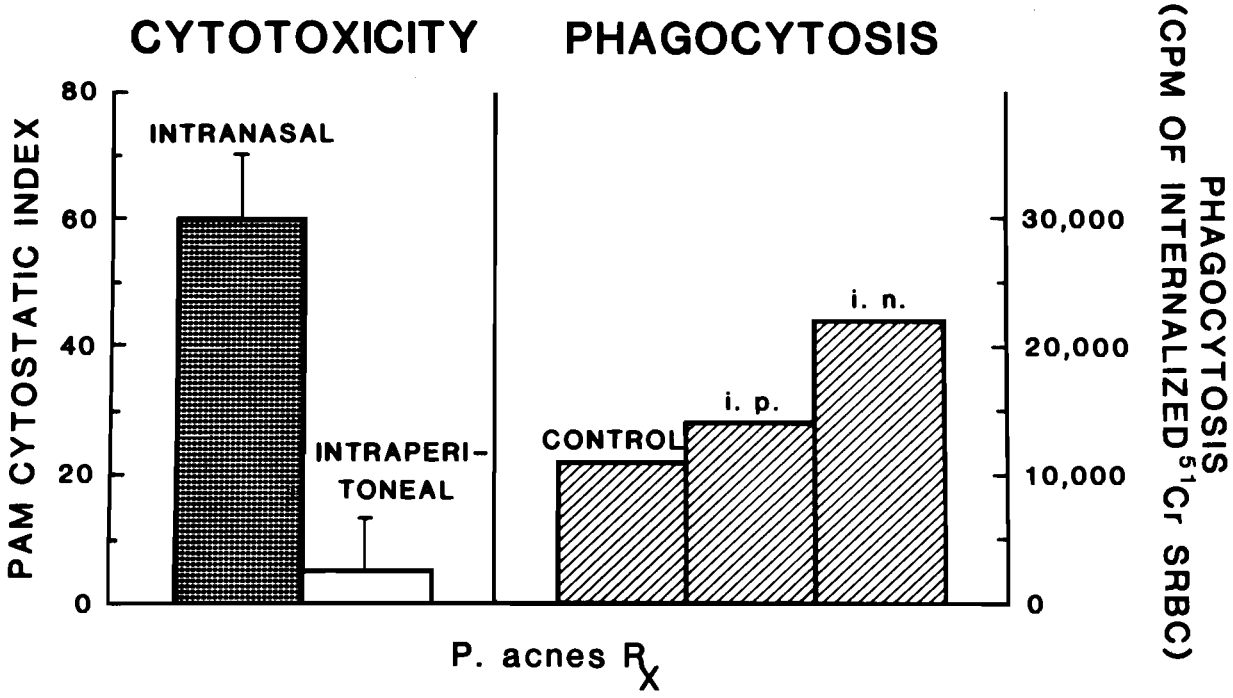

Fig. 1. Stimulation of alveolar macrophage function following intranasal and intraperitoneal administration of *P. acnes*.

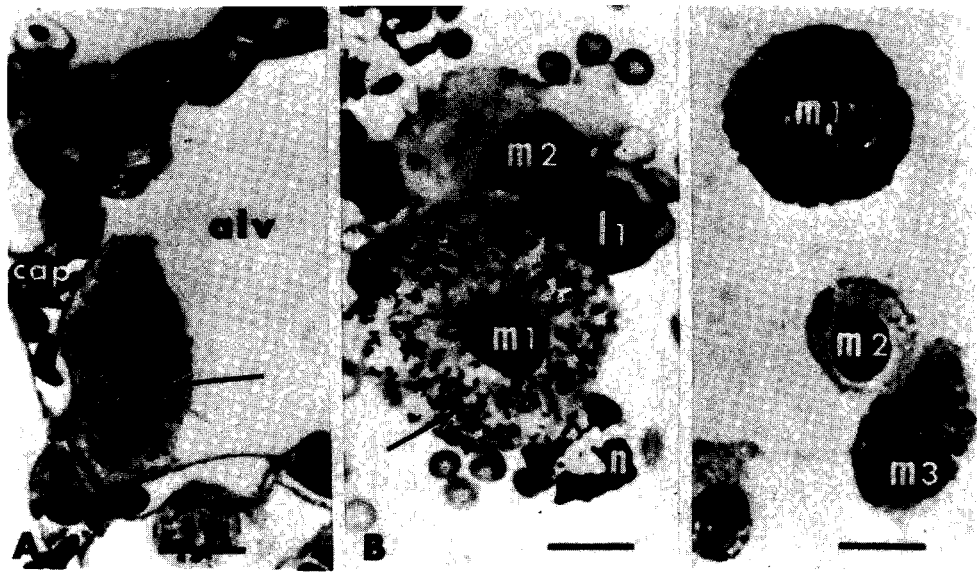

Fig. 2. Alveolar macrophages from C3H mice 3 days after i.n. inoculation with *P. acnes*. (A) Epoxy-embedded section (1 μm thick) stained with toluidine blue; putative *P. acnes* bacilli (arrow) are present in the cytoplasm of an alveolar macrophage (m); an alveolar lumen (alv) and erythrocytes within a capillary (cap) are also indicated. (B) A cytocentrifuge preparation of lung lavage cells stained with Jenner-Giemsa; an alveolar macrophage (ml) containing numerous putative *P. acnes*, bacilli (arrow) is present as well as an alveolar macrophage (m2) with no *P. acnes*, a neutrophil (n) and lymphocyte (l). (C) A cytocentrifuge preparation stained with Taylor's method for gram-positive and/or gram-negative bacteria; alveolar macrophages containing various numbers of gram-positive bacteria are indicated (many in m1, none in m2, a few in m3). Bar=10μm for all photographs.

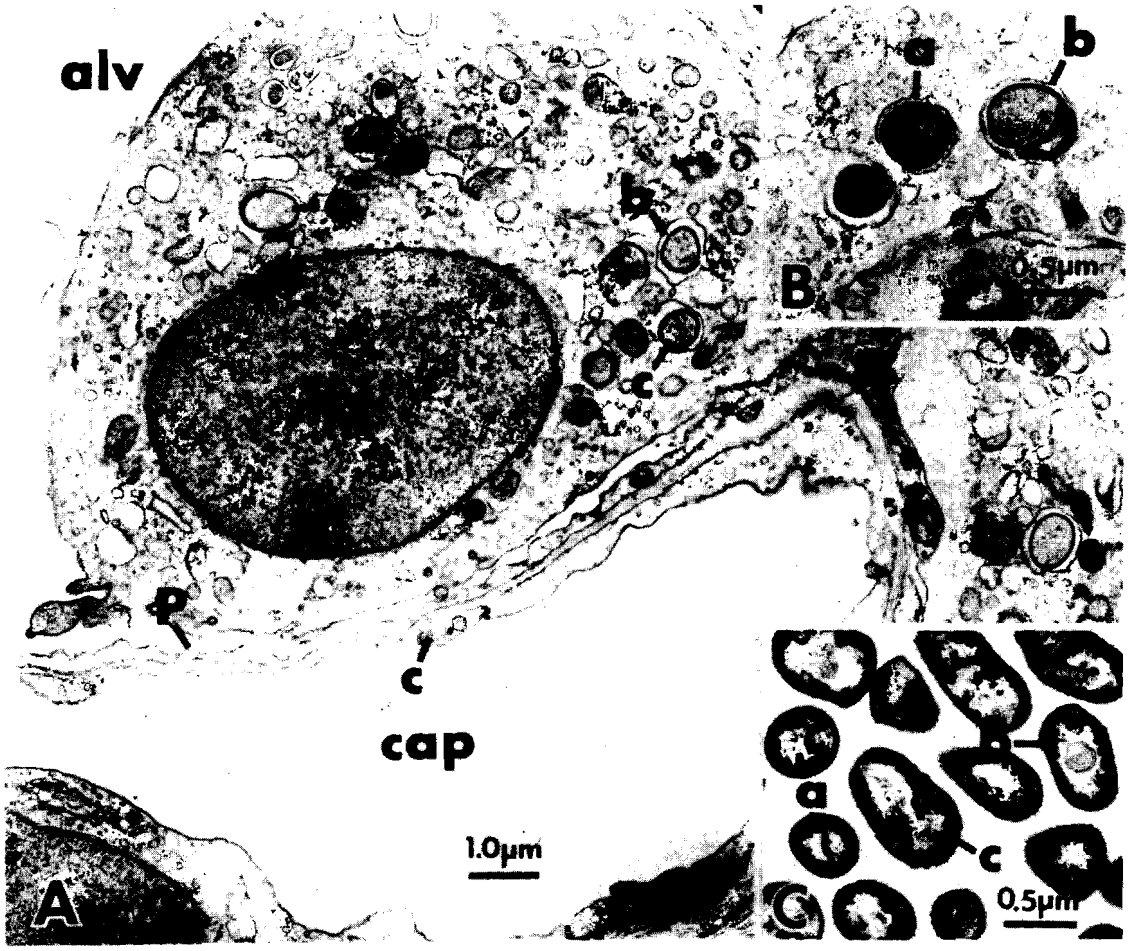

Fig. 3. (A) Electron micrograph of an alveolar macrophage (M) containing phagocytosed particles (a-c) 3 days after i.n. inoculation (14 mg/kg of *P. acnes).* Note also the alveolar lumen (alv), cytoplasm of a type I pneumocyte (P), nucleus (n) and cytoplasm (c) of a capillary endothelial cell and a capillary lumen (cap.) (B) Electron micrograph of phagocytosed particles (a,b) in the cytoplasm of an alveolar macrophage. (C) Electron micrograph of *P. acnes* organisms after pelleting. Note the profiles in cross-section (a), longitudinal section (b) and fission (c).

(Figure 2C) demonstrate the heterogeneous response of PAMs to i.n. inoculations of *P. acnes.* Cells observed in the Taylor's preparations are essentially the same morphologically as those seen in the Jenner-Giemsa slides except that the putative bacilli of the latter appear several fold smaller (compare figures 2B and 2C). Some cytocentrifuged cells demonstrate hundreds of bacilli distributed throughout the cytoplasm (Fig. 2B, magenta; Fig. 2C, blue-black) while others contain few or none (Figs. 2B and 2C). These bacilli were observed in the PAM cytoplasm for at least 10 days following i.n. inoculation. Maroon deposits were observed throughout the cytoplasm of some cytocentrifuged pulmonary macrophages which were stained for acid phosphatase. Other cells were virtually deposit-free.

Aside from the fact that many PAMs in i.n. -treated animals engulfed *P. acnes,* no other histological differences were noted among control, i.n., or i.p.-treated animals. Epithelium and underlying tissues of treated intrapulmonary bronchi, bronchioles, respiratory bronchioles, alveolar ducts, alveolar sacs and alveoli (viz., type I and type II pneumocytes) appeared normal as did the thickness of the alveolar septa.

Electron microscopy

Electron microscopy demonstrates that most alveolar macrophages lie within the alveolar lumina immediately adjacent to alveolar septa. Some with numerous pseudopodia contacting the underlying epithelial cells appear tightly adhered; others are loosely attached. Those cells from mice receiving i.n. but not i.p. inoculations of *P. acnes* were observed to contain phagocytosed particles (Figs. 3A and 3B) apparently in various stages of degradation. Some closely resemble profiles of *P. acnes* organisms after pelleting (compare «a» in Fig. 3B with «a» in Fig. 3C).

Because of the morphological heterogeneity of PAMs observed in control and experimental animals, it

is not possible from these studies to be certain about the relative abundance or complexity of lysosomes, endoplasmic reticulum, Golgi and other organelles in the various groups. We did notice in some cells of both groups 1) a peripheral organelle-free zone of ectoplasm 2) euchromatic nuclei, 3) pinocytotic and coated vesicles (acanthosomes), 4) Golgi complexes of variable complexity, 5) agranular endoplasmic reticulum, 6) numerous mitochondria, 7) phagosomes, 8) primary lysosomes, 9) secondary lysosomes, 10) «chain» vesicles (i.e., a row of vesicles located adjacent to the plasmalemma) and 11) pseudopodia and ruffling of the plasma membrane. We further noted that it was not uncommon to observe in the same PAM profiles of *P. acnes* and a well developed Golgi complex and endoplasmic reticulum.

Discussion

Light and electron microscopic analysis of murine lungs three days after intranasal inoculation with *P. acnes* revealed that pulmonary alveolar and airway macrophages (PAMs) were the only cells with *P. acnes* bacilli in their cytoplasm. Bacilli were not observed in pulmonary interstitial macrophages, granulocytes, lymphocytes or pulmonary parenchymal cells such as type I and type II pneumocytes. We have demonstrated PAM activation as assayed by enhanced phagocytosis, increased cytotoxicity, increased cytostasis and enhanced natural resistance to influenza virus (Gangemi et al., 1983) three days after intranasal instillation of *P. acnes*. These functional changes were observed during the time in which *P. acnes* bacilli were observed in PAM cytoplasm. *P. acnes* bacilli were never observed in PAMs isolated from i.p.-treated mice and no enhancement of functional activity was observed following treatment by this route of inoculation. One interpretation of these results is that recognition and subsequent endocytosis of *P. acnes* by PAMs triggers a series of biochemical events which ultimately result in PAM activation. The inability of the PAM to efficiently degrade *P. acnes* may result in the production of a persistent activational signal.

PAMs containing *P. acnes* often possess a well developed Golgi complex and many profiles of rough endoplasmic reticulum suggesting that the cells are undergoing extensive protein synthetic activity. This is further morphological evidence that many of the PAMs which we observed were activated. Although macrophages typically migrate through connective tissue proper, it is unusual for them to move over an epithelial surface, but this is what airway and alveolar macrophages do in order to maintain alveolar sterility and the numerous profiles of pseudopodia and chain vesicles observed in many suggest a significant amount of surface activity. In this regard, numerous lipid droplets are observed. They may be related predominately to the continuing replacement of segments of the surface membrane pinched off during phagocytosis (Bowden, 1972). Because PAMs are motile cells, it is rare for them to be tightly bound to their substrate, however, deep infoldings between adjacent alveolar macrophages and underlying type I pneumocytes are occasionally observed. Finally, significant intracellular digestion is evidenced in PAMs y an expanded lysosomal compartment including myeling figures, multivesicular bodies and residual bodies.

Due to the morphological heterogeneity of both the experimental and control macrophage populations, we cannot state unequivocally without doing morphometric analysis that the above-mentioned cytological features characterize the entire population of *P. acnes*- treated macrophages. Some of these features were also observed in a few of the control macrophages as were coated vesicles, «Birbeck»-like granules (significance unknown) and numerous elongated mitochondria. We expected to see many mitochondria since alveolar macrophages are known to generate ATP for phagocytosis and other cellular functions via oxidative phosphorylation. Other populations of macrophages such as synovial macrophages and peritoneal macrophages which reside in low oxygen environments depend on glycolysis (which occurs in the cytoplasmic matrix) to generate ATP for their functional activities and, therefore, tend to possess a smaller number of mitochondria. However, peritoneal macrophages subjected to treatment by *P. acnes* do share a number of cytological features with similarly treated PAMs including hypertrophy of the GERL and the presence of numerous lipid droplets (Pugh-Humphreys and Thomson, 1979).

There was no evidencce of movement of *P. acnes* through the respiratory epithelial lining into the underlying connective tissue proper. Therefore, it is not surprising that no other histological differences were noted among control, i.n., or i.p.-treated lungs. For example, in contrast to the findings of Al-Izzi and Maxie (1982) in calves, we saw neither consistent lymphoid hyperplasia around the bronchi and the bronchioles nor thickned alveolar septa in *P. acnes* -treated mice.

References

Al-Izzi S.A. and Maxie M.C. (1982). Effect of *Corynebacterium Parvum* on bone marrow macrophage colony production, peripheral blood leukocytes, and histologic changes of tissues in calves. Am. J. Vet. Res. 43, 2244-2447.

Bowden D.H. (1972). The alveolar macrophage and its role in toxicology. Crit. Rev. Tox. 2, 95-124.

Brown J.H. and Brenn L. (1931). A method for the differential staining of gram-positive and a gram-negative bacteria in tissue sections. Bull. Johns Hopkins Hosp. 48, 69-73.

Christie G.E and Bomford R. (1975). Mechanisms of macrophage activation by *Corynebacterium Parvum*. Cell Immunol. 17, 141-149.

Collet A.J (1979). Preservation of alveololar type II pneumocyte lamellar bodies for electron microscopic studies. J. Histochem. Cytochem. 27, 989-996.

Fidler I.J. (1981). The in situ induction of tumoricidal activity on alveolar macrophages by liposomes containing muramyl dipeptide is a thymus-independent process. J. Immunol, 127, 1719-1720.

Gangemi J.D., Hightower J.A., Jackson R.A., Hammer M.H. Welsh M.G. and Sigel M.M. (1983). Enhancement of natural resistance to influenza virus in lipopolysaccharide-responsive and -nonresponsive mice by *Propionibacterium acnes*. Inf. Immun. 39, 726-735.

Hocking W.G. and Golde D.W. (1979). The pulmonary alveolar macrophage. N. Engl. J. Med. 301, 580-587.

Li C.Y., Yam L.T. and Lam K.W. (1970). Acid phosphatase isoenzyme in human leukocytes in normal and pathologic conditions. J. Histochem. Cytochem. 18, 473-481.

Preece A. (1972). A Manual for Histologic Technicians. 3rd ed. Little, Brown and Company. Boston p 241.

Pugh-Humphreys R.C.P. and Thomson A.W. (1979). An ultrastructural study of peritoneal mononuclear phagocytes from *Corynebacterium Parvum* -injected mice. Br. J. Exp. Path. 60, 259-268.

Spurr A.J. (1969). A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruc. Res. 26, 31-43.

Taylor R.E. (1966). Modification of the Brown and Brenn gram stain for the differential staining of gram-positive and gram-negative bacteria in tissue sections. Amer J. Clin. Path. 46, 472-474.

Yam L.T., Li C.Y. and Lam K.W. (1971). Tartrate-resistant acid phosphatase isoenzyme in the reticulum cells of leukemic reticuloendotheliosis. N. Eng. J. Med. 284, 357-360.

Accepted February 10, 1987