

Differential Induction of Tumor Necrosis Factor Alpha in Ovine Pulmonary Alveolar Macrophages following Infection with *Corynebacterium pseudotuberculosis*, *Pasteurella haemolytica*, or Lentiviruses†

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Soluble mediators such as tumor necrosis factor alpha (TNF- α) may be important in the pathogenesis of many chronic pulmonary infections. We examined the ability of *Corynebacterium pseudotuberculosis*, *Pasteurella haemolytica*, and ovine lentiviruses (OvLV) to induce TNF- α secretion by pulmonary alveolar macrophages (PAM). Bronchoalveolar lavage cells, composed of greater than 90% PAM, were obtained from normal sheep. Bronchoalveolar lavage cells were cultured for 2, 24, 48, 72, or 168 h in endotoxin-free RPMI medium (with 10% autologous serum) or in medium containing one of the following additives: lipopolysaccharide, 1- μ m polystyrene beads, *C. pseudotuberculosis*, *P. haemolytica*, or one of two plaque-cloned OvLV, 85/28 or 85/34. Lipopolysaccharide, *C. pseudotuberculosis*, and *P. haemolytica* induced TNF- α activity in PAM cultures as early as 2 h after inoculation, as assessed by a colorimetric cytotoxicity assay. This activity could be blocked by rabbit anti-recombinant bovine TNF- α serum. In contrast, medium alone, polystyrene beads, and productive infection by OvLV did not induce TNF- α activity in PAM cultures. Bacterial pathogens which infect pulmonary macrophages may elicit the secretion of TNF- α within the lungs and lead to the cachectic state associated with chronic pneumonia.

Tumor necrosis factor alpha (TNF- α) is a 17-kDa protein produced by activated cells of the monocyte-macrophage lineage (5). It is a potent mediator of inflammation and has been implicated in the promotion of a variety of immune-mediated lesions (5). TNF- α may play a protective role in infectious processes, presumably by activating macrophages and thereby enhancing the killing of intracellular pathogens (9, 17). However, it is also likely that this monokine mediates pathology in microbe-induced shock and in chronic infectious diseases in which macrophage activation is a characteristic feature (5, 7, 9). Although a range of pathogens comprising viruses, bacteria, and protozoal parasites (3, 4, 30) can stimulate TNF- α secretion by human mononuclear phagocytes, little is known about the role of TNF- α in naturally occurring pulmonary diseases.

A cardinal feature of pulmonary diseases induced by chronic lentiviral and bacterial infections in sheep is cachexia, and dual infection by these agents often results in a condition known as "thin ewe syndrome" (14). The pulmonary lesions in sheep with these dual bacterial and lentiviral infections consist of a combination of lymphoproliferative interstitial pneumonia and bronchopneumonia (13, 21). This constellation of lesions resembles that found in pediatric and adult AIDS patients with opportunistic and nonopportunistic pulmonary infections, which are a major cause of death in humans with lentiviral infections (18, 21, 23). Thus, dual infection of ovine pulmonary alveolar mac-

rophages (PAM) with bacteria and lentiviruses represents a potential model for examining the role of cytokines in lesion development in secondary pulmonary infections associated with human immunodeficiency virus (HIV).

Although there is a plethora of pathophysiological alterations that could lead to the debilitated state in chronic pneumonic infections, one mechanism may involve the hypersecretion of cytokines, such as TNF- α and interleukin-1, with catabolic activities from infected mononuclear phagocytes. Indeed, repeated experimental inoculations of recombinant TNF- α in cattle result in a reversible debilitating condition which is characterized by depression, anorexia, and cachexia and which mimics the chronic disease state (6). PAM are the primary target cells of *Corynebacterium pseudotuberculosis* (11) and ovine lentiviruses (OvLV) (20), and there is a marked influx of PAM in cases of pneumonic pasteurellosis that often further complicates cases of thin ewe syndrome (10). We have demonstrated that OvLV-induced lymphoproliferative interstitial pneumonia is associated with elevated levels of locally produced interferon, which may promote lymphoproliferation (21). Previous studies in humans documented TNF- α secretion by PAM in response to microbes that are associated with pulmonary infections in AIDS (19, 23, 30). To better understand the role of cytokines in the interaction between bacteria and lentiviruses in ovine lungs and in the pathogenesis of chronic pulmonary diseases of comparative biological interest, we examined the TNF- α -inducing capabilities of *C. pseudotuberculosis*, *Pasteurella haemolytica*, and lentiviruses in cultures of ovine PAM.

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MATERIALS AND METHODS

Cells. PAM were obtained by postmortem bronchoalveolar lavage (12) from clinically normal yearling Rambouillet sheep that tested negative for antibodies to OvLV (31) and *C. pseudotuberculosis* (22). Two clinically normal sheep, 12-3 and 9-25, used in experiments to examine the effects of *P. haemolytica* showed low and high levels of seropositivity, respectively, for antibodies to *P. haemolytica* (15). Lavage cells from the normal lungs were composed of greater than or equal to 90% PAM, as assessed by morphology, nonspecific esterase staining (32), and reactivity with monoclonal antibody IL-A29 (12). PAM were suspended in endotoxin-free RPMI medium (Sigma Chemical Co., St. Louis, Mo.) supplemented with L-glutamine (2 mM), gentamicin (50 μ g/ml), and 10% autologous heat-inactivated serum. The cells were cultured at 37°C with 5% CO₂ in 24-well tissue culture plates.

Bacterial preparations. Stock cultures of *C. pseudotuberculosis* (ATCC 19410) or *P. haemolytica* type A1 (isolated from a case of bovine pneumonia) were streaked onto complete blood agar plates. This serotype of *P. haemolytica* has been used to examine the interaction between ovine lung lavage cells and the bacterium (9a). After 48 h at 37°C, single colonies were collected and placed in endotoxin-free RPMI medium with 10% autologous heat-inactivated serum but without antibiotics. Following 6 h at 37°C, these suspensions were used as standard cultures to inoculate PAM cultures.

OvLV preparations. Two plaque-cloned OvLV, 85/28 and 85/34, with differential pathogenicities for PAM (20) were grown and their titers were determined in goat synovial membrane cells (kindly provided by W. P. Cheevers, Washington State University) as previously described (20). Supernatants for which titers were determined were used to inoculate PAM cultures.

Stimulation of PAM. PAM were cultured in 1 ml of supplemented medium in 24-well plates (Costar, Cambridge, Mass.) at a concentration of 10⁶/ml and stimulated for 2, 24, 48, 72, or 168 h with the following stimuli: (i) medium alone; (ii) latex beads (0.03 to 3.12 μ m; 1:1,000 stock; Duke Scientific, Palo Alto, Calif.); (iii) endotoxin (lipopolysaccharide [LPS]) (*Escherichia coli* serotype O55:B5; 0.00125 to 12.5 μ g/ml; Sigma); (iv) *C. pseudotuberculosis* or *P. haemolytica* (1/100 or 1/1,000 dilution of a standard suspension culture); and (v) OvLV 85/28 or 85/34 (multiplicity of infection, 0.1). To test the effect of antibiotics on TNF- α production induced by bacteria, we did not include gentamicin, which has in vitro activity against both species of bacteria (29), in some cultures. At each time, supernatants were harvested, centrifuged at 15,000 \times g for 10 min, and frozen at -70°C until assayed. Forty-eight-hour cultures were used to compare the levels of TNF- α induced by pathogens on the basis of results from preliminary experiments. Bacterial and viral infections of macrophages were confirmed by examination of Wright-Giemsa-stained cytopins, immunocytochemical staining of cytopins with monospecific rabbit serum against *C. pseudotuberculosis* (prepared in our laboratory) or caprine arthritis-encephalitis virus p-30 (kindly provided by W. P. Cheevers), or electron microscopic examination of cell pellets or monolayers prepared en face as previously described (25). Seven independent experiments with seven different PAM donors were performed.

TNF- α assay. TNF- α activity in supernatants was assayed colorimetrically by its cytotoxicity for the WEHI-164, clone 13, mouse fibrosarcoma cell line essentially as described

previously (16). In brief, 50,000 WEHI cells in 100 μ l were cultured with 100- μ l volumes of test supernatants or log dilutions of standard recombinant bovine TNF- α (rBoTNF- α ; kindly provided by Ciba-Geigy, SA) and 10% normal rabbit serum or 10% anti-TNF- α rabbit serum (prepared in our laboratory by four monthly injections of 50 μ g of purified rBoTNF- α ; neutralizing titer, 1/500). The final concentration of the supernatant in all assays was 50%. After 20 h of incubation at 37°C, 25 μ l of MTT (5 mg/ml in phosphate-buffered saline; Sigma) was added. Following 2 h of incubation at 37°C, 100 μ l of the supernatant was carefully removed and 100 μ l of extraction buffer containing sodium dodecyl sulfate and *N,N*-dimethylformamide (16) was added. Following overnight incubation, color development was quantitated at 595 nm in a model 3550 microplate reader (Bio-Rad, Richmond, Calif.) with extraction buffer as the blank. Levels of TNF- α activity were quantitated with a standard curve derived from the rBoTNF- α values. Standard curves were generated for each assay of PAM supernatants. All data are expressed as percent cytotoxicity compared with negative controls (medium or medium plus additives without PAM) and were calculated as follows: [(OD of negative control - OD of test well)/OD of negative control] \times 100, where OD is optical density. All assays were performed at least twice with triplicate wells of each sample supernatant in each experiment.

In preliminary experiments, we used WEHI-164 cells, the parent of the clone 13 cell line, as targets in the bioassay and found them to be 3 to 4 logs less sensitive than clone 13 cells to lysis by rBoTNF- α (data not shown). Although the TNF- α sensitivity of WEHI-164 cells was increased by pretreatment with actinomycin D, WEHI-164 clone 13 cells were used throughout the remaining studies.

Immunoblotting. Standard rBoTNF- α at a concentration of 0.65 mg/ml was mixed with a buffer containing 62 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate, 5% 2-mercaptoethanol, 7.5% glycerol, and 0.01% bromophenol blue, and the mixture was boiled for 5 min. Approximately 10 μ g of rBoTNF- α was separated on a 12% mini-polyacrylamide gel electrophoresis gel (Bio-Rad) by standard procedures. Separated proteins were blotted onto Immobilon-P membranes (Millipore, Bedford, Mass.) blocked in 3% nonfat dry milk in phosphate-buffered saline for 1 h and probed with a 1/200 dilution of normal rabbit serum or anti-rBoTNF- α rabbit serum and then with a 1/200 dilution of peroxidase-conjugated goat anti-rabbit immunoglobulin G (Pel-Frez, Rogers, Ark.). Recognized proteins were visualized with TMB substrate (Kirkegaard and Perry, Gaithersburg, Md.).

Statistical methods. Student's *t* test was used to determine the significance of percent cytotoxicity among the various treatment groups.

RESULTS

Specificity of the WEHI-164 cell bioassay for ovine TNF- α . To eliminate the possibility that the additives themselves were nonspecifically toxic for WEHI-164 clone 13 cells, we incubated samples of additives at the concentrations used to inoculate PAM cultures for 24 h without PAM and tested them for their direct effects on the fibrosarcoma cells. No cytotoxic activity was noted when additives alone were added to the WEHI-164 cell assay (data not shown). To confirm that the lysis of WEHI-164 cells was due to the action of ruminant TNF- α , we used a monospecific rabbit antiserum against rBoTNF- α to block cytotoxicity. This

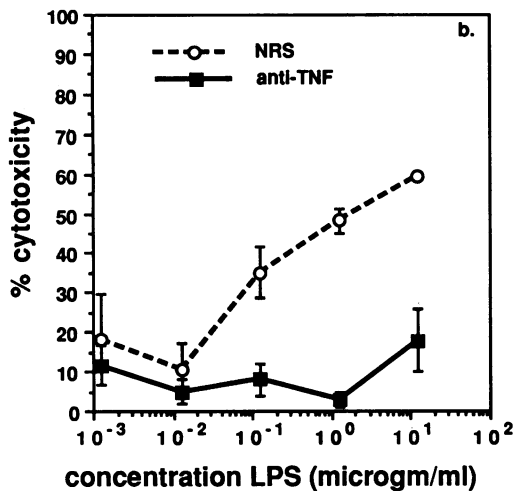
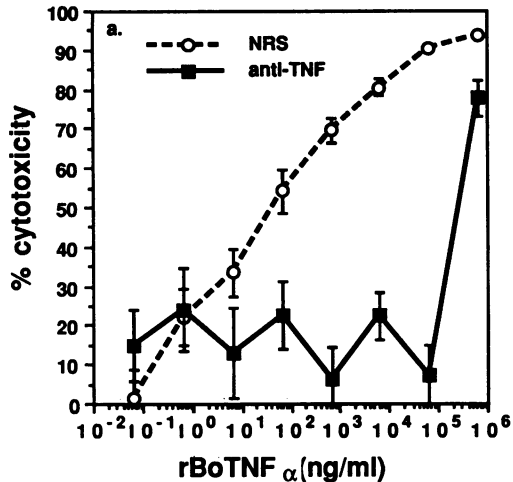


FIG. 1. (a) Lysis of WEHI-164 clone 13 cells by rBoTNF- α . Lysis of target cells was blocked by the inclusion of monospecific anti-rBoTNF- α rabbit serum (final concentration, 10%). Bars represent means of triplicate wells for each dilution tested \pm standard errors. (b) Dose-dependent induction of TNF- α activity by LPS in 48-h PAM cultures from one sheep. Lytic activity was blocked by the inclusion of monospecific anti-rBoTNF- α rabbit serum, indicating homology between ovine and bovine TNF- α . NRS, normal rabbit serum.

antiserum reacted with a single protein of approximately 16 kDa and a dimer of approximately 32 kDa on immunoblots of the rBoTNF- α standard (data not shown). At a final concentration of 10%, this antiserum effectively blocked lysis due to the presence of rBoTNF- α at concentrations ranging from 0.065 to 65,000 pg/ml (Fig. 1a). Lytic activity in the WEHI-164 cell assay was induced in ovine PAM in a dose-dependent fashion by coculturing for 48 h with LPS at doses ranging from 0.00125 to 12.5 μ g/ml (Fig. 1b). Lytic activity in supernatants from ovine PAM cultures stimulated with this range of LPS doses was effectively blocked by the anti-rBoTNF- α serum, indicating the presence of TNF- α and the specificity of the antibody for both bovine and ovine TNF- α (Fig. 1b).

Induction of TNF- α release by *C. pseudotuberculosis* and *P.*

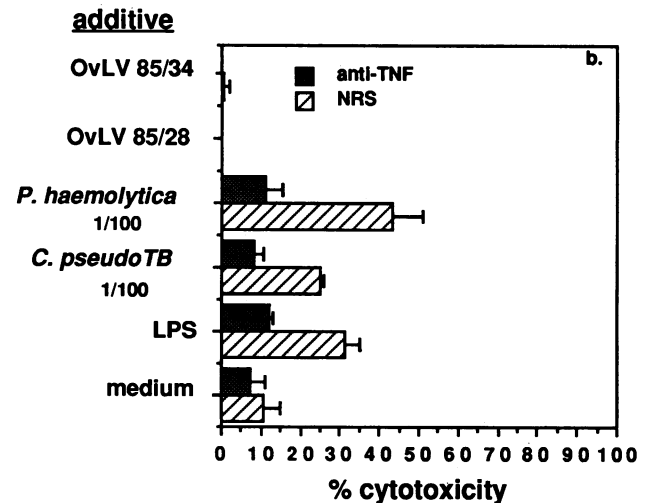
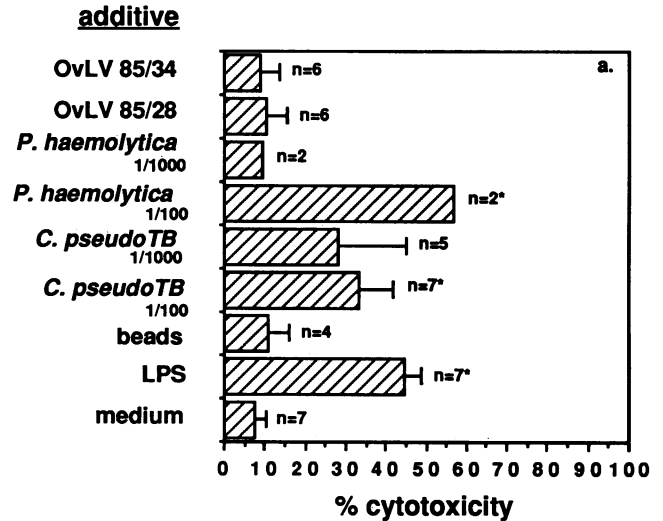


FIG. 2. (a) Microbe-induced TNF- α activity in PAM cultures derived from sheep and harvested 48 h after the initiation of culturing with additives. When compared with the negative (medium) and positive (LPS) control cultures, PAM cultures infected with *C. pseudotuberculosis* (*C. pseudoTB*) and *P. haemolytica* but not OvLV 85/28 or OvLV 85/34 had significant TNF- α activity. Bars represent means of triplicate wells for each animal tested \pm standard errors. n, number of different sheep tested; *, significant differences. Each assay was conducted twice, and the final concentration of supernatant in each well was 50%. Results of one experiment are shown. (b) Blocking of microbe-induced TNF- α activity by the inclusion of TNF- α -specific antiserum in the assay, showing that lysis was due to the action of TNF- α . NRS, normal rabbit serum.

haemolytica. Compared with the low levels of cytotoxicity in supernatants from PAM cultures with medium alone, there was significant ($P < 0.02$) WEHI-164 cell lytic activity in PAM cultures inoculated with *C. pseudotuberculosis* or *P. haemolytica* (Fig. 2a). The levels of cytotoxicity induced by these bacterial species were comparable to those induced by LPS in PAM cultures. Lytic activity in these cultures was completely blocked by TNF- α -specific antiserum (Fig. 2b). TNF- α activity was induced by both bacterial species in

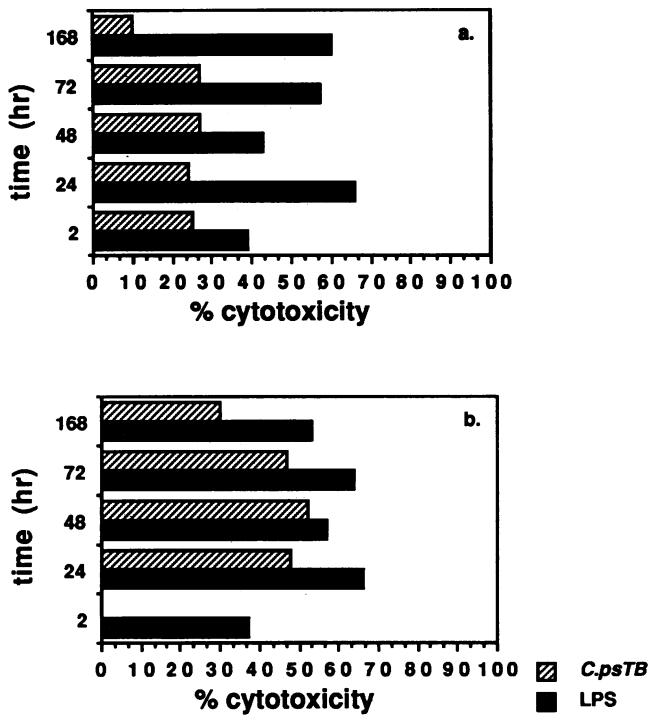


FIG. 3. Kinetics of TNF- α release in cultures of ovine PAM from two sheep, 650 (a) and 641 (b), indicating variable responses to LPS and *C. pseudotuberculosis* (*C. psTB*) determined in WEHI-164 cell assays performed concurrently. No TNF- α activity was detected in PAM cultures inoculated with latex beads or OvLV clones.

PAM cultured in the presence and absence of gentamicin (data not shown).

Lack of TNF- α activity in lentivirus-infected PAM cultures. In contrast to supernatants from PAM cultures treated with LPS and the two bacterial species assessed, supernatants from PAM cultures inoculated with OvLV 85/28 or 85/34 did not contain significant levels of TNF- α activity compared with cultures incubated with medium alone (Fig. 2).

Kinetics of TNF- α release from PAM. The kinetics of TNF- α release in PAM cultures inoculated with various additives were determined with PAM derived from four sheep (Fig. 3). LPS induced high levels of TNF- α in PAM from all four sheep. This induction was detectable as early as 2 h after stimulation in two of the four sheep and at 24 h in two of the four sheep. LPS-induced TNF- α activity peaked at 24 h but remained high throughout the culture period.

Phagocytosis of *C. pseudotuberculosis* (Fig. 4a) induced TNF- α activity in PAM cultures from all four sheep. This induction was detectable as early as 2 h after inoculation (Fig. 3). The levels of *C. pseudotuberculosis* phagocytosis-induced TNF- α activity peaked from 48 to 72 h after culturing and varied with regard to the degree of response among individual lambs. This induction occurred under equivalent culture conditions and doses of bacteria, reflecting individual animal variations (Fig. 3). In contrast, phagocytosis of latex beads did not stimulate TNF- α activity at any time after inoculation.

Lytic activity that could be blocked by the TNF- α -specific antiserum was not detected in PAM cultures inoculated with OvLV 85/28 or 85/34 at any of the times tested, despite immunocytochemical and ultrastructural evidence of infec-

tion of up to 40% of PAM by OvLV at 168 h after inoculation (Fig. 4b). Syncytium formation typical of the cytopathology induced by lentiviruses was also apparent in these cultures (Fig. 4b).

DISCUSSION

The interaction between bacteria and lentiviruses and ovine PAM is a model relevant to better defining the role of cytokines in the pathogenesis of chronic pulmonary disease associated with HIV infections (21). Furthermore, we have shown that infection of PAM with the bacterial organisms *C. pseudotuberculosis* and *P. haemolytica*, which are commonly isolated from cases of chronic pneumonia in sheep, results in TNF- α release in vitro. Failure of latex bead ingestion to induce TNF- α indicates that specific components of the bacteria and not simply the process of phagocytosis are responsible for this effect. In contrast to infection with the bacterial pathogens examined, productive infection of PAM with OvLV did not result in TNF- α secretion by PAM.

Induction of TNF- α secretion in vitro and in vivo has been reported for both gram-negative and gram-positive bacteria or their products in a variety of species (1, 2, 7, 30). Our results demonstrate the applicability of WEHI-164 clone 13 cells to the bioassay of ovine TNF- α induced by bacterial pathogens. LPS and several mycobacterial antigens have been implicated as specific bacterial components that induce TNF- α secretion (1, 2, 30). The presence of TNF- α in PAM cultures infected with *C. pseudotuberculosis* and *P. haemolytica* and containing antibiotics suggests that these bacteria need not be alive to induce TNF- α secretion and that some of their soluble products are effective in inducing TNF- α . It is probable that the LPS of *P. haemolytica* induces TNF- α in ovine PAM (1, 2); however, other specific constituents of *C. pseudotuberculosis* and *P. haemolytica*, such as the exotoxins of these agents, that stimulate the secretion of TNF- α remain to be identified. It has been proposed that vaccination with antigens that stimulate TNF- α secretion to induce an antibody-mediated blockade of TNF- α secretion or activity may be a rational approach to reducing the effects of disease caused by intracellular parasites such as plasmodia (26). Thus, the identification of TNF- α -inducing constituents of the intracellular pathogens *C. pseudotuberculosis* and *P. haemolytica* would be useful in determining how such antigens may be used in prophylaxis against chronic bacterial infections.

We found considerable variability in TNF- α production in response to LPS and bacterial pathogens among sheep. Opsonizing antibody that mediates enhanced phagocytosis and processing may be irrelevant to TNF- α production, since all sheep used here were seronegative for *C. pseudotuberculosis*. The induction of TNF- α by LPS suggests that the mechanisms involved in TNF- α induction may in fact be independent of phagocytosis, especially in the case of bacteria that secrete exotoxins. The variability in TNF- α secretion may be attributable to differences in the pulmonary macrophage subpopulations among the sheep. Several subpopulations of PAM that differ not only morphologically but also in interleukin-1 production have been described in rats and other species (28). It would be worthwhile to determine whether similar functional differences that may account for differences in monokine secretion following stimulation with microorganisms exist among ruminant PAM. Alternatively, individual differences in susceptibility to infection or in TNF- α secretion in response to the agents tested could occur

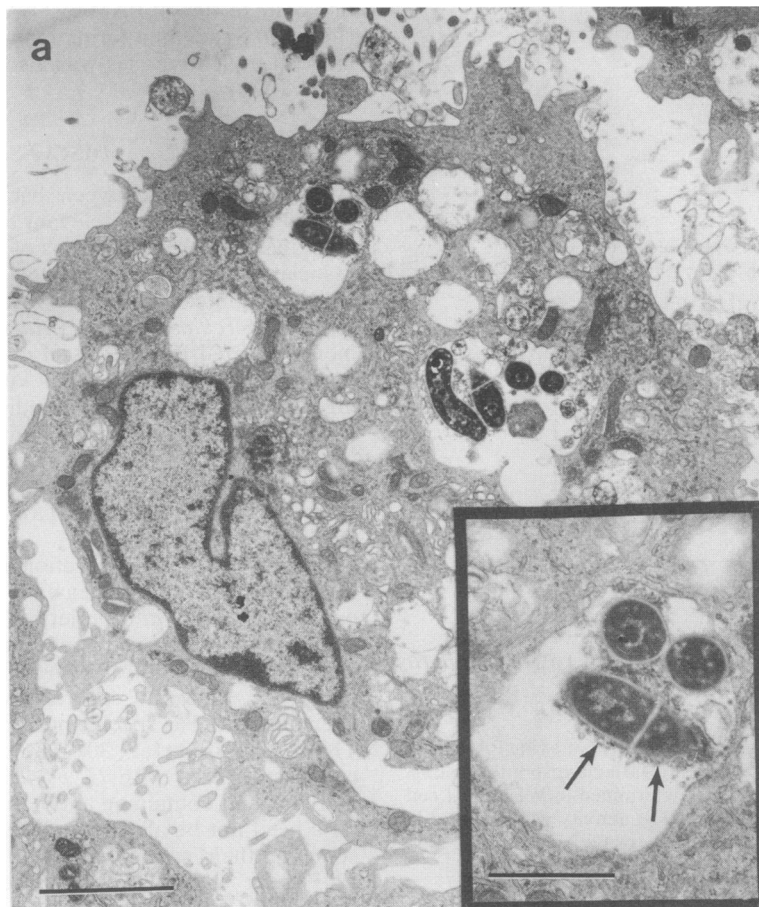


FIG. 4. (a) Low-magnification transmission electron micrograph of small vacuolated PAM containing *C. pseudotuberculosis* (1/100 dilution of a 6-h culture; 12 h after infection) in phagosomes. Bar, 2.5 μ m. (Inset) Higher magnification of apparently viable *C. pseudotuberculosis* cells, one of which is undergoing division. Flocculent irregular electron-dense material external to the cell wall is indicated by arrows. Uranyl acetate and lead citrate were used to stain the en face preparation. Magnification, $\times 10,000$. (b) Low-magnification transmission electron micrograph of small syncytial PAM infected with OvLV 85/34 (multiplicity of infection, 0.1; 7 days after infection) showing occasional nascent or mature lentivirus particles associated with plasmalemma (arrowheads). Bar, 2.5 μ m. (Inset) Nascent lentivirus with nucleocapsid (large arrowhead). Uranyl acetate and lead citrate were used to stain the cell pellet. Bar, 100 nm.

at the level of polymorphisms in TNF- α genes (8), a possibility which raises interesting questions regarding the control of an individual animal's response to a specific pathogen.

The failure of OvLV to induce TNF- α secretion by the infected target cells is in agreement with recent studies of HIV infections in human peripheral blood mononuclear phagocyte populations examined under endotoxin-free conditions (24). Our use of two different biological clones of OvLV with different pathogenicities for ovine PAM suggests that strain differences in the infecting lentiviruses do not account for the failure to induce TNF- α secretion. Although OvLV themselves apparently do not induce TNF- α secretion, the local production of TNF- α stimulated by bacteria during dual pulmonary infections may be central to the pathogenesis of chronic ovine lung disease, mediating both lentiviral replication and recruitment of target PAM. Bacteria play an important role as cofactors in HIV-associated pneumonias (23, 27). Substantial data indicate that pathogens isolated from pneumonic lungs in AIDS patients stimulate monokine secretion and that TNF- α , interleukin-1, and interleukin-6 induce HIV expression in infected human mononuclear phagocytes (27). Recently, we demonstrated

that rBoTNF- α enhances lentivirus expression in infected alveolar macrophages (11a), indicating one mechanism of bacterial-viral synergism in an established model of lentivirus-induced pulmonary disease.

To our knowledge, this is the first demonstration of TNF- α secretion by ovine mononuclear phagocytes in response to nonspecific and specific stimuli. Further studies are needed to evaluate the importance of local TNF- α production *in vivo* during infection with pulmonary pathogens in this and other systems. It will be of particular interest to evaluate TNF- α levels in lung tissue from infected animals and to determine whether there is circulating TNF- α in sheep that become cachectic because of chronic lung disease. The role of TNF- α and other monokines in effecting the outcome of the intracellular replication of *C. pseudotuberculosis* and *P. haemolytica* is currently being investigated.

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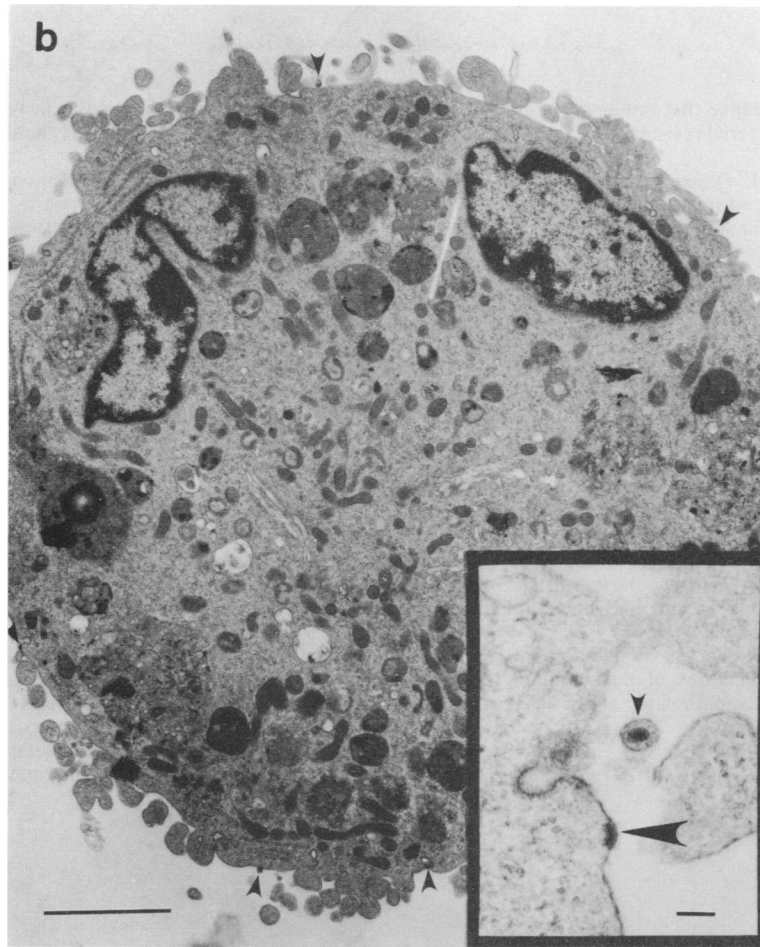


FIG. 4—Continued.

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