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Replication of Bovine Parainfluenza-3 Virus in Bovine Alveolar Macrophages : Effects on Phagocytosis and Phagosome-Lysosome Fusion

Richard Allen Hesse

REPLICATION OF BOVINE PARAINFLUENZA-3 VIRUS IN BOVINE ALVEOLAR MACROPHAGES: EFFECTS ON PHAGOCYTOSIS AND PHAGOSOME-LYSOSOME FUSION

BY

RICHARD ALLEN HESSE

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This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

David A. Benfield, Ph.D. Date

Thesis Advisor

Ernest J. Hugghins, Ph.D. Date

Head, Biology Department

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I dedicate this thesis to my wife, Debby, who has remained by my side throughout these difficult years. Her support and understanding have enabled me to achieve goals which I could not have reached alone.

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Abstract

RICHARD ALLEN HESSE

Bovine parainfluenza-3 virus was found to replicate in alveolar macrophages in vitro. Cytopathic changes include spindle cell and giant cell formation, cellular detachment, and diffuse cell lysis progressing to total destruction of the monolayer.

Direct immunofluorescent microscopy demonstrated viral antigens in greater than 90% of the cells three days after virus inoculation. Virus titers in culture supernatants increased by a factor of 1.6×10^6 $TCID_{50}$ by six days post inoculation. Phagocytosis assays showed no statistical differences of glass adherent cells in virus infected and control macrophages. When the number of glass adherent cells available for assay are taken into account (normalization) differences in phagocytic values are significant at 5 and 7 days post inoculation. Phagosome-lysosome fusion assays demonstrated marked reduction of fusion activity of virus infected macrophages compared to control macrophages. Fusion values of infected cells were 51% and 53% that of control cells at days 4 and 6, respectively. Normalization of these values reduced the fusion rate to 37% of control macrophages.

INTRODUCTION

Bovine respiratory disease (BRD) results in substantial economic loss to the North American cattle industry (7, 15). It has been estimated that 40% to 80% of bovine disease involves the respiratory system (7). Understanding and control of BRD remains elusive in spite of volumes of scientific literature on this disease complex and the availability of a multitude of vaccines.

Most investigators agree that BRD frequently results when cattle are subjected to environmental stresses or various etiological agents (7, 20). Stress incurred in shipment and/or virus infection are thought to predispose the bovine lung to secondary bacterial invasion. Bovine parainfluenza-3 virus (BPI-3) and infectious bovine rhinotracheitis virus (IBR) have been implicated as primary pathogens which may compromise the animal and result in pneumonic pasteurellosis (7, 15, 20).

Relatively little is known about viral-bacterial interactions with the bovine pulmonary defense systems; the mucociliary apparatus and alveolar macrophages play an important role in the outcome of the disease process (10). Quantitative studies of viral-bacterial interaction in the murine system have demonstrated severe defects in <u>in situ</u> bactericidal mechanisms without alterations in bacterial transport rates in spite of extensive destruction of bronchial

ciliated epithelium (4). Similar results in clearance rates were obtained in BPI-3 virus infected calves challenged with P. hemolytica, Lopez drew parallels between the murine and bovine in vivo systems and suggested that BPI-3 virus infection interfered with the phagocytic activity of alveolar macrophages (8).

Relatively little is known about the interactions of BPI-3 virus and cultured bovine alveolar macrophages (BAM). Rossi et al demonstrated that monocytes derived macrophage cultures exhibited cytopathic effect (CPE) after BPI-3 inoculation (16). However, it is not known if this virus will undergo productive replication in BAM or what effect it will have upon BAM functions. This study examines the interactions of BPI-3 and cultured BAM with regard to CPE, quantitation of progeny virus, and the quantitation of macrophage processes of phagocytosis and phagosome-lysosome fusion.

MATERIALS AND METHODS

Bovine Alveolar Macrophages (BAM) and Bovine Embryonic Lung (BEL) Cells. Lungs from four freshly slaughtered steers were obtained from the Department of Animal Science at South Dakota State University. Trachea and lung were removed and pulmonary lavage, based on the method of Fox (2), was conducted within 30-60 minutes of the time of death. The lungs were filled via the trachea with phosphate buffered saline, pH 7.2,

and gently massaged. Three to 5 liters of lavage fluid were required for each lung and about half of the fluid was recovered. Lavage fluids were centrifuged at 300 X g for 20 minutes at 4°C and cell pellets were resuspended in tissue culture medium (TCM) consisting of Eagle minimum essential medium (EMEM, Gibco Laboratories, Grand Island, NY) containing 10% fetal calf serum (FCS), gentocin (100 ug per ml), fungizone (2.5 ug per ml) and 25 mM HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid). Cell suspensions were stored at 5°C until all lungs were processed (2.5 hours to 30 minutes). Immediately prior to seeding, the cells were centrifuged again as before and resuspended in 25 ml of TCM. The total number of cells was determined with a hemocytometer and the BAM concentration was adjusted to 1 X 10^6 per ml TCM. Cells from each of four lungs were processed separately and .8 ml of the BAM suspensions was added to one chamber of 4-chamber Lab-Tek slides (Lab-Tek Division, Miles Laboratories, Inc., Naperville, IL). The cells were allowed to attach for 2 hours at 37°C in a humid atmosphere containing 5% CO_2. After incubation, the slides were washed with EMEM, the media was replaced and the cells were reincubated. After 18 hours incubation, a count of the cells adherent to the glass was made by gently scraping them off the slide and counting the cells with a hemocytometer. The average

number of adherent cells per chamber was used to establish the virus multiplicity of infection, and to calculate the number of yeast cells to achieve a 20 yeast/BAM ratio in BAM function assays. A BEL cell-line was established in our laboratory by standard cell-culture methods. The BEL cells were seeded in TCM to reach confluency by the time the BAM slides were inoculated with the virus.

Seed Virus. Bovine parainfluenza-3 virus (BPI-3) SF-4 strain was obtained from the American Type Culture Collection and inoculated in BEL. After extensive (>90%) cytopathic effect had appeared, the culture supernatant was collected, clarified by centrifugation at 1,000 X g for 15 minutes and small aliquots were stored at -70°C. The titer of this virus stock was 3.2 X 10^6 median tissue culture infective dose$_{50}$ ($TCID_{50}$) per ml.

Virus Inoculation. Bovine alveolar macrophages and BEL monolayers grown in 4 chamber Lab-Tek slides and maintained for 24 hours were inoculated with 600 $TCID_{50}$ of BPI-3 virus in 0.2 ml of EMEM supplemented with 2% FCS and gentocin. After a 1 hour adsorption period at 37°C in a humid atmosphere containing 5% CO_2, 0.6 ml of TCM was added to each chamber. Sham inoculated cells exposed to virus free diluent served as controls. At 24 hour intervals after inoculation, the culture supernatant was removed and stored at -70°C until virus titration. Viral titers of culture supernatant were determined

by microtiter assay in BEL cells. The BAM cultures were observed for CPE and selected areas were photographed at the same time samples of the supernatant were withdrawn.

Immunofluorescence and Wright Staining. Chambered slides for direct immunofluorescence assay (DFA) were air dried, fixed in acetone at room temperature for 10 minutes and stored at -20°C until tested. Fluorescein isothiocyanate labeled bovine anti-BPI-3 serum (kindly provided by M. L. Snyder, National Veterinary Services Laboratory, Ames, IA) was used to detect BPI-3 viral antigens in virus infected and sham-infected BAM and BEL cultures. Bovine alveolar macrophage slides for light microscopy were rinsed in PBS, air dried, fixed in methanol and stained with Wright stain.

Preparation of Yeast Suspensions. Candida glabrata (a laboratory strain) was grown for 18 hours at 37°C in trypticase soy broth. The yeasts were centrifuged, resuspended in 5 ml of EMEM with antibiotics and cell counts were made with a hemocytometer. A portion of this suspension was diluted to 1.6 X 10^7 yeasts per ml in EMEM containing an adult bovine serum with a C. glabrata agglutinin titer of 1:2,560. The final dilution of this serum in EMEM was 1:45,000 ± 800.

Phagocytosis Assay. The ability of BPI-3 infected and control macrophages to phagocytize yeast was assessed by differential acridine orange (AO) staining (19). Culture

supernatants were decanted and 0.4 ml of the yeast suspension (20 yeasts/BAM) was layered onto each chamber of the slide. The slides were incubated for 30 minutes at 37°C; chambers were removed, BAM monolayers were washed 5 times in EMEM, fixed in Carnoy's fluid, air dried and stored at -20°C. Immediately prior to staining, the monolayers were rehydrated by sequential dips in 80%, 70%, and 50% ethanol and distilled water, and equilibrated in McIlvaine's buffer pH 3.8. Staining was accomplished by flooding the slide with a 0.01% solution of AO in McIlvaine's buffer. The slides were rinsed and wet mounted in the buffer and observed with a Zeiss epifluorescent microscope at 600 X magnification. Bovine alveolar macrophages having at least 2 yeasts associated with them were considered phagocytic. One hundred to 130 BAM were observed and scored.

Phagosome-Lysosome Fusion Assay. The effects of BPI-3 infection on macrophage phagosome-lysosome fusion were assessed by a modification of the methods described by Jakab et al (5). Lysosomes in BAM were labeled by adding 0.4 ml of an AO solution containing 12.5 ug per ml in EMEM to each chamber. The slides were then incubated for 20 minutes at 37°C. Excess AO was removed by decanting and washing in EMEM. The BAM monolayers were challenged with 0.4 ml of the described yeast suspension and incubated for 30 minutes at 37°C. The yeasts were decanted and washed 5 times, EMEM was added to the chambers and the

slides were incubated an additional 60 minutes at 37°C. The chambers and gaskets were removed, the slides were dipped in EMEM, wet mounted, and observed with a Zeiss epifluorescent microscope at 600 X magnification. Phagosome-lysosome fusion was evaluated by the staining of intracellular yeasts: all phagosomes containing green, yellow or orange yeasts were considered fused with the pre-labeled lysosomes. Fusion patterns of BPI-3 infected and control BAM cultures were quantitated by randomly counting 100-130 macrophages and determining the percentage of phagosome-lysosome fusion that had occurred.

Data Analysis. Differences in phagocytosis and in phagosome-lysosome fusion of BAI-3 infected and control BAM were analyzed by the paired student T-test, $P<0.05$ was considered significant. Data were normalized by counting BAM on photographs of random areas of the chambers and comparing densities of BPI-3 infected and control BAM cultures at a given assay date. The number of control BAM adherent to glass was assigned a value of 100%. The normalized data were calculated by using the formula:

$N = X\ (Y/Z)$ where

N = normalized % of phagocytic viral-infected BAM, or normalized % of phagosome-lysosome fusing virus-infected BAM;

X = % of phagocytic viral-infected BAM, or

% of phagosome-lysosome fusing viral-infected BAM;

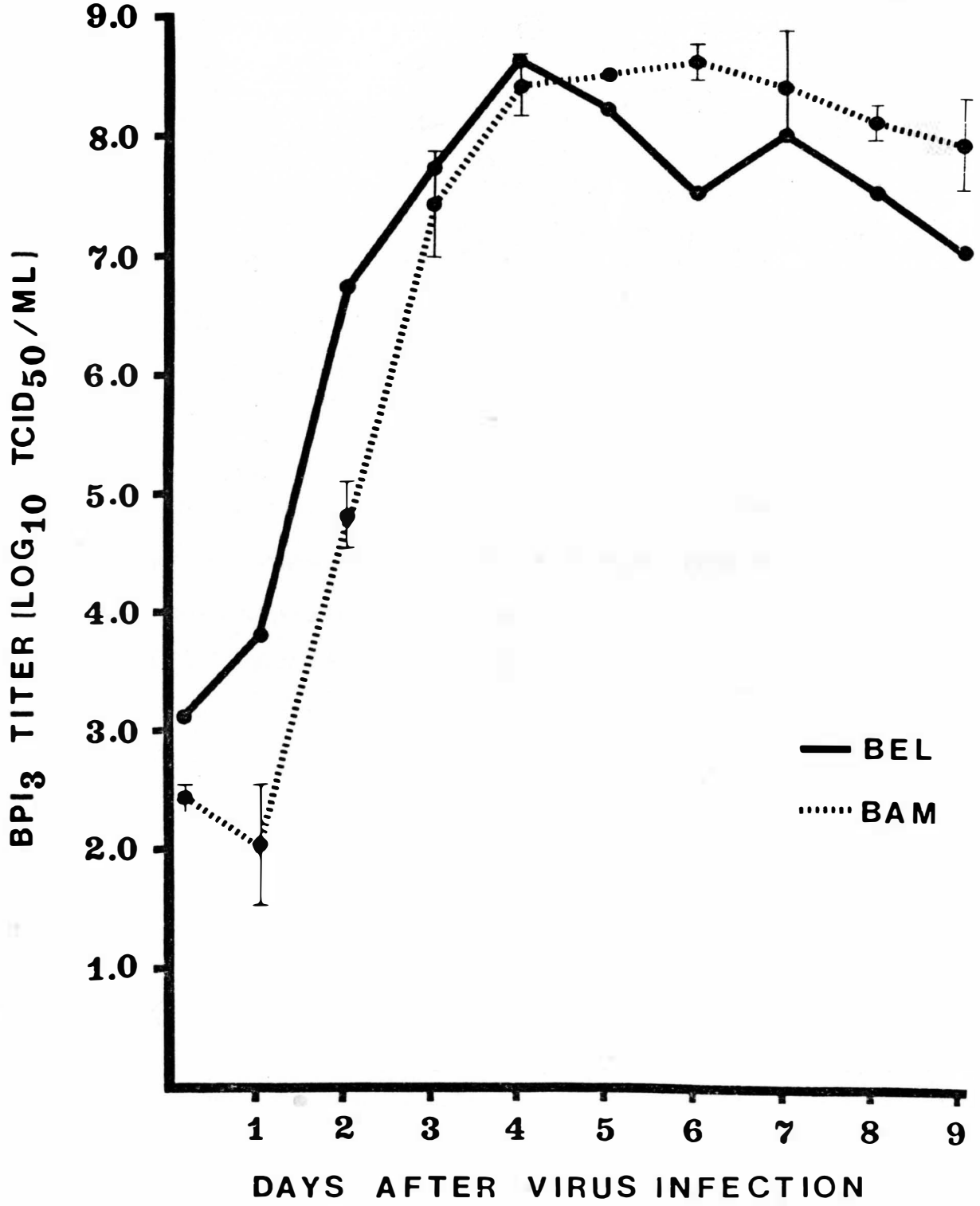
Y = number of viral-infected BAM adherent to glass;

Z = number of control BAM adherent to glass.

RESULTS

Replication of BPI-3 Virus in Cultured Alveolar Macrophages. Cultures of BAM and BEL were inoculated with BPI-3 virus at a multiplicity of infection of approximately 0.001 $TCID_{50}$/cell. As illustrated in Fig. 1, productive viral replication occurred in both cell types. The BPI-3 replicated at a more rapid rate in BEL cells. Peak viral titers, however, were equivalent in both BAM and BEL cultures. Mean viral titers from BAM culture fluid increased form 2.4 $\log_{10}$ $TCID_{50}$ on day 0 to 8.6 $\log_{10}$ $TCID_{50}$ on day 6. Daily microscopic examination of virus infected BAM cultures revealed extensive morphological changes including giant and spindle cell formation, cytoplasmic streaming, cellular detachment, and cell lysis. The cytopathic effect of BPI-3 on BAM cultures is illustrated in Fig. 2. Observations were made with a Zeiss inverted microscope equipped with a Hoffman modulation system. Wright stained monolayers of infected BAM showed extensive numbers of giant cells and cytoplasmic streaming. These giant cells exhibited peripherally arranged nuclei, some of which contained as many as 80 nuclei per cell (Fig. 3A). The number of BPI-3 infected BAM remaining

Figure 1. Replication of bovine parainfluenza virus (BPI-3) in cultured bovine alveolar macrophages (BAM) and bovine embryonic lung (BEL) cells. Data points of the BAM curve are the mean ± standard error (SE) of results of titrations of culture media of four different sets of BAM. Data on the BEL curve represent a single replication curve.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1
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7
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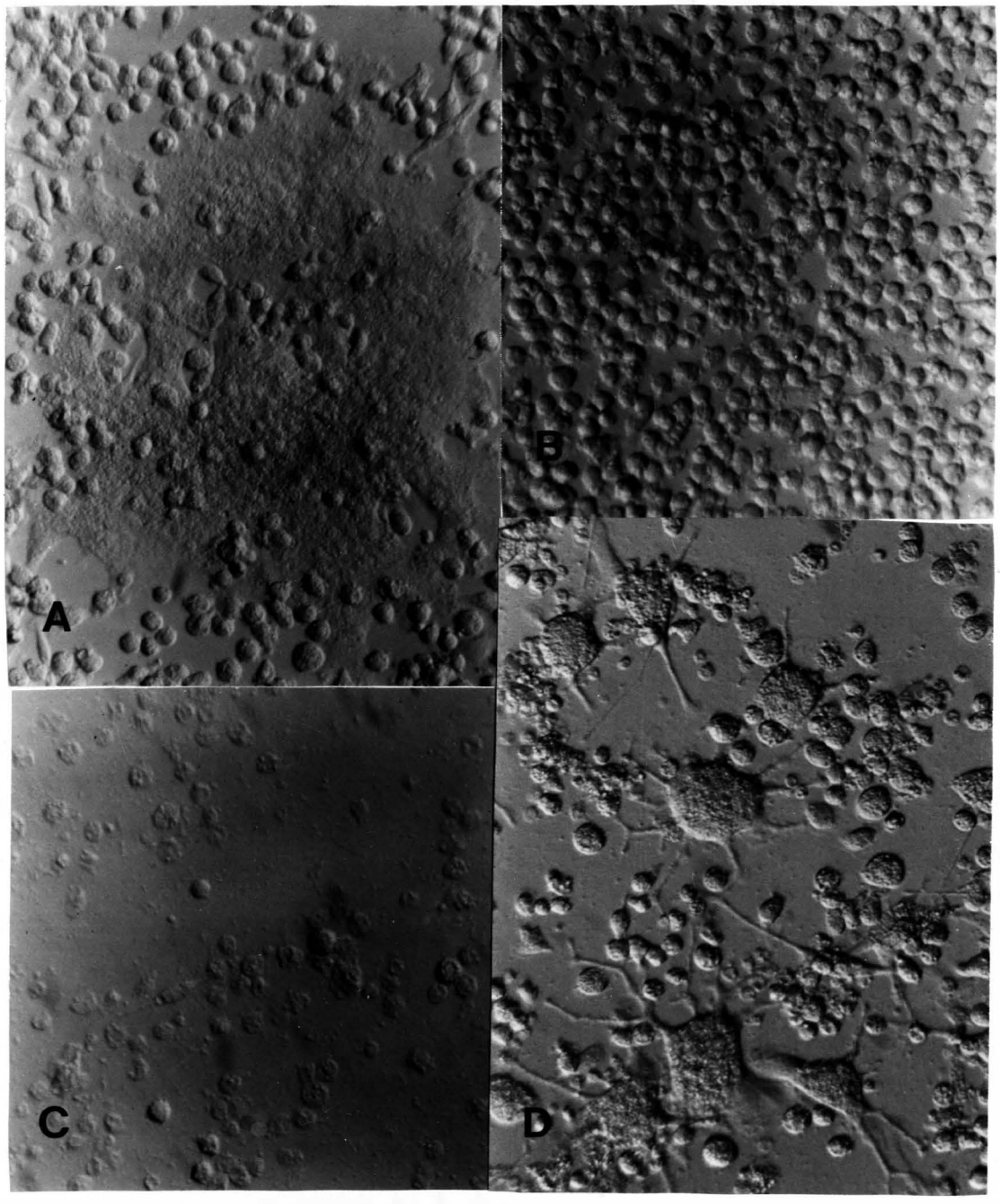
9
DAYS AFTER VIRUS INFECTION

Figure 2. Cytopathic effect of BPI-3 on cultured BAM. A: Virus induced fusion of large numbers of BAM 48 hours post inoculation (PI). B: Sham-inoculated control BAM. C: Extensive cellular detachment and cell lysis at 7 days PI resulting from viral infection. (X 375). D: Giant cells of virus infected BAM at 72 hours PI exhibiting cytoplasmic streaming.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adherent to the glass declined significantly as the viral infection progressed. The number of control BAM adherent to glass remained constant over the same period. Direct immunofluorescence of BPI-3 infected BAM detected viral antigens in 1% to 5% of the cells as early as 24 hours post inoculation (PI). Greater than 90% of the BAM were DFA positive by day 3. At 24 hours PI, the direct fluorescent antibody positive cells exhibited a diffuse, very fine granular cytoplasmic fluorescence. At 3 days PI and thereafter, fluorescence was characterized by intense staining of the cytoplasm and cell membrane.

Photomicrographs in Fig. 4 show the typical staining characteristics of BAM that have phagocytized or undergone phagosome-lysosome fusion of C. glabrata. Differential acridene orange stained cells (Fig. 4A) were used to determine the phagocytic ability of virus or sham-inoculated BAM. The AO staining method was superior to the traditional Wright stain in that it consistently enabled the observer to view the phagocytized yeast. Overstaining or understaining was not a problem using the differential AO technique. Vital AO staining of BAM lysosomes and subsequent fusion with phagosomes (Fig. 4B) was used to assess the ability of virus infected or control BAM to undergo phagosome-lysosome fusion.

Bovine Alveolar Macrophage Function Assays. The percent

Figure 3. BPI-3 induced giant cells. A: Wright stain of infected monolayers at 48 hours PI showing peripherally arranged nuclei. X 400. B: Vital-acridine orange (AO) stained giant cell with large number of randomly distributed nuclei showing phagocytosis and phagosome-lysosome fusion. (X 375).

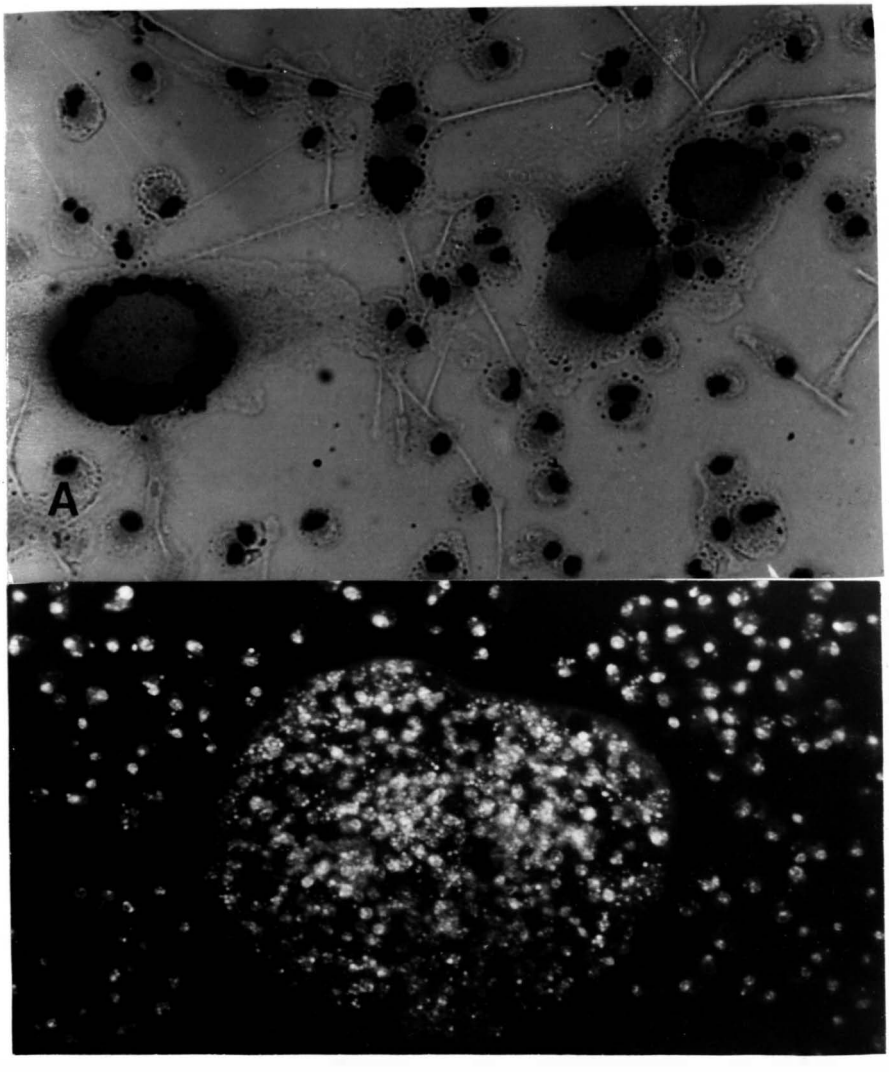
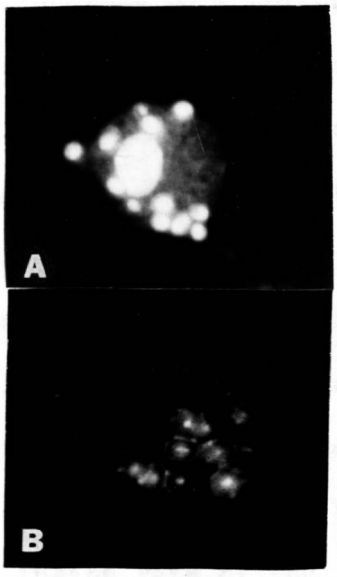

A

Figure 4. Phagocytosis and phagosome-lysosome fusion by BAM. A: Phagocytic BAM fixed and stained by differential AO technique. B: Viable BAM with AO-labeled lysosomes ingesting C. glabrata. Note yeasts in fused (light) phago-lysosomes and non-fused (dark) phagosomes (X 1500). The photomicrograph was obtained by the simultaneous use of bright-light and fluorescent light. Fused phago-lysosomes appeared green, yellow, or orange colored because of the AO staining of the phagocytized C. glabrata yeasts. Non-fused phagosomes (visualized by bright light) containing unstained C. glabrata appeared dark.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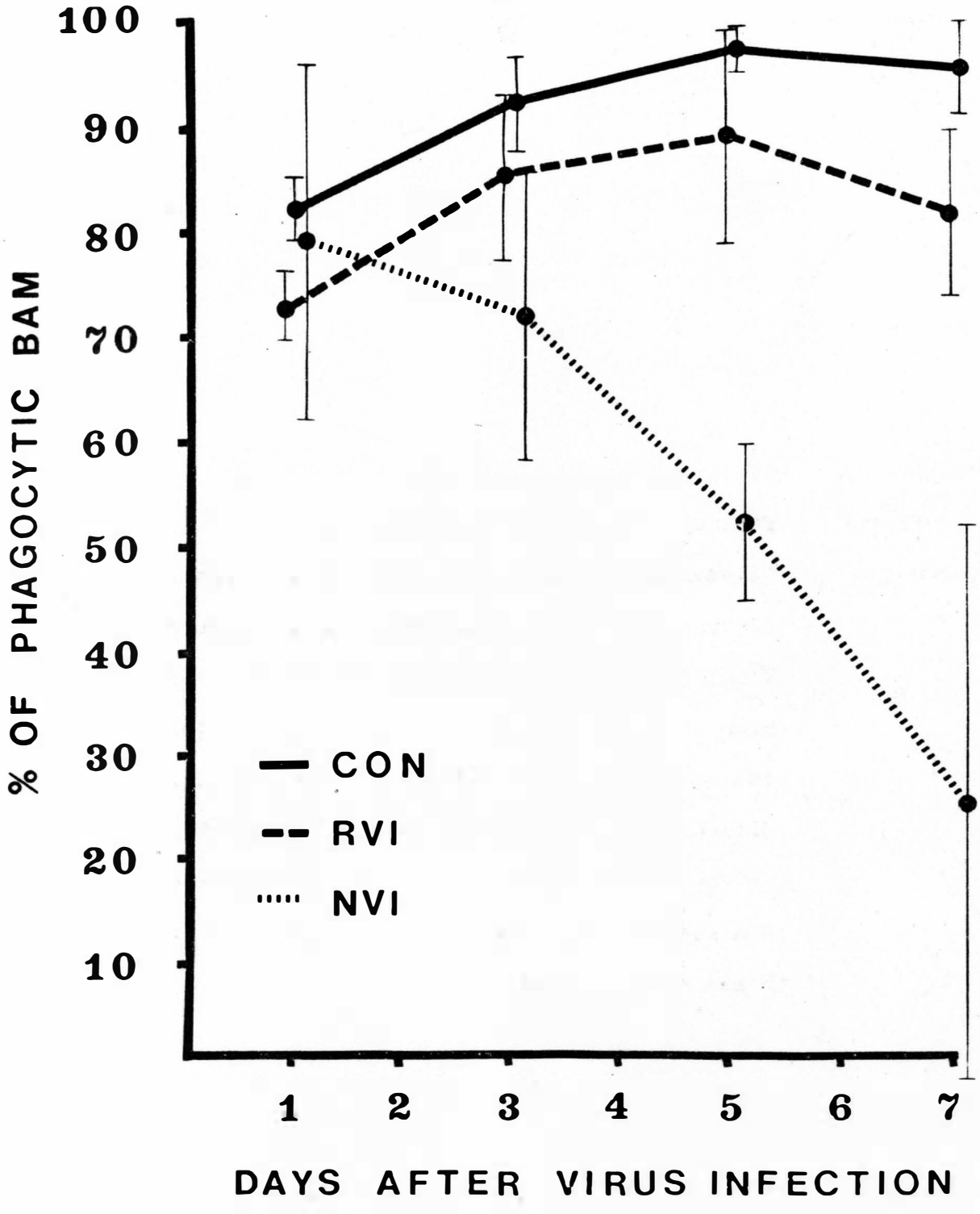
of control and BPI-3 infected macrophages phagocytizing C. glabrata are shown in Fig. 5. Phagocytic values of control macrophages ranged from 82%-96%. The viral infected BAM ranged from 73%-88%. Differences between these two groups were not significant. However, when the reduced number BPI-3 infected BAM cells adherent to glass were normalized, the phagocytic values dropped precipitously. Normalized phagocytic values were significantly less ($P \leq 0.01$) than control values showing 55% of cells phagocytizing at day 5 and 25% at day 7.

Phagosome-lysosome fusion assays demonstrated marked reduction of fusion activity of virus infected macrophages as compared to control macrophages. Fusion values (Fig. 6) of BPI-3 infected macrophages were 51% and 53% that of control cells at days 4 and 6, respectively. Normalization of these values reduced the fusion rate of the infected BAM to 37% of the control BAM.

DISCUSSION

There is little or no information available about bovine parainfluenza-3 virus and bovine alveolar macrophage interaction in vitro. Since macrophages may play an important role in the outcome of a disease; it is therefore useful to determine if a virus associated with the disease will replicate in them. Productive replication of cytomegalovirus, rubella virus and herpes simplex virus in macrophages has been demonstrated, but

Figure 5. Phagocytosis by sham-inoculated control BAM and BPI-3 infected BAM. CON: Percent of phagocytic control BAM. RVI: Raw (non-normalized) percent of viral infected phagocytic BAM. NVI: Normalized proportion of virus infected BAM. Each point is the mean ± SE of results obtained in four different sets of BAM. Differences between CON and RVI values are not significant. Differences between CON and NVI values are significant ($P<0.01$) on days 5 and 7 after viral infection.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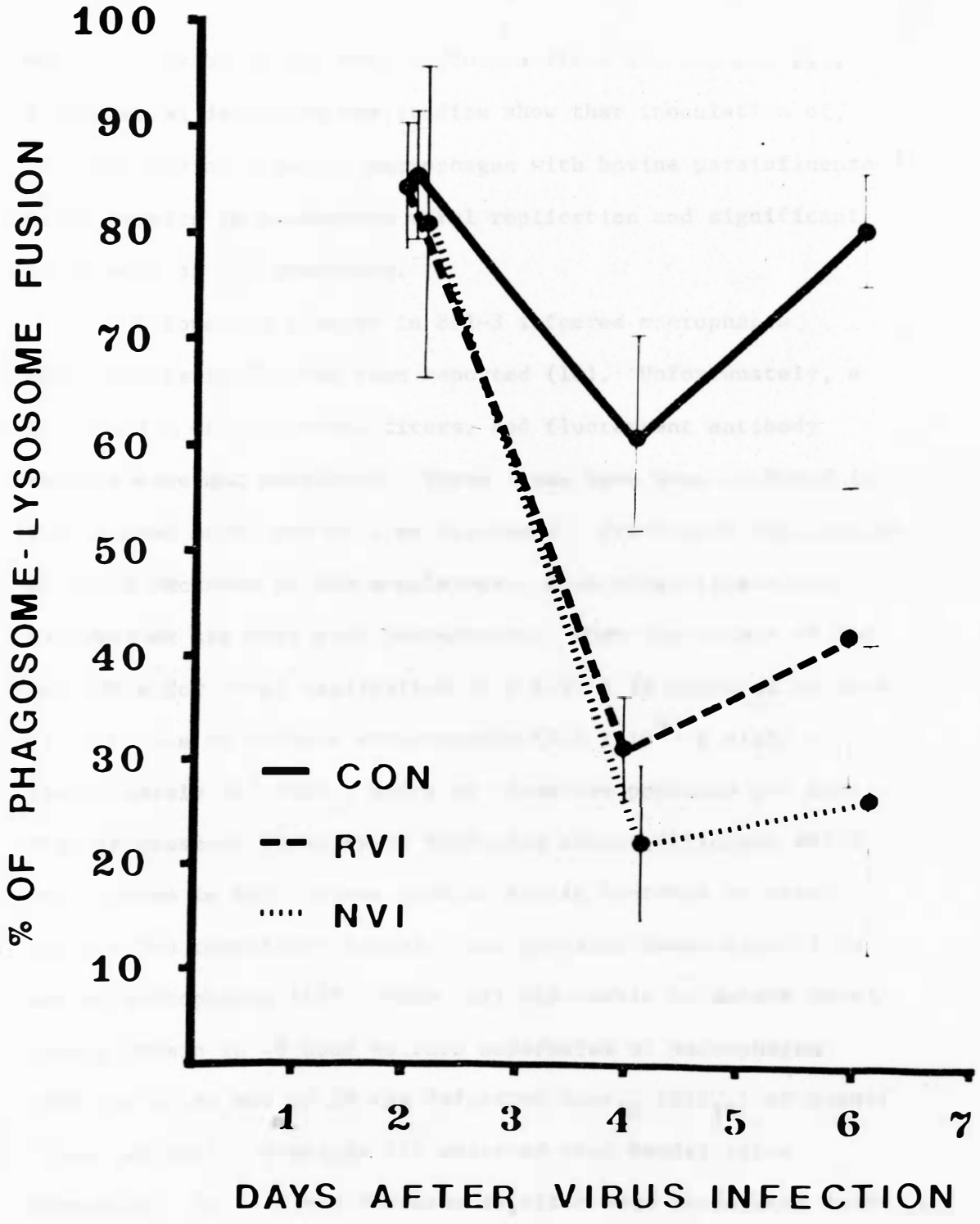
DAYS AFTER VIRUS INFECTION

Figure 6. Phagosome-lysosome fusion activity of sham-inoculated control BAM and BPI-3 infected BAM. CON: Percent of fusing control BAM. RVI: Raw (non-normalized) percent of virus infected fusing BAM. NVI: Normalized proportion of virus infected fusing BAM. Each point is the mean ± SE of results obtained in four different sets of BAM. Differences between CON and RVI and values were significant ($P<0.05$) on day 4 and ($P<0.01$) on day 6 PI. Differences between CON and NVI values were significant ($P<0.01$) on days 4 and 6 after viral infection.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DAYS AFTER VIRUS INFECTION

increase in titer during the same period.

Cytopathic changes of BPI-3 infected BAM were pronounced. Morphological changes included giant and spindle cell formation, cytoplasmic streaming, cellular detachment and extensive cell lysis. Alveolar giant cell formation is a characteristic histological lesion observed in BPI-3 infected calves (20). These changes are generally not observed in cultured murine macrophages infected with Sendai virus (1, 12); however, giant cell formation and some degeneration was observed when 50 EID_{50} per cell was inoculated onto monolayers of murine macrophages (12).

Fluorescent antibody results in this study are in contrast with those obtained with Sendai virus infected murine macrophages. Our results show that 1 to 5% of the BPI-3 inoculated BAM were FA positive by day 1 and 95% were FA positive by day 3, an indication that secondary replication of BPI-3 in BAM. This is in contrast to the observations of Mims (12), who reported that infected murine macrophages were FA positive, but the percent of positive cells did not increase beyond the percent recorded at 24 to 48 hours. This indicated that no secondary replication of Sendai virus occurred in murine macrophages.

The results of BAM phagocytosis assays (Fig. 5) may be interpreted two different ways. Phagocytic values of viral

infected BAM that remained adherent to glass were not significantly different from those of the control macrophages. Since most of the infected BAM that remain adherent to glass after day 3 were shown to be virus infected by FA, it appeared that virus infection did not significantly affect the attachment or phagocytosis of C. glabrata. However, when the number of infected BAM available for assay were taken into consideration (normalization), differences in phagocytic values were significant ($P<0.01$) at days 5 and 7. This result should be expected as BPI-3 replicates in BAM these cells are lysed and detach from the glass. This obviously reduces the number of BAM available for phagocytosis. One might speculate that the raw phagocytic values (non-normalized data) would be representative of what occurs in vivo because the bovine lung is a dynamic system, i.e. replacement macrophages are readily available to replenish those destroyed by BPI-3. If this speculation is correct and one compares the results of the BPI-3-BAM in vitro system to the Sendai virus, murine macrophage in vivo system differences become apparent. Phagocytic values in the murine system were significantly reduced as the infection progressed (5, 23, 24) and there appeared to be an inverse relation between the percent of FA positive cells and the percent of phagocytic cells (25). This inverse relationship between the percent of DFA positive BAM and the percent of phagocytic BAM did not hold

in the BPI-3-BAM *in vitro* system.

Phagosome-lysosome fusion is one of the components of the intracellular microbicidal process that has been used to measure viral induced macrophage dysfunction (5, 13, 14). The data in this study indicate significant inhibition of phagosome-lysosome fusion of virus infected vs. control BAM. Fusion values of BPI-3 infected macrophages were 51% and 53% of control cells at days 4 and 6. Normalization of these values reduced the fusion rate to 37% of control BAM. It is interesting to note that giant cells were fully functional with regard to phagocytosis and phagosome-lysosome fusion. This indicates that early in the infection, BPI-3 will induce membrane fusion without affecting macrophage function. A phagosome-lysosome fusion defect has been described by Jakab (5) in the Sendai virus-murine alveolar macrophage system. In other studies of this defect, it was determined that extracellular lysosomal enzyme activity in lung fluids increased while macrophage lysosomal enzymes decreased (24), and misdirected release of lysosomal enzymes resulted in cytolysis of the alveolar macrophages (18).

Synergism has been demonstrated between Sendai virus and *Pasteurella pneumotropica* in the respiratory tract of mice (3). A similar synergistic effect could occur with BPI-3 and *Pasteurella sp.* resulting in bacterial pneumonia (6). *In vitro*

investigations with alveolar macrophages have dealt primarily with P. hemolytica (10, 11, 22) the organism most responsible for severe respiratory disease in cattle. Investigations of viral-bacterial interactions in bovine alveolar macrophages have not been reported. Data presented in this report indicate that although phagocytosis is not significantly impaired in BPI-3 infected BAM these macrophages are unable to efficiently process the ingested yeast. Extrapolation of these in vitro data to spontaneous respiratory disease would suggest that BPI-3 infection of calves could result in significant impairment of the ability of alveolar macrophages to deter secondary bacterial infection.

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0.000 *

10 X
75.57 =
1 300.200 *

10 X
0.75 =
13.500 *

10 X
9.14 =
104.520 *

10 X
2.00 =
36.000 *

1 300.200 +
13.500 +
104.520 +
36.000 +
150.000 + health ins.
35.000 +
75.000 + foreign student fee
1.500 +
1 835.780 *

9 months Tuition + fe

1 835.780 +
54.840 +
12.000 +
4.500 +
153.420 +
2 360.540 *

12 months Tuition + fees

0.000 *

9 X
372.50 =
3 352.500 *

— Liv
Exp
— O

12 months living expenses

3 352.500 +
1 117.500 +
4 470.000 *

9 X
35 =
315.000 *

0.000 *

0.000 *

Other

315.000 +
105.000 +
420.000 *

3 352.500 +
315.000 +
1 835.780 +
5 503.280 *

— To
9 M

Total 12 months

2 360.540 +
4 470.000 +
420.000 +
7 250.540 *