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SUBHUMAN PRIMATE MODEL FOR THE STUDY OF INFECTION INDUCED BY 'K--ETC(U)
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Squirrel monkeys were instilled intratracheally with 700 Klebsiella pneumoniae organisms and developed lobar pneumonia in about 24 h. Characteristic clinical findings were fever, anorexia, and coughing. Laboratory findings included leukocytosis or leukopenia, with the latter more prominent in ultimately fatal infections; bacteremia and shedding of bacteria into the pharynx. Infected monkeys showed increased plasma lysozyme activity as well as increased plasma ceruloplasmin, haptoglobin and α_1-antitrypsin. Mortality rate was 60%; mean

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time to death was 50.5 h. Pathologically, the disease spread by means of the pores of Cohn and other pathways that generally did not involve airways as a means of dissemination until about 30 h. The squirrel monkey seems to be a better model for human respiratory K. pneumoniae infection than does the rat or mouse.

Subhuman Primate Model for the Study of Infection Induced by *Klebsiella pneumoniae*

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Running head: *K. PNEUMONIAE* IN MONKEYS

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on the Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

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ABSTRACT

Squirrel monkeys were instilled intratracheally with 700 Klebsiella pneumoniae organisms and developed lobar pneumonia in about 24 h. Characteristic clinical findings were fever, anorexia, and coughing. Laboratory findings included leukocytosis or leukopenia, with the latter more prominent in ultimately fatal infections; bacteremia and shedding of bacteria into the pharynx. Infected monkeys showed increased plasma lysozyme activity as well as increased plasma ceruloplasmin, haptoglobin and α_1-antitrypsin. Mortality rate was 60%; mean time to death was 50.5 h. Pathologically, the disease spread by means of the pores of Cohn and other pathways that generally did not involve airways as a means of dissemination until about 30 h. The squirrel monkey seems to be a better model for human respiratory K. pneumoniae infection than does the rat or mouse.

Mice and rats are the experimental animals most commonly employed for the study of respiratory Klebsiella pneumoniae infection (1, 3, 10). Clinical and biochemical studies are difficult to carry out in these animals, however, because of their small size. Recently, we have found that squirrel monkeys (Saimiri sciureus) developed pneumonia after intratracheal instillation of Streptococcus pneumoniae (2), and also were susceptible to K. pneumoniae administered by this route (4). Therefore, in anticipation of studies of aerosol therapy, we have carried out experiments designed to characterize the squirrel-monkey model in greater detail. This report includes clinical, histopathological, and selected biochemical observations.

MATERIALS AND METHODS

Test organism. Techniques for growing, storing and enhancing the virulence of the A-D strain of type 1 K. pneumoniae have been described previously (3). Inocula (1.5 ml) for the infection of monkeys contained 600 to 800 viable organisms and were prepared as previously described (4).

Test animals. Healthy, juvenile, male squirrel monkeys weighing from 0.5 to 1.0 kg were used. They were housed individually in wire-bar cages and allowed free access to commercial monkey chow and water. Their diet was supplemented with fresh fruit several times weekly. During experiments, fruit was eliminated and each monkey was limited to 6 biscuits daily to facilitate estimation of food consumption.

Intratracheal inoculation (i.t.). This method of infecting monkeys has been described previously (4). The dose administered, 700 organisms, was determined to be approximately one LD_{50} (4).

Preparation of samples for histological examination and estimation of bacteria concentration in tissue. In a separate experiment to determine histopathology, a blood sample was obtained, monkeys were killed by intravenous injection of pentobarbital, and the abdominal and thoracic cavities were opened. For estimation of bacterial concentration, samples of selected organs were removed aseptically, weighed, homogenized in trypticase soy broth (TSB) and brought to a final volume of 4.0 ml. Appropriate dilutions were made in TSB and plated on trypticase soy agar. After incubation, colonies were counted and concentrations calculated. The remaining portion of the organs were fixed with 10% neutral buffered

formalin, cut in 6-μm sections and stained with hematoxylin and eosin or Brown and Hopps stains. Sections were then examined by light microscopy.

Clinical determinations. Once daily, 0.75 ml blood was obtained from the saphenous vein for determination of total and differential leukocyte concentration, hematocrit, and bacteremia. Bacteremia was determined by spreading 0.1 ml of blood on the surface of a trypticase soy agar plate, incubating for 18 h at 37 C and counting colonies produced. Additional daily determinations, or observations, included rectal temperature, respiratory rate, pharyngeal swabbing for Klebsiella, weight, activity, sneezing or coughing, dyspnea, and food consumption. Three base-line determinations were made on each of the above parameters, and testing continued for 8 days after infection. The volume of the daily blood sample was increased to 1.5 ml on two separate days prior to infection as well as on days 1, 2, 3, 6 and 8 for the determination of plasma lysozyme, α_1-antitrypsin, ceruloplasmin, and haptoglobin. Prior experimentation indicated that this bleeding schedule would cause a 20% drop in hematocrit value and no other changes. Lysozyme activity was measured by the method of Osserman and Lawlor (8). The concentration of three other serum proteins was estimated by radial immunodiffusion procedures using kits obtained from Behring Diagnostics (American Hoechst Corp., Somerville, NJ). Although these kits were designed for determination of human proteins, preliminary investigation showed sufficient cross-reactivity with rhesus, cynomolgus, and squirrel monkeys

to permit assay of proteins in these species.

Experimental design. Sixteen monkeys were infected and serially killed in groups of 4 at 6, 24, 30 and 48 h for histopathological examination and estimation of tissue bacterial concentrations. Three groups of 5 monkeys each were infected after three base-line determinations and then were assessed for 8 days to evaluate clinical course of illness and mortality. Simultaneously, three groups of 5 monkeys each were sham-inoculated with sterile trypticase soy broth. The results of replicate experiments were not different for each of the parameters under investigation so the data from the three groups were combined.

RESULTS

Histological and bacteriological studies. The concentration of K. pneumoniae in selected tissues at various times after infection is presented in Fig. 1. The 6-h period was arbitrarily chosen as a period early in infection; at 24 h clinical signs were just becoming apparent, and at 30 h illness was clearly visible. The 48-h time was chosen because we anticipated that first deaths would occur shortly thereafter. One of the 4 monkeys reserved for the 48-h period died 2 to 3 h early. The bacteriological data are presented in Fig. 1. The data from liver and kidney are not shown because concentrations in these organs were almost identical to those in the spleen at all time periods. The data indicated that bacteria multiplied in the lungs and were carried in small numbers by the blood to other tissues. Of interest was the relatively small number of bacteria isolated from blood even at 48 h. At 24 h a few hundred bacteria were isolated from brain and stomach of each of the 4 monkeys (not shown).

Histological examination of the 4 monkeys 6 h after inoculation with K. pneumoniae revealed minimal to mild lobular alveolitis restricted, in every instance, to a single diaphragmatic lobe. This lesion was characterized by an infiltration of alveolar spaces by a few neutrophils and extravasated red blood cells (Fig. 2A).

Twenty-four hours after inoculation, on gross examination, the 4 monkeys examined had patchy grayish-red areas of consolidation confined to the diaphragmatic lobe on one side. Histologically, the inflammation had progressed to an early moderate lobar pneumonia confined to a single

lobe. Alveolar spaces, ducts, and some respiratory bronchioles in affected areas were filled with edema fluid, fibrin, and a cellular infiltrate composed primarily of neutrophils and a few macrophages (Fig. 2B). Numerous encapsulated gram-negative pleomorphic bacilli that were consistent with K. pneumoniae were present in the edematous exudate (Insert). Interlobular septae were moderately distended with fibrinous fluid and were infiltrated with neutrophils. Lymphatic channels within the septae were moderately dilated.

Monkeys at 30 h after inoculation had total consolidation of one diaphragmatic lobe with patchy areas of involvement in the middle lobes of the same side. Microscopically, these monkeys had a lobar pneumonia that involved the entire diaphragmatic lobe and portions of the middle lobe. The lesion at this time was characterized by an intense outpouring of neutrophils, some macrophages, and a fibrinous edema that gave the lung parenchyma a solid appearance. The lesion was accompanied by foci of alveolar septal necrosis. Interlobular septae were markedly distended by an inflammatory exudate and contained severely dilated lymphatics (Fig. 2C). The exudate continued onto the pleural surface of the affected lobes. In addition to the lobar pneumonia, one monkey in this group had an intense neutrophilic infiltrate within the lumen and walls of bronchioles. The airways in other animals contained variable amounts of exudate, but the walls of these airways were generally uninvolved (Fig. 2D).

The inflammatory process at 48 h after inoculation was qualitatively similar to that seen at 30 h, but more extensive. A lobar pneumonia involving diaphragmatic and middle lobes with patchy areas of inflammation

extending into the apical lobes was seen in 2 of the monkeys.

There was a mild to moderate splenitis in 5 of 12 monkeys observed 24 h or later after infection. Similar splenic changes are reported in rats experimentally infected with K. pneumoniae (3).

A patchy interstitial pneumonia was present in 13 of the 16 monkeys that were necropsied. This lesion was subacute to chronic in nature and was apparently unrelated to the experimental infection. In several monkeys the interstitial involvement was associated with adult Filaroides spp. In only one monkey was K. pneumoniae found in association with this lesion.

Clinical and laboratory observations. All of the 15 noninfected control monkeys survived without overt illness, whereas 9 of the 15 infected animals died (60%) with a mean time to death of 50.5 h.

Illness, first observed at 24 h, consisted of lethargy, anorexia and dyspnea. Signs of illness were pronounced in virtually all monkeys by 30 h. Principal clinical and laboratory findings are presented in Figs. 3 and 4. After infection, significant increase in temperature was observed throughout the experiment with the exception of day 5 (Fig. 3).

The total leukocyte concentration varied widely during this study (Fig. 3); however, the counts obtained from animals that ultimately died were significantly different than those of infected monkeys that survived (Table 1). Monkeys that survived the infection had leukocytosis; those that ultimately died, leukopenia. Virtually all infected monkeys showed marked neutrophilia on day 1 (not shown). This value returned to within normal limits in 2 to 3 days in monkeys that

survived, but relative neutrophilia persisted in those that died.

Klebsiella were isolated from the pharynx of all inoculated monkeys by the second day (Fig. 3). The frequency of isolation declined thereafter and pharyngeal cultures were negative for K. pneumoniae after day 5. Bacteria were isolated from peripheral blood samples from more than 80% of the monkeys 24 h after inoculation and from all monkeys on the third and fourth days. Bacteremia was not detected after day 6. A greater number of bacteria was detected in the blood of monkeys that died than in those that survived (Table 2).

Increased respiratory rates were also characteristic of this infection (Fig. 4), reaching peak values on days 2 to 4 and then slowly declining. All infected monkeys had inspiratory and expiratory dyspnea throughout the test period.

Food consumption and body weight data are also shown in Fig. 4. The loss of weight was more pronounced than the anorexia, possibly indicating that some of the loss of weight was catabolic in origin.

Biochemical reactions. Plots of changes in four plasma proteins are presented in Fig. 5. Unfortunately, the variation between monkeys was very great and statistical analysis often failed to discern differences. When the data was normalized to preinfection base-lines, however, ceruloplasmin seemed to be the protein most affected by infection. Concentrations were significantly higher ($P < 0.05$) on the first day after infection and were still significantly elevated ($P < 0.005$) on day 8. The pattern for α_1-antitrypsin seems to be

about the same as that of ceruloplasmin, but infected animals differed significantly from controls only on days 2, 3 and 6. The values for haptoglobin and lysozyme activity also were quite variable, but differed from controls on days 2 and 3. When the plasma protein values of surviving and dying monkeys were compared, only lysozyme exhibited a significant difference (Table 3), and this difference was seen only at one time period.

DISCUSSION

Intratracheally-instilled K. pneumoniae (700 cells) caused an acute lobar pneumonia in squirrel monkeys. Clinically, the disease was characterized by fever, anorexia, coughing, leukocytosis or leukopenia, bacteremia and shedding of bacteria into the pharynx. The monkeys also showed increased concentrations of plasma α_1-antitrypsin, ceruloplasmin, and haptoglobin as well as increased plasma lysozyme activity. Mortality occurred in 60% of infected animals with a mean time to death of about 50 h.

Pathologically, the disease was characterized by initial multifocal involvement of the alveoli (6 h) in the diaphragmatic lobe that spread to involve major portions of the same lobe by 24 h. The lesion at 24 h was still confined to peripheral tissues with involvement of alveolar spaces, ducts and a few respiratory bronchioles. This observation suggests that initially the disease spread by way of the pores of Cohn and other alveolar duct-bronchiolar pathways including the canals of Lembert rather than by the airways. Inflammatory exudate was not seen in larger bronchioles and bronchi until 30 h. Airways themselves were not involved. It is assumed that spread of pneumonia to adjacent lobes occurred when the inflammatory exudate was either expelled up airways during respiration or moved by mucociliary action. Usually, involvement was unilateral with the disease originating in the diaphragmatic lobes and extending in time to the apical lobe on the same side.

The most important question concerning this work is how well the disease in the model simulates that in humans. Clinically, the lobar form of the disease in humans as described by Julianelle (6) is characterized by abrupt onset, variable fever (frequency low), cough, extensive rusty sputum production, neutrophilia, and bacteremia in about 60% of patients. Extension of infection to other tissues is infrequent. Generally, our clinical and laboratory findings are consistent with these observations. We have not seen sputum production, but this lack may be due to difficulties in observation. Conversely, the observation that 100% of monkeys have bacteremia in contrast to 60% of human patients may be the result of more frequent blood sampling in the monkeys. The association of leukopenia and persistent bacteremia with mortality, as seen in monkeys, has also been reported in humans (6, 7).

When pathological lesions are considered, the similarity between human and simian models is even more striking. In both species, the lesions may be described as lobar pneumonia with severe edema of interlobular septa and distended lymphatic channels. Airways are relatively free of involvement early in the disease in man (5, 11). The principal difference between the two species seems to be the low frequency of abscess formation in the squirrel monkey. Although the necrosis of alveolar septae that is believed to be a prerequisite for abscess formation in man (5, 11) also occurs in the monkey, abscess formation was seen very rarely in our studies, even in monkeys in which the infection had caused death. The absence of abscess formation,

however, may be due to the rapidity with which death occurred in monkeys.

Specific plasma proteins were measured in the expectation of using alterations in concentration as prognostic indices as we have done in the rat (3). Too much variation was encountered among monkeys, however, to permit use of concentration estimates as prognostic indices except with lysozyme. The changes that were observed, however, are consistent with the presence of inflammatory disease; the possible significance of these metabolic changes is discussed in detail elsewhere (9).

Although the squirrel monkey appears to be a good model for acute pneumonia due to K. pneumoniae, the question of the utility of this model compared to the mouse and rat arises. The low price and small size of small rodents is particularly appealing because more animals can be employed and thus statistical analysis can be more readily utilized. However, intranasal injection of organisms into mice and rats results in bronchopneumonia (2, 3) rather than in lobar pneumonia. Intratracheal instillation has also been reported to produce lobar pneumonia in rodents (10). However, the use of gastric mucin as an adjuvant in the rodent study (10) may have had profound effects. The squirrel monkey, in contrast, mimics man in the form that the Klebsiella pneumonia takes, and, in addition, more readily allows the sequential measurement of conventional clinical signs such as fever, appetite, respiratory rate, weight change, blood and throat cultures and hematology in the same animal than does the rodent. Also, the cross reactivity between certain human and monkey plasma proteins not only allows measurement of changes in specific acute phase reactants, as opposed to fractions thereof, but

may, assuming that the right protein or combination of proteins can be found, allow for additional quantitation of the severity of disease and the success of therapy.

With subhuman primate models for lobar pneumonia, we can now more thoroughly study the pathogenesis of such pneumonia, evaluate the effective uses of chemotherapy and devise new approaches to therapy, both antimicrobial and supportive, with more confidence that our findings will be relevant to man.

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TABLE 1. Total leukocyte concentration in dying and surviving monkeys during K. pneumoniae infection.

Day after infection	Leukocytes/mm^3 (x 10^3)		P[a]
	Surviving monkeys (n)	Dying monkeys (n)	
Base-line	6,275 (6)	6,955 (9)	
1	12,633	6,522 (9)	<0.01
2	12,750	2,233 (3)	<0.01
3	8,117	2,900 (1)	
4	7,000	6,500 (1)	

[a] By t-test.

TABLE 2. Bacteremia in K. pneumoniae-infected monkeys

Day after infection	Number of bacteria/ml of blood (geom. mean) Surviving monkeys (n)	Dying monkeys (n)	P[a]
1	3.9×10^1 (6)	5.6×10^2 (9)	<0.05
2	7.4×10^1 (6)	1.5×10^3 (3)	<0.05
3	2.3×10^2 (6)	5.4×10^2 (1)	
4	6.3×10^0 (6)	5.0×10^3 (1)	
5	0 (6)	No survivors	

[a] By t-test, computed on log transformation.

TABLE 3. Lysozyme concentration in K. pneumoniae-infected monkeys

Day after infection	Plasma lysozyme concentration (µg/ml)		P[a]
	Surviving monkeys (n)	Dying monkeys (n)	
Base-line	1.6 (6)	1.8 (9)	
1	2.2 (6)	2.4 (9)	
2	3.4 (6)	5.5 (3)	<0.05
3	2.6 (6)	4.6 (1)	
6	2.8 (6)	No survivors	
8	3.4 (6)		

[a] T-test.

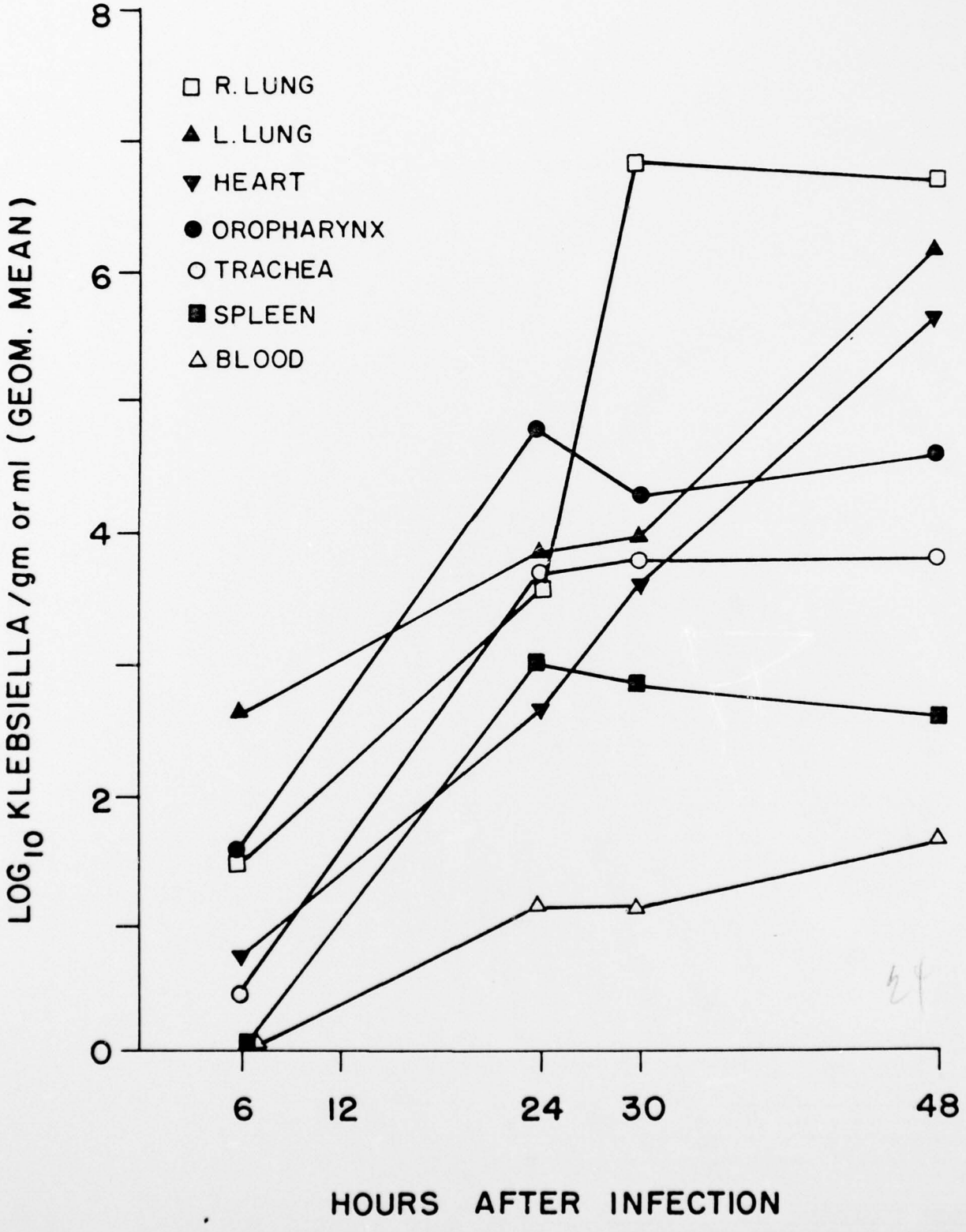
FIG. 1 Concentration of K. pneumoniae in selected tissues at selected times after intratracheal instillation. Values presented are the geometric mean of 4 monkeys at each time.

FIG. 2 (A) Infiltration of alveolar spaces by a few neutrophils and extravasated red blood cells (6 h postinfection) x 190.
(B) Alveolar spaces filled with edema fluid, fibrin, and a cellular infiltrate composed primarily of neutrophils and a few macrophages (24 h) x 190. Inset: Gram-negative bacterial rods within alveolar exudate. x 950.
(C) Interlobular septa markedly distended by fibrinous edema and a neutrophilic infiltrate (24 h) x 190.
(D) Neutrophilic exudate within alveolar spaces and the lumen of a bronchiole without involvement of the wall of the airway (30 h) x 190.

FIG. 3 Rectal temperature of infected and control monkeys.
Vertical bars are the standard errors of the mean and are shown only where significant differences exist.
Middle - Total leukocyte counts as above.
Bottom - Isolation of Klebsiella from blood and throat.

FIG. 4 Top - Respiratory rate in infected and control monkeys.
Vertical bars are standard errors of the mean.
Middle - Appetite as measured by biscuit consumption.
Bottom - Body weight during infection.

FIG. 5 Values of four selected plasma proteins during infection. Standard error bars omitted for clarity.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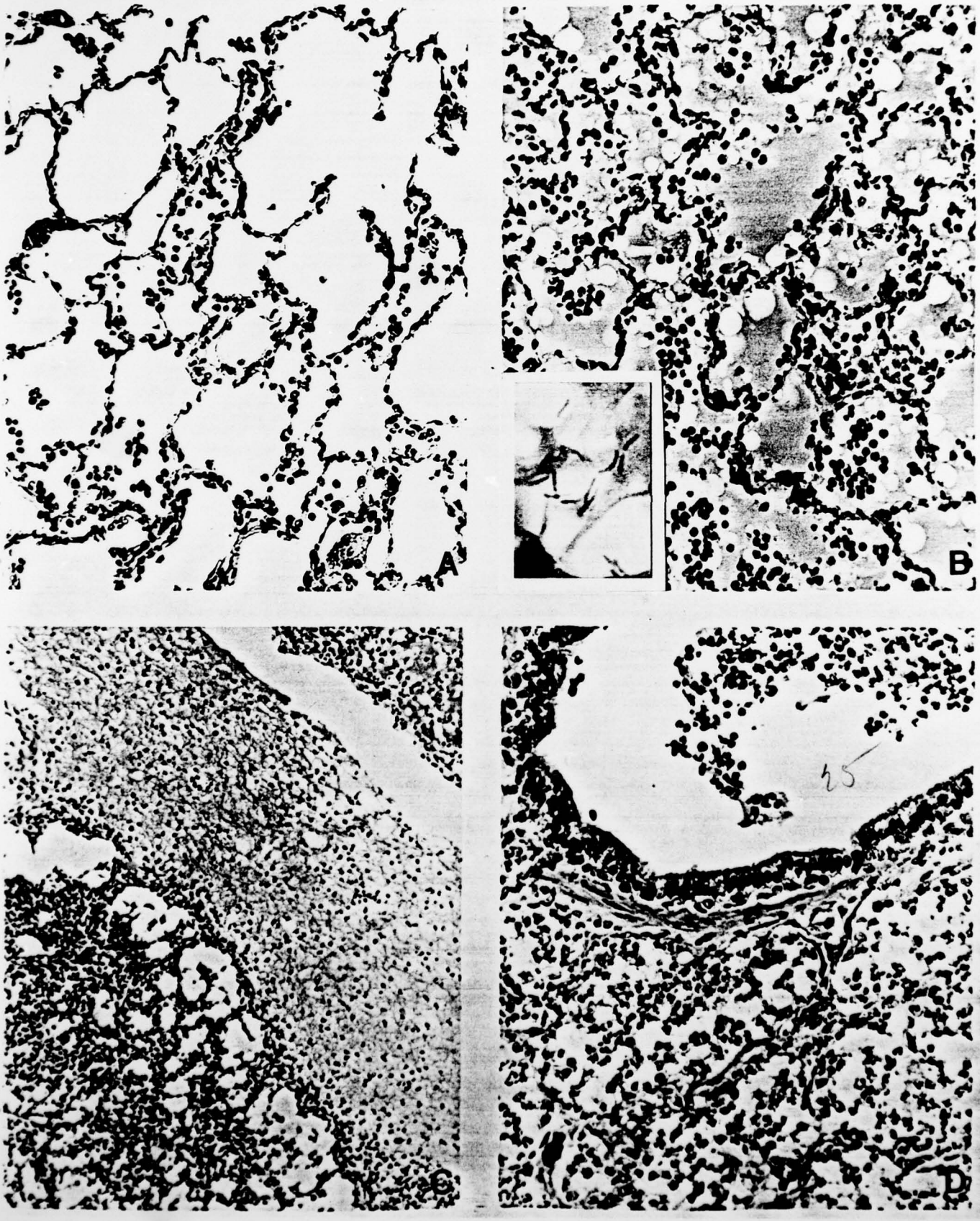
HOURS AFTER INFECTION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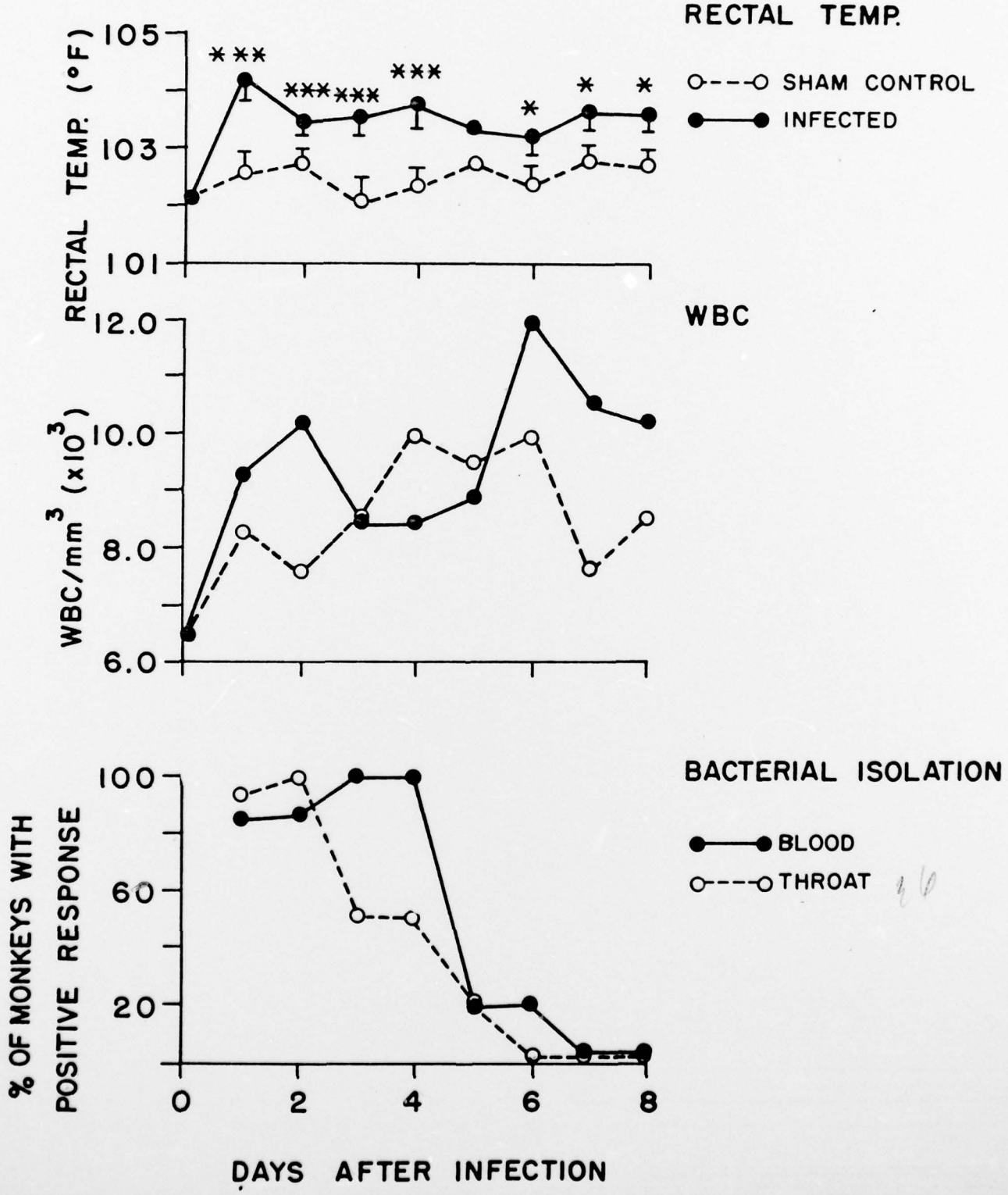
RECTAL TEMP.
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BACTERIAL ISOLATION
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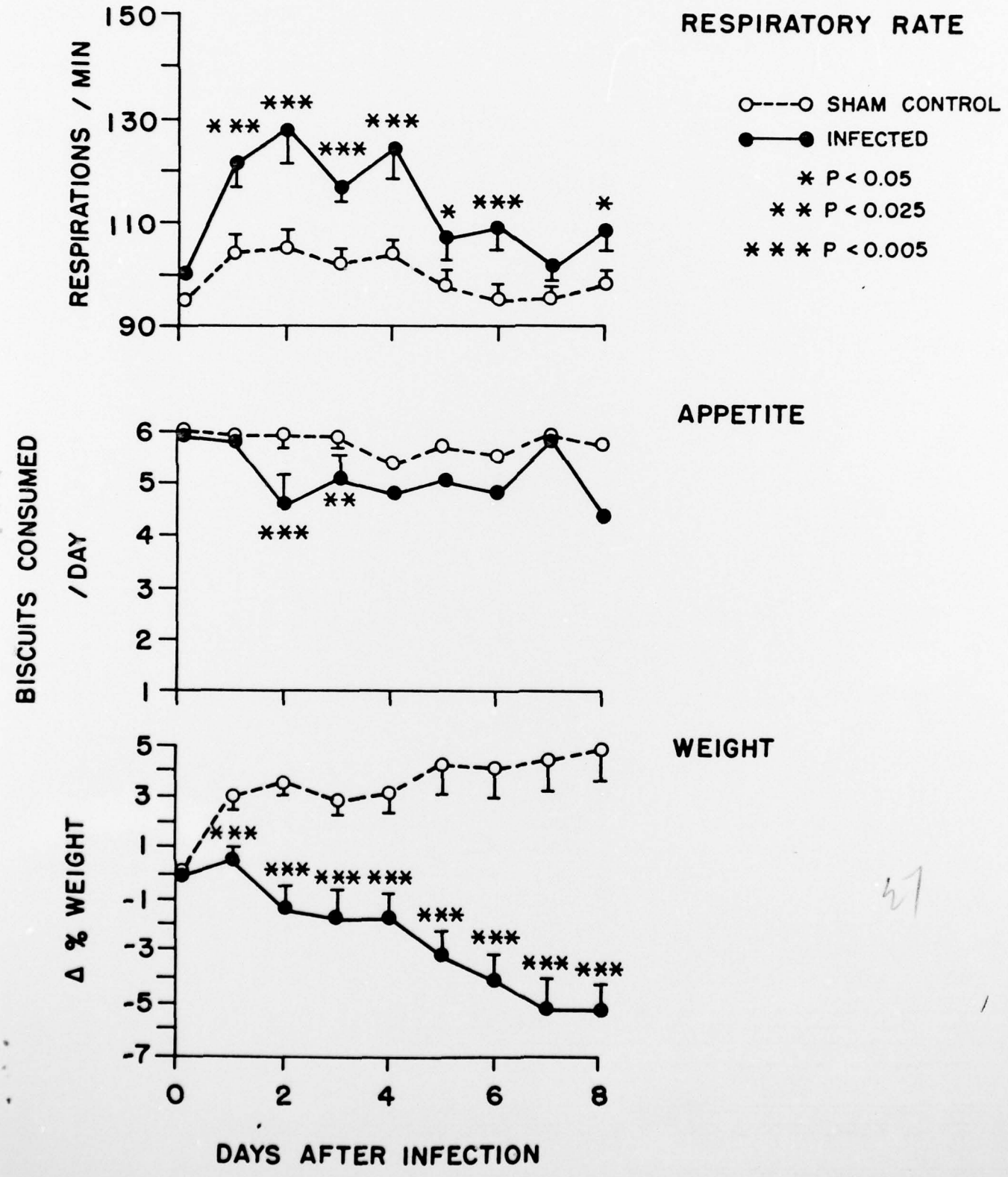
DAYS AFTER INFECTION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APPETITE
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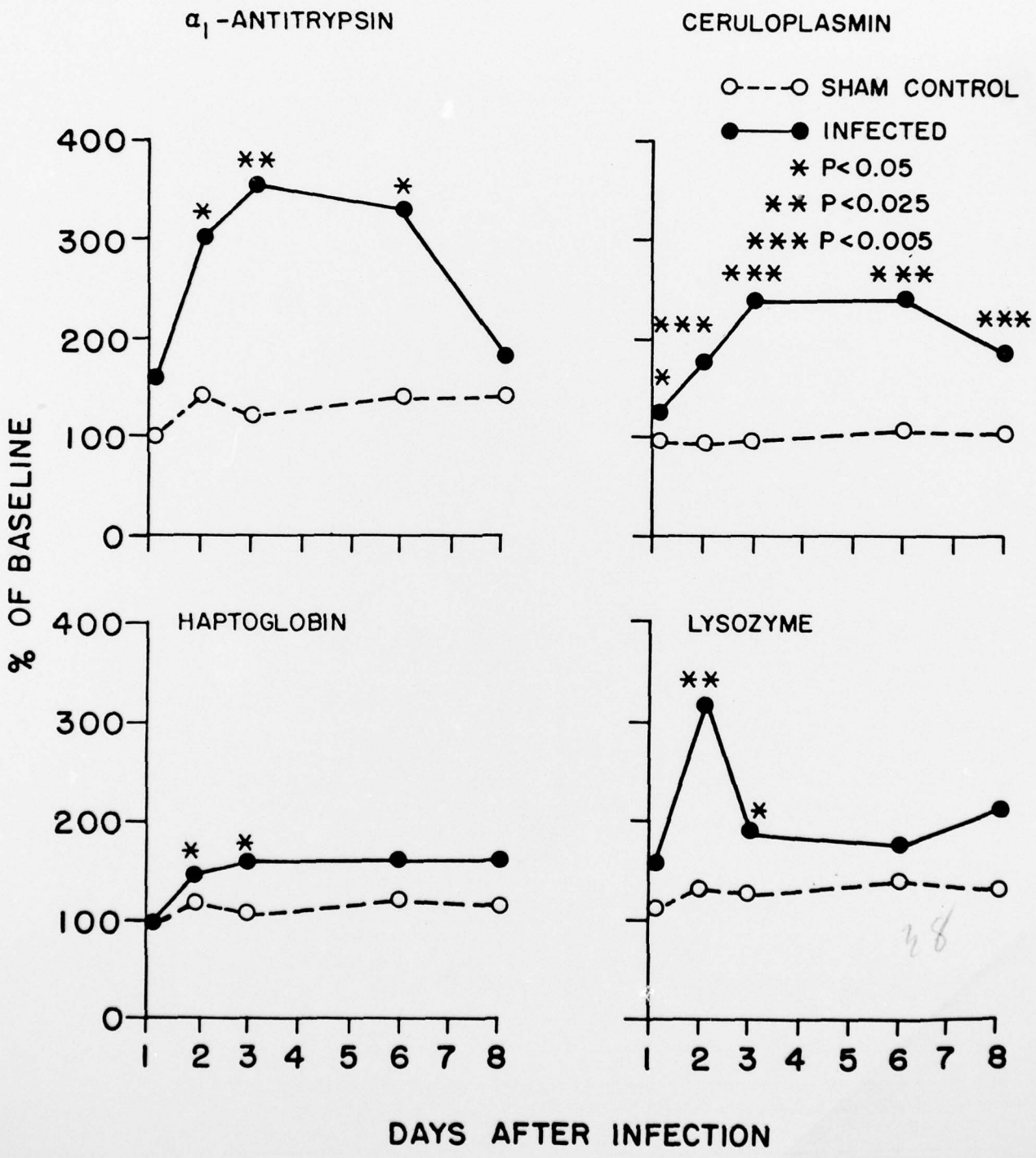
DAYS AFTER INFECTION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DAYS AFTER INFECTION