INFECTION AND IMMUNITY, Mar. 1980, p. 960-968
0019-9567/80/03-0960/09$02.00/0

Vol. 27, No. 3

Alveolar Macrophage Ingestion and Phagosome-Lysosome Fusion Defect Associated with Virus Pneumonia

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Virus-induced suppression of pulmonary phagocytic defenses is associated with defects in the intracellular processing of bacteria by alveolar macrophages. To determine whether the intracellular defect is related to a failure in phagosome-lysosome fusion, mice were infected with a sublethal dose of Sendai virus, and the capacity of phagocytic cells, obtained by lung lavage, to exhibit phagosome-lysosome fusion was quantitated during the course of the viral infection. Lysosomes of alveolar macrophages were prelabeled with acridine orange, the cells were challenged with *Candida krusei*, and fusion was determined with fluorescence microscopy by the discharge of the dye into the yeast-containing phagosome. Ultrastructural cytochemical studies verified the validity of the fluorescent fusion assay. Simultaneous experiments were performed to determine whether the viral infection also suppressed phagocytic ingestion by alveolar macrophages. Phagosome-lysosome fusion was progressively inhibited during the viral infection, reaching a low at day 7 when only 13 ± 3% of the phagocytic cells fused as compared with 97 ± 3% in cells from uninfected control animals; respectively, 55 ± 5% as compared with 74 ± 2% of the phagocytic cells contained yeasts. Thereafter, phagosome-lysosome fusion progressively increased reaching near normal levels (92 ± 3%) on day 17 of the infection. At the same time period, phagocytic uptake was enhanced to a level where 97 ± 3% of the cells contained yeasts. These data demonstrated that virus-induced suppression of intrapulmonary killing of bacteria involves functional lesions that retard the ingestion of inhaled organisms by alveolar macrophages and inhibit intracellular processing by degradative lysosomal enzymes by interfering with phagosome-lysosome fusion.

Pulmonary virus infections are known to predispose to bacterial pneumonia (8, 14). The mechanisms of these viral-bacterial interactions have been studied in a model of murine Sendai virus pneumonia (8) which closely resembles that induced by influenza virus infection in humans (6, 13). Quantitative measurements of bactericidal and physical transport mechanisms in the lung during virus pneumonia have demonstrated severe defects in in situ bactericidal mechanisms without alterations in bacterial transport rates, despite histological evidence of destruction of bronchial ciliated epithelium (9). One mechanism identified for the bactericidal dysfunction is a defect in the intracellular processing of the organism (11). This study examines the effect of viral infection on one of the subcomponents of the intracellular phagocytic process, phagosome-lysosome fusion, to relate viral-induced suppression of intracellular killing to abnormalities in phagosome-lysosome fusion by alveolar macrophages.

MATERIALS AND METHODS

Animals. Male Swiss albino mice (CD-1 strain, 18 to 20 g) were used in these experiments. The animals were housed in filter-topped cages and fed food and water ad libitum.

Viral infection. Mice were infected by aerosol inhalation with a sublethal dose of Parainfluenza 1 (Sendai) virus by previously described methods (9).

Collection of free alveolar cells. Virus-infected and noninfected mice were anesthetized with an intraperitoneal injection of sodium pentobarbital and exanguinated by cardiac puncture. The lungs were excised in toto from each mouse and lavaged four times with a total of 5 ml of normal saline. The lavaged cells were centrifuged at 400 × *g* for 10 min and suspended in 1.0 ml of the original lavage fluid. An 0.5-ml portion (containing approximately 2.5 × 10^5 cells) of the suspension was pipetted into each of two chambers of a four-chamber Lab-Tek tissue culture chamber slide for the phagosome-lysosome fusion assay, whereas the remaining 0.5-ml portion was pipetted into a chamber of a separate culture slide for the phagocytic-index assay. Adherent cells were allowed to settle and spread on the glass surface at 37°C for 40 min.

Phagosome-lysosome fusion assay. Fusion patterns in cells from virus-infected and noninfected lungs were visualized by dark-field fluorescent microscopy by a modification of the methods described by Hart and Young (5). Forty minutes into the incubation

period, 20 μl containing 1 μg of freshly prepared acridine orange solution was added, respectively, to each of the cell chambers. After tilting the chamber slides several times to mix chamber content, the slides were reincubated for another 20 min. Thereafter, each chamber was washed four times with 0.4 ml of prewarmed normal saline to remove the nonadherent cells and excess acridine orange.

After being washed, the monolayers were challenged in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered medium 199 plus 10% fetal calf serum (tissue culture medium [TCM] 199 + S) with a suspension of *Candida krusei*. The yeast was grown overnight in Trypticase soy broth, washed two times with normal saline, and suspended in the tissue culture medium to a concentration of approximately 2×10^7/ml; 0.3 ml of this suspension was added to each cell culture chamber, giving a ratio of yeast to alveolar macrophages of about 20:1. After incubation for 1 h at 37°C, the chambers were washed four times with prewarmed saline to remove the excess yeasts, and the slides were wet mounted with a cover slip and examined with a Zeiss fluorescent microscope at 1,000× magnification. Phagosome-lysosome fusion patterns were quantitated by randomly selecting and counting 50 macrophages containing intracellular *C. krusei* and determining whether the prelabeled lysosomes had fused with the yeast-containing phagosomes as previously described (5). Controls for the inhibition of phagosome-lysosome fusion were performed with the "lysosomotropic" drug suramin previously demonstrated to inhibit the normal fusion process (5). Suramin (250 μg in 20 μl) was added to the culture chambers containing the lavaged cells from noninfected mice 10 min after the addition of acridine orange and removed from the monolayers with the dye. In all other respects, the fusion assay was identical to suramin-untreated cells.

For the ultrastructural cytochemical studies of phagosome-lysosome fusion, the macrophages were suspended in TCM 199 + S and challenged with viable *C. krusei* for 1 h at 37°C. The cells were then pelleted at 200 × *g* and fixed with 2% glutaraldehyde, postfixed with osmium and processed for acid phosphatase cytochemistry with a modified Gomorri technique (1) utilizing beta-glycerophosphate as a substrate and lead acetate as an electron-dense coupler.

Phagocytic index assay. Cell cultures were treated identical to those in the fluorescent fusion assay with the exception that acridine orange was not added to the chambers. After the excess yeasts were washed from the monolayers, the slides were air dried, fixed with 100% methanol, stained with Wright-Giesma stain, and examined. The stained preparations were examined under the light microscope at 1,000× magnification, and the percentage of cells containing yeasts was recorded. One hundred phagocytic macrophages were also scored by the total number of *C. krusei* associated with them.

RESULTS

Patterns of phagosome-lysosome fusion with normal murine alveolar macrophages were similar to those described with murine peritoneal macrophages by Hart and Young (5) (Fig. 1a–e). Briefly, the orange-fluorescing lysosomal granules assembled around the unstained yeast cells. Next, these periphagosomal granules disappeared and a bright orange confluent rim of fluorescence appeared around the individual yeasts, indicating the discharge of lysosomal content into the phagosomes. Gradually, the dye permeated into the yeast cells, coloring the whole organism a vivid green. Over the next 30 min the intensity of the green fluorescence faded until, sometime later, dark holes were seen, suggesting the degradation of the yeast by the lysosomal enzymes. These stages overlapped, since after 1 h of incubation of the yeasts and the alveolar macrophages periphagosomal lysosomes, fluorescent rims and colored yeast were all observed.

Suramin did not interfere with the uptake of the yeast cells by alveolar macrophages; 1 h after the start of ingestion, periphagosomal lysosomes were predominant, with fluorescent rims and colored yeast being inconspicuous (Fig. 1f). Concurrent controls showed the normal fusion pattern.

Acridine orange-stained alveolar macrophages from uninfected mice appeared homogeneous when compared with the lung cell population from virus-infected animals. The normal macrophages varied in size; however, the range of these sizes varied only by approximately twofold. Such macrophages contained a distinct pale green nucleus and many small, round, orange-fluorescing lysosomal granules; 97 ± 3% of the ingested *C. krusei* were fused in these phagocytes.

One day after Sendai virus infection, polymorphonuclear leukocytes (PMNs) appeared in the lavaged-cell population. The multilobed nucleus of these cells was also pale green, but with less intensity in the coloration than the nucleus of the alveolar macrophages. The cytoplasm contained a few orange-staining lysosomal granules which, at this time period, readily fused with the ingested yeast cells. However, the brightness of green-colored *C. krusei* was limited and difficult to discern from the individual lobes of the nucleus. Furthermore, the intracellular yeasts that had not fused were also difficult to separate visually from the convolutions of the nucleus of the mature PMNs or the center of a doughnut-shaped nucleus of immature PMNs. Because of these difficulties in quantitating phagosome-lysosome fusion in PMNs, the fusion patterns of these phagocytic cells were only observed for qualitative changes throughout the viral infection.

Although few morphological changes were observed in the alveolar macrophage population on day 1 of the virus infection, major changes

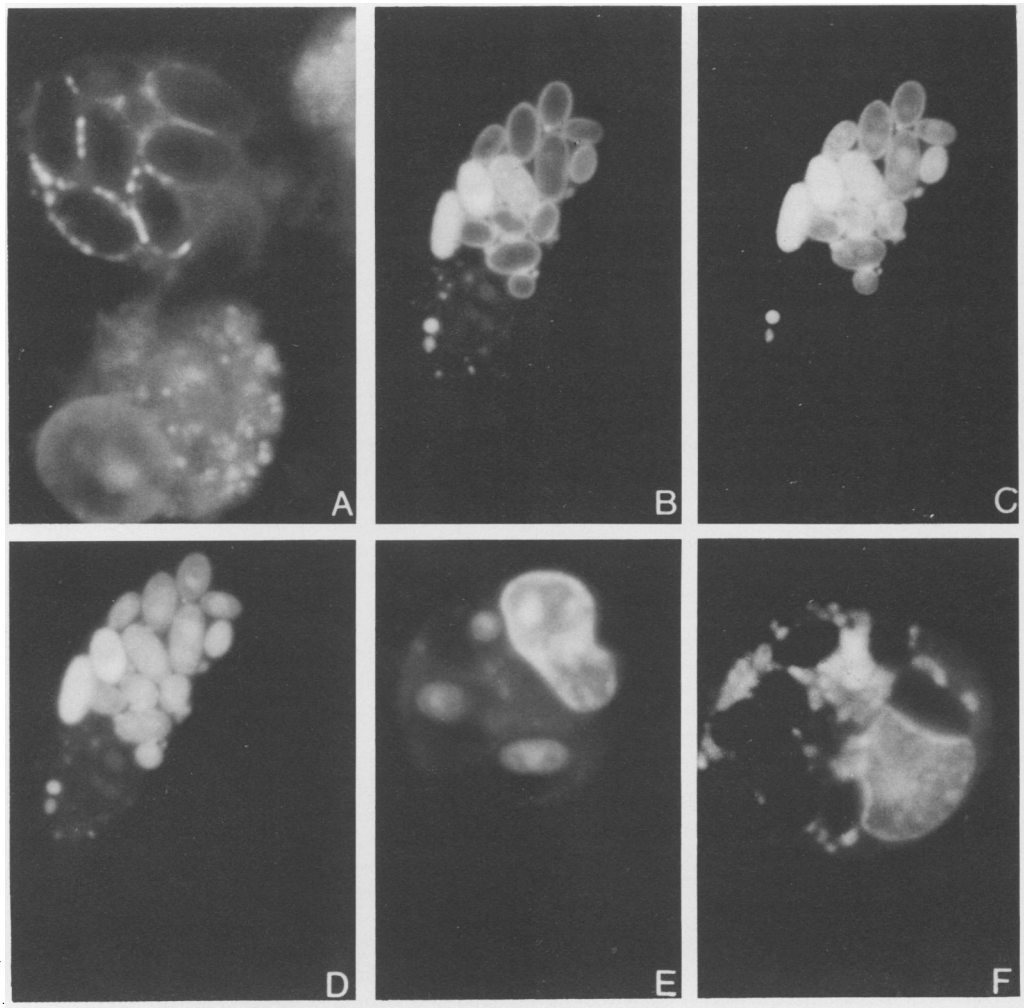

FIG. 1. *Viable alveolar macrophages with acridine orange-stained lysosomes after incubation with live C. krusei. The normal pattern of macrophage response includes: the orange-fluorescing lysosomal granules assembling around the unstained yeasts (A); a vivid orange confluent rim of fluorescense around the individual yeasts (B); and the dye permeating into the yeast cells (C), coloring the whole organism green (D). Since there is considerable overlap in the normal fusion pattern, the more brightly staining yeasts in B, C, and D have most likely been ingested earlier. Thirty minutes after incubation (E), the coloration of the yeasts is fading, and the phagolysosomal membrane appears loose around the organism. Inhibition of the normal fusion process by suramin is shown in F.*

occurred on day 3. At this time period, the intracytoplasmic orange granules no longer appeared small and round but were larger and pleomorphic. Some of the cells themselves were enlarged to approximately three to four times the size of normal alveolar macrophages. Such large cells aggregated in clumps and readily ingested the yeasts, but phagosome-lysosome fusion was not observed in these phagocytes. In addition to the large alveolar macrophages, smaller mononuclear cells were also observed. These cells, approximately the size of a macrophage from an uninfected lung, contained a pale green nucleus and large, round, canary yellow-staining granules. Single and double green-colored yeasts were observed primarily in these monocytes (Fig. 2a) which were included in the tabulation with the more familiar alveolar macrophages. Phagosome-lysosome fusion patterns were observed in the ever-increasing number of PMNs.

Five days into the viral infection, the large aggregating alveolar macrophages were still nonfusion, whereas the smaller mononuclear phag-

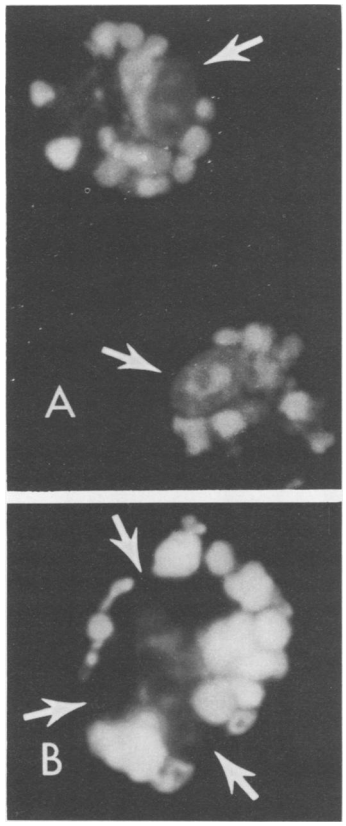

FIG. 2. *Mononuclear phagocytes with large canary yellow-staining intracytoplasmic granules. Cells were lavaged from lungs on days 3 (A) and 7 (B) of the viral infection. Note the lightly stained yeast particles (A) and the unstained yeasts (B).*

ocytes, first observed on day 3, contained numerous colored yeasts. Some colored yeasts were also observed on occasion in PMNs; however, the majority of PMNs appeared to be degranulated since very few of them contained any orange-staining granules.

Two days later, at the height of the virus-induced inhibition of phagosome-lysosome fusion, when only 13 ± 3% of the intracellular yeasts showed the normal fusion pattern, the morphological picture was as follows: (i) most of the cell population consisted of the smaller mononuclear cells containing uncolored yeasts (Fig. 2b); (ii) the orange-staining granules in the alveolar macrophages appeared to have increased in size since day 3; such cells primarily contained unstained yeasts (Fig. 3); and (iii) PMNs were plentiful; however, very few of them contained any intracytoplasmic orange granules. At this time period, <20% of the phagocytic macrophages showed the normal fusion pattern.

Gradually, from day 9 of the viral infection through the end of the assay period (day 17), fewer of the PMNs and mononuclear cells were observed. On the other hand, the proportion of alveolar macrophages increased, and the orange-staining granules became smaller and rounder with time. At the later time intervals, a proportion of the alveolar macrophages were consistently larger and more spread out on the surface of the chamber slide. Such cells consistently contained more colored yeasts than did the smaller alveolar macrophages.

The cytochemical studies of the phagosome-lysosome fusion process are presented in Fig. 4, 5, and 6. In these micrographs, the acid phosphatase reactivity products appear as dark granules in the lysosomes and within phagolysosomes, indicative of phagosome-lysosome fusion (Fig. 4). Three days after viral infection, acid phosphatase could not be found in the yeast-containing phagosomes of a large number of macrophages (Fig. 5). Those macrophages obtained during the day 7 of the viral infection also showed no evidence of phagosome-lysosome fusion (Fig. 6).

Quantitated fluorescent fusion patterns in free lung cells (less the PMNs) obtained during the course of the viral infection are presented in Fig. 7. The number of ingested yeasts that were not fused increased with time, reaching a peak at day 7 when 87 ± 3% of the intracellular yeasts were colorless. Thereafter, the fusion patterns gradually returned to normal until, at day 17, only 8 ± 3% of the yeasts had not fused.

The percentage of phagocytic alveolar macrophages and the total number of *C. krusei* associated with 100 phagocytic cells is presented in Fig. 8. In the uninfected control animals, 74 ± 2% of the lavaged alveolar macrophages were phagocytic, each containing approximately two yeasts.

During days 1 and 3 of the viral infection, both quantitated parameters increased slightly; however, only the increase in the percentage of phagocytic macrophages was significant ($P < 0.05$). At day 5, the values declined, reaching a low on day 7, when only 50 ± 5% of the alveolar macrophages were phagocytic and had ingested only one yeast cell. Thereafter, from day 9 of the viral infection through the end of the assay period on day 17, a progressive increase in phagocytosis and the total number of ingested *C. krusei* was observed; at day 17, 90 ± 2% of the cells were phagocytic, containing 428 ± 27 yeast cells per 100 alveolar macrophages.

DISCUSSION

Bacterial multiplication associated with virus infections is related to defects in in situ bacteri-

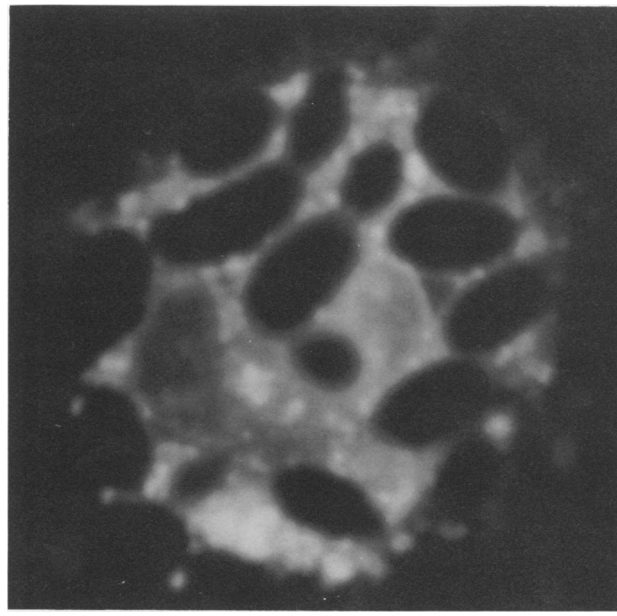

FIG. 3. *Alveolar macrophage lavaged from the lungs during day 7 of the viral infection. This actively phagocytic cell contains numerous unstained yeast particles in its cytoplasm. Note the smaller, intracytoplasmic granules (orange) as compared with the larger granules (canary yellow) observed in the phagocytic cells in Fig. 2.*

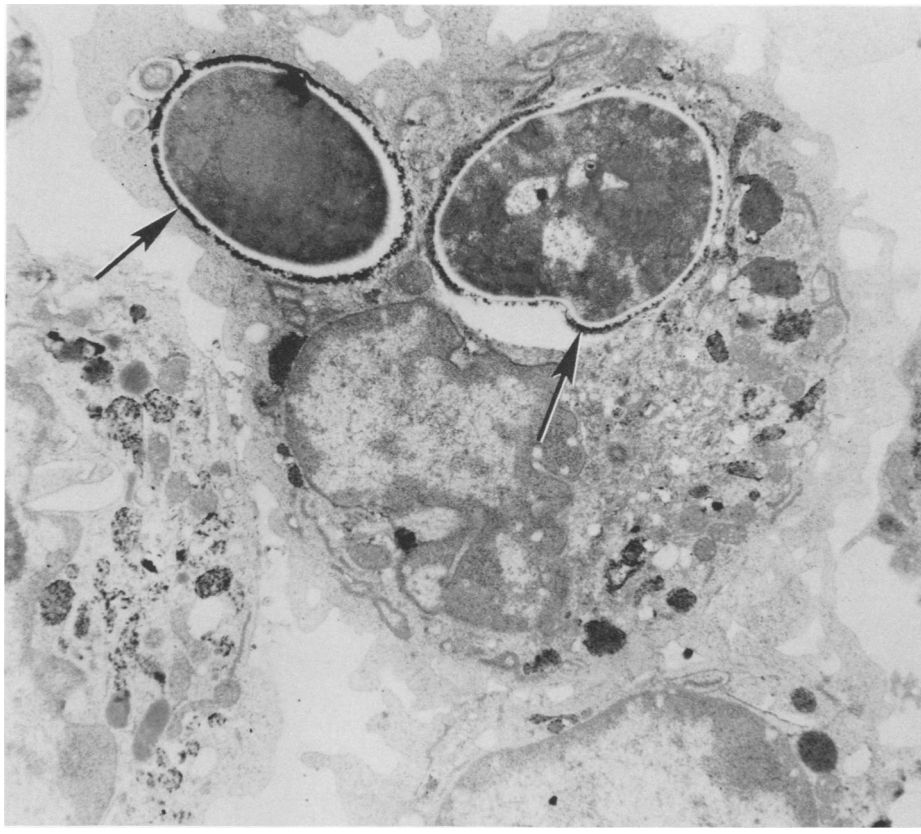

FIG. 4. *Electron micrograph of a lung macrophage from a normal, noninfected mouse challenged in vitro with C. krusei for 1 h. The macrophage contains two ingested yeast cells, both of which are surrounded by electron-dense acid phosphatase reaction product (arrows), indicating that lysosomes have fused with the phagosomes.*

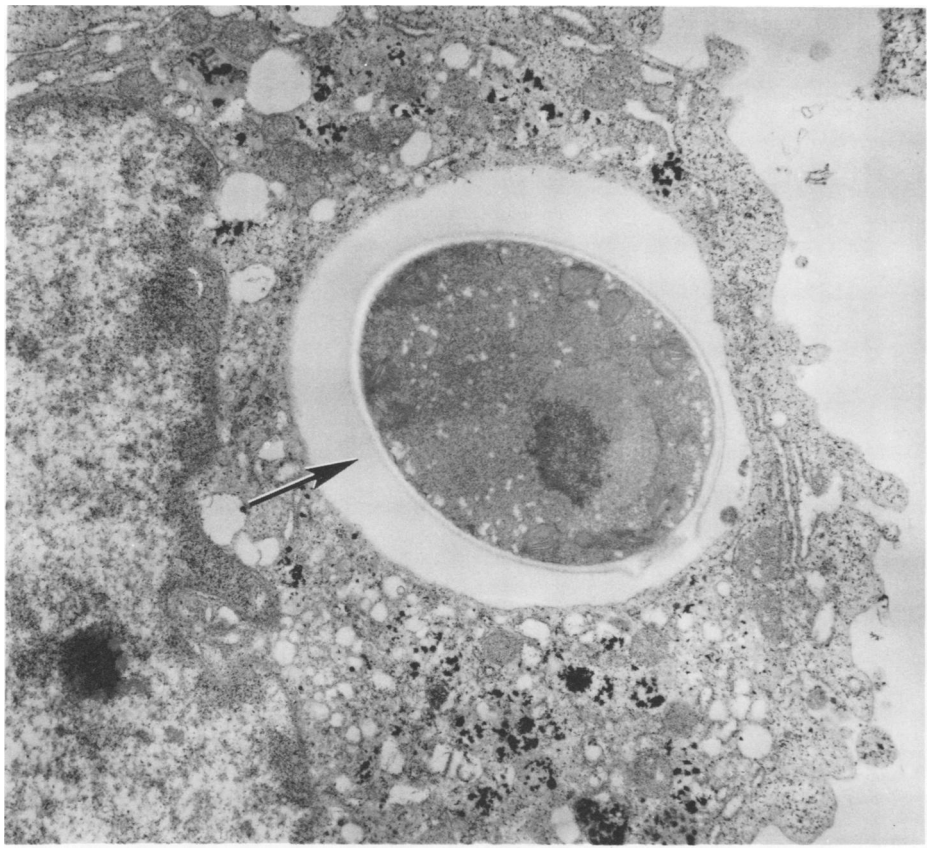

FIG. 5. *Lung macrophage from a mouse infected 3 days earlier with Sendai virus. The phagosome (arrow) surrounding the ingested yeast cell is devoid of any acid phosphatase reaction product, indicating the absence of lysosome-phagosome fusion.*

cidal (phagocytic) mechanisms of the lung (9). During the period of maximal suppression of pulmonary antibacterial activity, the alveolar macrophages in virus-infected lungs are capable of ingesting the bacteria, but intracellular processing of the organisms is severely depressed (11). The experiments herein examined a subcomponent of the intracellular process, phagosome-lysosome fusion, to determine whether the viral-induced defect was related to an inhibition of the fusion process. In addition, the engulfment phase of the phagocytic process was also examined to document alterations in the capacity of the cells to ingest bacteria. Finally, to obtain a more complete understanding of the virus-induced bactericidal defect, the two phagocytic parameters were examined throughout the course of the viral infection.

The data clearly demonstrate that dynamic events occur in the lung during the course of the virus infection as reflected by the lavaged phagocytic cell population. Quantitative studies (G. A. Warr, and G. J. Jakab, manuscript in preparation) have shown a 10-fold and a 500-fold increase in the macrophage and neutrophil populations lavaged from the lungs during the first 7 days of the viral infection. Herein, qualitative changes were also observed in this heterogeneous cell population. Early in the infection, when the alveolar macrophages began to lose their fusion capacity, a rapid influx of fusion-competent polymorphonuclear leukocytes was observed, followed shortly thereafter by fusion-competent mononuclear phagocytic cells. Then, on day 7 of the infection, during the period of maximal suppression of pulmonary bactericidal activity, all three of the pulmonary phagocytic cell types exhibited little or no phagosome-lysosome fusion. With the resolution of the viral infection, the alveolar macrophage population slowly regained their normal fusion capacity. Concurrently, fewer of the polymorphonuclear leukocytes and monocytes were recovered from the lungs. These observations indicate that com-

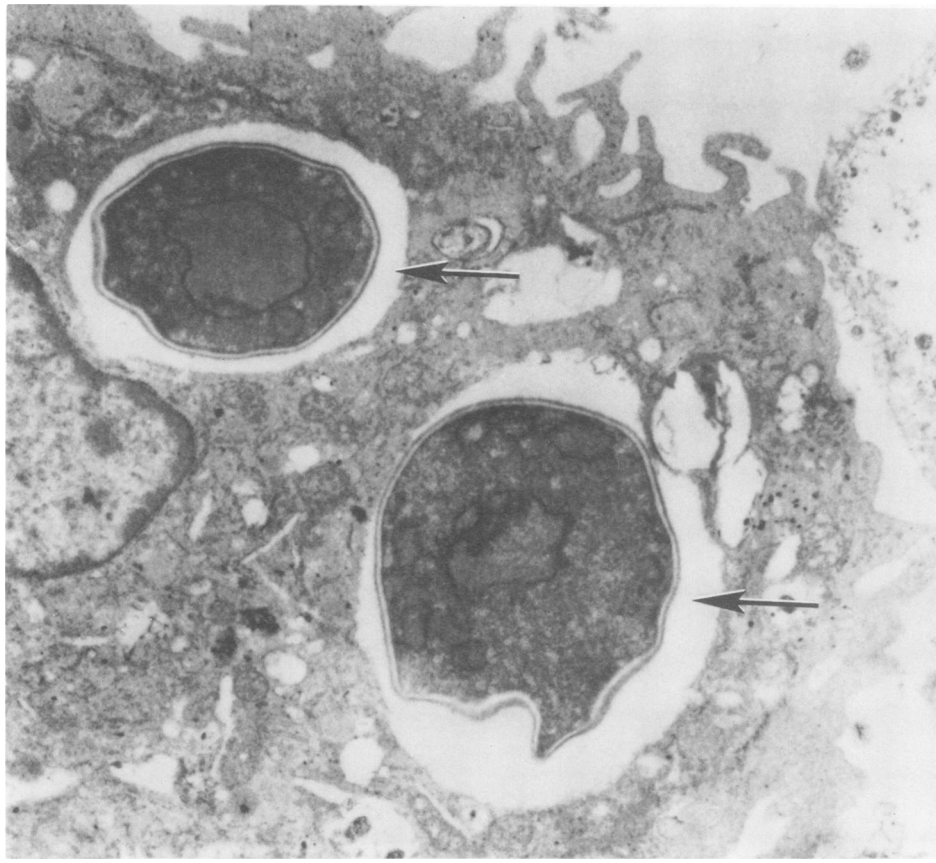

FIG. 6. *Lung macrophage from a mouse infected 7 days earlier with Sendai virus contains two ingested yeast cells. The phagocytic vacuoles surrounding both yeast cells (arrows) are devoid of acid phosphatase reaction product.*

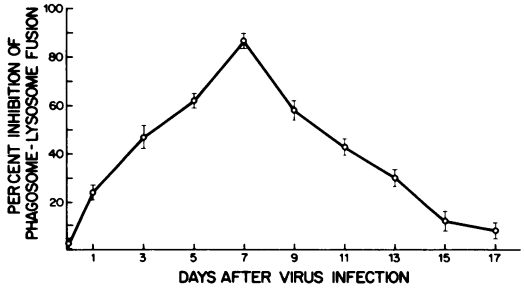

FIG. 7. *Phagosome-lysosome fusion pattern in phagocytic cells obtained by lung lavages during the course of the viral infection. Each point represents the mean ± standard error of 8 to 10 individual determinations.*

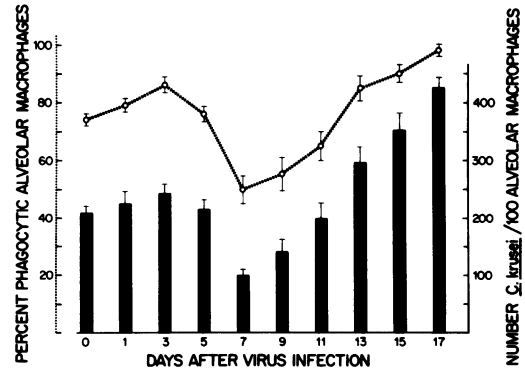

FIG. 8. *Engulfment of C. krusei by phagocytic cells obtained by lung lavage during the course of the viral infection. Each point represents the mean ± standard error of 8 to 10 individual determinations.*

pensatory phagocytic cells are recruited into the lungs during the initial course of the viral infection. These early phagocytes appear to be functionally competent, as measured by their ability to ingest cells and by the ability of their lysosomes to fuse with the yeast-containing phagosome. This latter intracellular process is then suppressed as the viral infection progresses.

The phagocytic monocytes with the "canary yellow" lysosomal granules were included in the tabulation of both ingestion and phagosome-lysosome fusion experiments. These cell types were not distinguishable from the alveolar macrophages with the Wright-Giesma stain used to quantitate phagocytic uptake. However, these monocytes differed prominently from the resident alveolar macrophages under dark-field fluorescence. The identity of these phagocytic mononuclear cells is not known, but it may represent blood monocytes entering the lung during the inflammatory process.

Ingestion of yeast cells by alveolar macrophages fluctuated both qualitatively and quantitatively during the course of the viral infection. An initial enhancement during the first 3 days was followed by a precipitous drop by day 7, not only in the number of alveolar macrophages that had ingested *C. krusei*, but also in the number of yeasts within these phagocytic cells. Thereafter, the percentage of phagocytic macrophages surpassed non-virus-infected control levels (74 ± 2%), reaching almost 100% at day 17. As the percentages of phagocytic alveolar macrophages increased, the total number of yeast cells engulfed far exceeded the number of noninfected controls. This is not obvious from the data presented in Fig. 8, since the quantitated categories of yeasts per phagocytic macrophage only included 1, 2, 3, 4, and >5. This set a limit of 500 *C. krusei* per 100 phagocytic macrophages. In reality, the majority of macrophages were engorged with yeast cells, making the yeast-to-macrophages ratio artificially low after day 11 of the infection. The morphological observations suggest that these macrophages were stimulated (12).

These fluctuations in phagocytic engulfment during the course of viral infection have been suggested by the data of others. Three days after experimental infection of guinea pigs with influenza virus, lavaged alveolar macrophages ingested bacteria at an enhanced rate as compared with noninfected control animals, even though the virus-exposed macrophages exhibited reduced intracellular killing (18). During day 5 of murine influenza, in vivo quantification of phagocytic function demonstrated an impairment in the ingestion of bacteria by macrophages with a concomitant proliferation of the organism within the macrophages (20). Finally, blood neutrophils from influenza patients showed enhanced bacterial ingestion rates 2 weeks after the acute phase of the disease (16).

In this study, the initial enhancement of phagocytic uptake by alveolar macrophages from animals with viral pneumonia reflects, in all probability, the additional defense capabilities recruited into the lungs by the inflammatory process. The subsequent enhancement is presumably associated with acquired immune mechanisms which augment macrophage phagocytic activity (7, 10, 12, 15).

The results clearly demonstrate that phagosome-lysosome fusion is progressively depressed, with peak inhibition correlating in time with maximal viral-induced suppression of pulmonary bactericidal activity. These studies were performed with macrophages lavaged from virus-infected lungs and challenged in vitro with *C. krusei*. An in vivo phagosome-lysosome fusion in virus-infected lungs against *Staphylococcus aureus* has also been recently demonstrated (17) corroborating the observations herein. Since a virus-induced in vivo inhibition in the intracellular processing of ingested bacteria has been documented (11, 18), the results herein might be interpreted as an explanation for the phagocytic defect. Suppression of phagosome-lysosome fusion would prevent the hydrolytic lysosomal enzymes to come in contact with the ingested organism (2–4), resulting in dysfunctions of intracellular processing (17, 19).

ACKNOWLEDGMENTS

This work was supported by NHLBI grants HL 16990 and HL 14212 (SCOR). G.J.J. is the recipient of an NHLBI Research Career Development Award (HL 00415). G.A.W. receives support from an NHLBI Research Fellowship award (HL 05405).

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