Milestones in Progression of Primary Pneumonic Plague in Cynomolgus Macaques

Running Title: Pneumonic Plague in Macaques

Frederick Koster,¹* David S Perlin,² Steven Park,² Trevor Brasel,¹ Andrew Gigliotti,¹
Edward Barr,¹ Leslie Myers,¹ Robert C. Layton,¹ Robert Sherwood,¹ C. R Lyons³

Lovelace Respiratory Research Institute, Albuquerque, NM¹;
Public Health Research Institute
at the International Center for Public Health, NJMS-UMDNJ, Newark, NJ²;
Depts. of Pathology and Internal Medicine,
University of New Mexico Health Science Center, Albuquerque, NM³

*Corresponding author: Lovelace Respiratory Research Institute, 2425 Ridgecrest Dr.,
Albuquerque, NM 87108
Phone: (505) 348-9614
Fax: (505) 348-8567
E-mail: fkoster@LRRI.org
ABSTRACT

Vaccines against primary pneumonic plague, a potential bioweapon, must be tested for efficacy in well-characterized non-human primate models. Telemetered cynomolgus macaques (Macaca fascicularis) were challenged by the aerosol route with approximately 100 ED₅₀ equivalent doses of Yersinia pestis (Y. pestis) strain CO92 and necropsied at 24 h intervals post exposure (pe). Telemetered heart rate, respiratory rate and temperature elevation above diurnal baseline identified the onset of the systemic response at 55-60 h pe in all animals observed for at least 70 h pe. Bacteremia was detected 72 h pe by Yersinia 16S rRNA-specific RT-qPCR and later by culture at the time of moribund necropsy. By 72 h pe multilobar pneumonia was established with diffuse septal inflammation consistent with early bacteremia, and all lung tissues had a high bacterial burden. No cytokines or chemokines were significantly elevated in serum at any time, and only IL-1β, CCL2 and CCL3 were elevated in lung tissue.

Inhalational plague in the cynomolgus macaque inoculated by the aerosol route mimics most clinical features of the human disease, and in addition mimics the disease progression from the anti-inflammatory phase to the pro-inflammatory phase described in the murine model. Defined milestones of disease progression, particularly onset of fever, tachypnea and bacteremia, will be useful in evaluating the efficacy of candidate vaccines.
Primary pneumonic plague is rarely acquired under natural conditions (5, 9, 21) but the virulence and ease of aerosolization makes *Yersinia pestis* (*Y. pestis*) a potential aerosolized bioweapon (13). The FDA ‘animal rule’ allows the use of large animal models to substitute for human testing if the model mimics human disease and is well characterized (11). Adequate characterization should include cause of death, phases of disease progression, and definition of secondary endpoints of success in addition to the primary endpoint of reduction in mortality.

Primary pneumonic plague has been well-described in the murine model following intranasal inoculation (3, 14, 20). The murine model mimics the human infection with respect to high mortality, focal consolidation in the lung and high level of bacteremia and dissemination to liver and spleen. The murine infection is described as a two-phase disease with an initial anti-inflammatory phase lasting 36 to 48 h after intranasal inoculation, followed by a rapidly progressing pro-inflammatory phase until death occurs at 4 days post-infection (3, 14).

Non-human primates are susceptible to aerosolized *Y. pestis* which produces a rapidly fatal disease similar to that seen in humans with primary pneumonic plague. Different species of NHP may display variable susceptibility and disease progression after exposure to *Y. pestis* aerosol (7, 10, 16), and a workshop has recommended a thorough characterization of the disease in multiple species (11). Following the initial report of the effective dose 50% (ED$_{50}$) for aerosolized *Y. pestis* strain CO92 in cynomolgus macaques (21), this report expands this model to examine in more detail the progression of disease in telemetered macaques.
METHODS

Animals. Both genders of cynomolgus macaques (Macaca fascicularis) of Indonesian origin were supplied by Covance and weighed 2.5–3.5 kg. Animals were screened for B-virus, Simian Immunodeficiency Virus (SIV), Simian Retrovirus (SRV), Simian T-cell Leukemia virus (STLV), and Simian Foamy Virus (SFV). Following quarantine, the macaques were collared and chair-trained to permit withdrawal of blood specimens without requirement for anesthesia, thus avoiding interruption of food intake. Telemetry monitoring devices (model T30F, Integrated Telemetry Services) were placed in an intramuscular pocket in the left abdominal wall with electrocardiographic leads placed on the lateral left and right chest wall, an intrapleural pressure transducer in the fifth intercostal space in the left chest, and a temperature probe in the battery pack placed in the lower abdominal musculature. The animals were moved into the ABSL3 laboratory at least 1 week before aerosol infection for acclimatization and collection of baseline physiological measurements. The protocol and all amendments were reviewed and approved by the LRRI Institutional Animal Care and Use Committee.

Aerosol exposure. Macaques were exposed to Y. pestis strain CO92 via head-only inhalation with a target dose of 100 ± 50 ED\textsubscript{50} of Y. pestis. One aerosol ED\textsubscript{50} in the cynomolgus macaque of Indonesian origin has been calculated to be 66 CFU (21). The term ‘ED\textsubscript{50} is used instead of LD\textsubscript{50} because animals are euthanized prior to natural death in order to reduce suffering. Fasted animals were anesthetized with 4 mg/kg Telazol approximately 15 min prior to aerosol exposure. The animal was placed in dorsal recumbancy in the plethysmography box, inserting its head through the dental dam head port into the head-only exposure apparatus contained in a Class 3 biosafety cabinet as previously described (21). The bacteria were nebulized using a Collison nebulizer (MRE-3 jet, BGI, Inc., Waltham, MA) and delivered to the
anesthetized monkey which was allowed to breathe freely. Real-time plethysmography (Buxco) targeted an inhaled volume of 5 L with actual exposure times ranging from 10 to 15 min. Aerosolized bacteria were sampled from the head exposure box into an all glass impinger (AGI-4; Ace Glass, Inc., Vineland, NJ) and concentrations were confirmed by quantitative bacterial culture. Purity of the aerosolized sample was assessed by colony morphology and growth on Congo Red-containing media. The target particle size mass distribution of between 1 and 3 µm was determined using a TSI Aerosol Particle Sizer (Model 3321; TSI, Inc, Shoreview, MN) which measured delivered aerosol over the range of 0.5 and 20 µm particles (4). Pathogen dose was calculated using the formula Dose = (C × V), where C was the concentration of viable pathogen in the exposure atmosphere, and V was the total volume inhaled based on Buxco plethysmography and exposure times.

Preparation of bacteria for inhalation. Separate bacterial suspensions were prepared for each animal. Briefly, a working stock cryovial was removed from frozen storage, thawed and used to inoculate five tryptose blood agar base with yeast extract (TBAB) slants that were allowed to incubate at 28°C ± 2°C for 72h. Following incubation and purity verification, 2 mL of sterile 1% peptone were added to each slant. The bacterial growth was harvested using sterile, cotton-tipped wooden swabs and pooled (10 mL total). The pooled suspension was centrifuged at 4100 rpm, 5-6°C, for 20 min. After disposal of the supernatant, the bacterial pellet was resuspended in 4 mL of 1% peptone and mixed thoroughly. Following standard ten-fold microbial dilution procedures, a 100-fold dilution of the suspension was prepared and analyzed at a wavelength of 600 nm using a spectrophotometer. The resulting optical density data were compared to the working stock standard growth curve and used to estimate the titer. This starting concentration was used to calculate the dilution scheme required to achieve the target
generator suspension concentrations. The generation suspensions were prepared based on target inhaled volumes of 3.5 liters (L). Challenge material was prepared in sterile brain heart infusion broth (BHIB) to a final volume of 10 mL. Prior to delivery to the Aerosol laboratory, 0.5 mL were removed for pre-bioaerosol analysis. Following removal of this aliquot, bacterial suspensions were weighed in order to establish a baseline for post-bioaerosol volume calculations.

Clinical observations. Beginning with the first exposure day, twice daily cage-side observations (at least 4 h apart) were performed on study monkeys, including mortality, activity, nasal discharge, sneezing, coughing, ocular discharge, inappetance/anorexia, diarrhea, neurologic signs or other abnormalities.

Telemetry. Vital signs and ECG were continuously recorded by implanted T30F or T31F telemetry devices (Integrated Telemetry Systems, Chicago, IL). Data was acquired every 0.01 s and displayed as hour-averaged temperature, respiratory rate and heart rate. Temperature was recorded in the battery pack implanted intramuscularly in the abdominal wall. Respiratory rate was detected either by intrapleural pressure changes (T30F) or by biopotential leads (T31F) placed subcutaneously over the diaphragm. Heart rate was recorded by subcutaneous limb leads counting R waves per minute. Respiratory and heart rates were calculated by the software (VR², D.I.S.S., Chicago, IL). Electrocardiogram (ECG) wave forms were recorded as raw data for real-time and subsequent review. Baseline temperature, respiratory rate, and heart rate were determined for each animal during the 4 days prior to aerosol challenge, and baseline hourly averages were used to calculate deviations from normal diurnal variation. Each animal and its continuously recorded telemetry data were inspected real-time every 4–6 h after the first sign of
illness to make a timely decision on euthanasia to reduce suffering. An animal was euthanized for the appearance of moribund disease based on the development of at least two of the following criteria, including body temperature less than 33°C, abnormal repolarization waveforms on electrocardiogram consistent with ischemia or acute heart failure, deep labored breathing, seizures, too weak to climb onto the perch, or unresponsive to stimulation with refusal to eat offered food.

**Quantitative bacteriology.** Venous blood (target volume of 1 mL) was drawn from the femoral vein and transferred to a tube containing EDTA. Anticoagulated blood was aliquoted for dilution and plating for quantitative bacteriology (CFU). Bacterial load was calculated by plating three dilutions of homogenized tissue or whole blood onto tryptic soy agar (TSA). The limit of detection was 200 CFU/mL blood. Following 72 h of incubation at 28°C, purity was verified and *Y. pestis* colonies were enumerated. Select colonies were placed into 1 mL cryovials containing sterile 1% peptone/15% glycerol and frozen at -80°C ± 10°C.

**Bacterial DNA quantitative real time PCR (qPCR).** For bacterial DNA purification, 100 µL of blood or 200 µL of homogenized tissue were immediately mixed with 900 µL of TRIZOL-LS buffer, or 800 µL of TRIZOL buffer, respectively, and frozen until extraction. A *Yersinia* genus 16S specific molecular beacon qPCR assay was developed for the detection of *Y. pestis* DNA with the following primer and probe sequences (5′-3′): CACACTGGGCTACACCTGAA (sense primer), TGACAAAGTGGTTGCTGAGG (anti-sense primer) and CGCGATCGACG (molecular beacon probe). A *Yersinia* genus 16S molecular beacon was labeled with fluorescein (FAM) and quenched with DABCYL. A second probe unique to the macaque GAPDH provided an internal extraction and positive amplification control. The primer and probe sequences are as follows:
CACACTGGGCTACACCTGAA (sense primer), TGACAAAGTGGTTGCTGAGG (anti-sense primer) and CGCGAT AGGGGTTGAGTTTAATACGC ATCGCG (molecular beacon probe).

The Macaca GAPDH molecular beacon was labeled with HEX and quenched with DABCYL.

The qPCR reaction mix contained the following: 2.0 µL of the extracted DNA, 1.0 µL of each primer (10 µM), 2.0 µL of each molecular beacon (5.0 µM), 25 µL of Brilliant® II QPCR Master Mix and PCR grade water to make the final reaction volume 50 µL. The qPCR assay was performed as a two color multiplexed reaction in a Stratagene Mx3005P QPCR System. The thermal cycling parameters included: 1 cycle of 10 min at 95°C, 45 cycles of 30 s at 95°C, 30 s at 50°C and 30 s at 72°C. Fluorescence (FAM & HEX) was measured during the annealing temperature. The lower limit of detection of the *Yersinia* genus 16S probe was 4 genome equivalents or 1 CFU of *Y. pestis* (data not shown, D. Perlin, unpublished data).

**Serum and tissue chemokines and cytokines.** Cytokines and chemokines were measured with Luminex bead assays using human antibody reagents (Biosource, Inc.) previously validated for specificity and sensitivity for macaque serum proteins. Serum was analyzed from each macaque prior to exposure, at necropsy, and at 48 h pi in 3 macaques planned to be necropsied at 96 h pi; one of these 3 also had a bronchoalveolar lavage specimen at 48 h pi suitable for analysis. From each of the 12 necropsies, two lung samples weighing approximately 200–300 µg wet weight were homogenized in PBS and frozen at -80°C until analysis. A total of 14 proteins including 9 cytokines and 5 chemokines were analyzed on each of 52 specimens from the 12 animals, for a total analysis producing 728 results. The chemokines measured included RANTES, MIP-1α, MIP-1β, MCP-1, and IL-8. The cytokines measured included IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p40, IFN-γ, GM-CSF, and TNFα. The assay for IL-1β and IL-6 has been validated (12) and the remainder of the assays were validated by the manufacturer.
Necropsy. When moribund euthanasia criteria were met or for timed necropsies, the animal was given 10 mg ketamine/kg of body weight intramuscularly and 2% isoflurane by mask until deep anesthesia, followed by exsanguinations through cardiac puncture, or in some cases by intravenous euthasol (pentobarbital sodium [86.7 mg/kg] plus phentoin [11.1 mg/kg]). Necropsy was immediately performed upon euthanasia, and tissues collected and weighed for bacteriologic and cytokine assays. Lung lobes were gently inflated with neutral buffered formalin to approximate normal volume prior to immersion fixation. Lung was designated as “lung-lesion” sample when collected from a discolored region that was also firm, in order to avoid areas of discoloration due to euthanasia artifacts. Lung was designated as “lung-nonlesion” sample when collected from an area that appeared normal without discoloration or firmness. Samples of lung for histopathology were taken immediately adjacent to areas sampled for bacteriology and cytokine analysis to permit parallel analyses. Lungs from animals necropsied at 24 h and 48 h after aerosol exposure had no apparent lesions and the two samples taken from each lung for parallel analyses were taken randomly from the caudal lobes. Tissue sections of lung, liver, spleen tracheobronchial lymph nodes and brain were processed routinely and paraffin embedded, sectioned at 4–6 µm thick, mounted on standard glass slides, and stained with hematoxylin and eosin for light microscopic examination. Lesions were graded subjectively by a single pathologist on a scale of 1–4 (1 = minimal, 2 = mild, 3 = moderate, 4 = marked).

Statistics. Changes in telemetered vital signs from baseline values were tested by Two-way ANOVA with repeated measures using Bonferroni post-tests. Changes in cytokine and chemokine levels in serum and tissue between necropsy groups were tested by One-way ANOVA using Bonferroni post-tests.
RESULTS

Four groups, each with three telemetered adult macaques, were aerosol challenged with *Y. pestis* at presented doses of 141 ± 32 ED$_{50}$ (group mean ± standard deviation). Animals were scheduled to be euthanized and necropsied 24, 48, 72, and 96 h pe; the latter group animals were actually moribund and necropsied at 70 h, 92 h and 94 h pe, respectively.

**Bacterial quantitation.** Small numbers of *Y. pestis* were cultured in only two of 6 lung samples at 24 h pe, while all blood, lymph node, liver and spleen cultures were negative at 24 h pe (Fig. 1). These low-level positive lung cultures may represent residual bacteria from the inhalation exposure, or may be small foci of persistent bacteria later expanding to pneumatic regions. All cultures were negative at 48 h pe. Bacteremia was detected in one of three macaques scheduled to be necropsied at 72 h pe, and was also detected in the three animals which were moribund at 70 h, 92h and 94 h pe. In contrast lung and tracheobronchial lymph node had substantial bacterial loads at 70 - 72 h pe with higher numbers of *Y. pestis* in visibly consolidated lesions compared to normal-appearing lung tissue, a difference that was no longer evident by 96 h pe.

The lower limit of detection when plating 100 µL of blood is 200 CFU/mL blood. To detect lower levels of bacteremia we used a real-time PCR molecular beacon probes specific for *Yersinia* 16 S rRNA subunit. A second probe to the macaque GAPDH provided an internal extraction and positive amplification control. The GAPDH CT values were a measure of extraction efficiency and ranged from 25 to 37. CT values for GAPDH >35 indicate inadequate nucleic acid recovery and were seen in only 3 of 36 samples. The lower limit of detection of the 16 S probe was 4 genome equivalents (Fig. 2A), equivalent to 4 CFU of *Y. pestis*. At 24 h pe all blood was negative and one of two culture-positive lung samples was positive by RT-PCR. At
48 h pe all samples were negative by culture and RT-PCR. At 72 h pe all 18 blood and tissue samples were positive by RT-PCR, including two bloods negative by culture. Among 18 samples from moribund animals, all were positive by both assays. Comparison of 20 tissue samples positive by both assays (Fig. 2B) found good quantitative correlation among samples from moribund animals ($r = 0.87$, $p < 0.01$) but not among samples collected at 72 h pe ($r = 0.2$, NS).

**Disease progression and telemetered physiology.** Telemetered data displayed as hour-averages for heart rate, respiratory rate and temperature superimposed on the pre-exposure diurnal variation is shown (Fig. 3) for two macaques observed until moribund status 96 h after aerosol exposure, and for all 6 macaques surviving until at least 70 h pe (Supplement Figure S1). Onset of abnormal body temperature defined by statistically significant departure from baseline diurnal value was seen at 60 h pe (Fig. 3) for both animals although temperature did not exceed 39°C until 68 h and 75 h pe, respectively. Among the 4 animals necropsied 70-72 h pe, three had initial temperature elevations at 55 – 60 h pe, although none had a temperature persist above 39°C. All 6 animals had tachypnea and 5 of 6 had tachycardia above baseline, including the animal that did not show temperature elevation. Onset of tachycardia and tachypnea appeared to coincide with the onset of fever as defined by departure from baseline, but markedly elevated heart and breathing rates were not seen until after 72 h pe. By 72 h pe most animals showed markedly depressed activity, withdrawn and immobile at the back of their cage in a hunched posture. As they became moribund, these animals were hypothermic, had decreasing respiratory rate and pulse, and were no longer able to balance on their perches.

**Cytokine and chemokine response.** Cytokines and chemokines were not elevated or not detectable in serum or lung tissue at 24 h and 48 h pe (Fig. 4), compared to pre-exposure levels.
Elevations of 3 cytokines and 3 chemokines were found in some lung tissue but never serum at 72 h pe. Few changes comparing 48 h and 72 h levels were statistically significant, however, likely due to the small group size of 3 animals for each comparison. IL-1β elevated in lung lesions (p < 0.001) and the chemokines CCL2 (MCP-1) and CCL3 (MIP-1α) elevated in most lesion and non-lesion lung samples (p < 0.01). In moribund animals tissue levels of cytokines and chemokines were not different from levels measured in non-moribund animals at 72 h pe. The other eight chemokines and cytokines that were measured were either not detected in any serum or tissue sample or not elevated following infection (Supplementary Material, Fig S2). One animal was clinically well and afebrile when necropsied at 72 h pe and had bacteremia with *Y. pestis* demonstrable only by RT-PCR. This animal had detectable but low levels of TNFα and IL-6 in lung tissue, and was the only animal with elevated IL-10 in pre-exposure serum.

**Pathology.** The gross appearance of the lungs was characterized by multifocal areas of pneumonia along with enlarged tracheobronchial lymph nodes (Fig. 5). Consolidated regions with pneumonia were apparent as palpably firm, moderately well-demarcated regions with variable amounts of dark red discoloration and hemorrhage. At 24 h pi, no lesions were found which could be attributed to *Y. pestis* infection. In the tissue samples examined histologically (Table, Fig. 6E) there was no detectable pathology, although two positive bacteriologic cultures (Fig. 1) likely represented small early foci of pneumonia. At 48 h pi, only one (#13422) of the 3 lungs suggested a possible early focus of infection, with alveolar thickening due to mononuclear cell infiltration (Fig. 6A), but no bacilli were seen. Occasional chronic inflammation was seen in the lung of some animals (table in Fig. 6E) is likely attributable to chronic changes found in these wild-caught macaques.
Timed necropsies reveal the explosive progression of disease between 48 h pe and 72 h pe (Figure 6 B-E). By 72 h pe distinct focal lesions were consistent with and confirmed histologically by fibrinosuppurative pneumonia and alveolar edema (Fig. 6B). In contrast the lung-nonlesion areas typically displayed multifocal, mild septal and/or alveolar infiltration with neutrophils and mononuclear cells (Fig. 6C). By 96 h pe, however, choice of a “non-lesion” sample often became difficult and those samples often demonstrated similar though less extensive pneumonia histologically (Fig. 6D and table in Fig. 6E). Culture also demonstrated wide distribution of bacteria by 96 h pe, likely due to the bacteremia distributing infection throughout all lung areas.

The areas of mixed alveolar inflammation/pneumonia were composed primarily of septal and alveolar neutrophils and macrophages. Areas of necrosis were present within severe foci of pneumonia, and extension to surrounding compartments (airways, vasculature) were present. The areas of pneumonia in these animals were typically deep within pulmonary parenchyma and not often associated with airways as is common with many classic, naturally acquired inhalation pneumonias (i.e., bronchopneumonia). This may be a result of secondary hematogenous spread to/within the lungs after initial phagocytosis by macrophages combined with the initial insult often occurring deep within the lung due to the well dispersed aerosol administered. Areas of alveolar edema were evidenced by eosinophilic background staining within alveolar airspaces. Basophilic, short rod bacteria typical of Y. pestis were visible both extra- and intracellularly in most cases of substantial pneumonia (the characteristic bipolar staining or “safety pin” appearance is often not prominent with tissue section H&E staining). In the cases where these bacteria were most abundant, they were typically scattered freely throughout the alveoli within
the edema fluid. Fibrinosuppurative pleuritis was seen occasionally with severe pneumonia, and was likely an extension of that process.

Liver and spleen were grossly unremarkable in this study and not examined histologically.

Related studies have demonstrated that liver of affected cynomolgus macaques is typically relatively unremarkable, characterized principally by mild to moderate sinusoidal leukocytosis (presence of an increased number of white blood cells within the hepatic sinusoids). In most cases the leukocytes are primarily neutrophils, with lesser numbers of monocytes and lymphocytes. Splenic histopathology is essentially similar to that seen in the liver, with a similar sinusoidal leukocytosis being the principal change. Scattered to moderate numbers of bacteria are occasionally evident within monocytes/macrophages in the spleen. The leukocytosis in the liver and spleen probably reflects to some extent a systemic increase in circulating white blood cells in response to disease. Similarly, the tracheobronchial lymph nodes (adjacent to the tracheal bifurcation/carina) usually display sinusoidal leukocytosis characterized principally by macrophages. Bacteria are often relatively frequent within the cytoplasm of monocytes/macrophages in this draining lymph node. None of these tissues have been observed to have focal inflammatory nodules or necrosis grossly or within areas examined histologically.

DISCUSSION

The purpose of characterizing non-human primate models of inhalational plague is for improved pre-clinical evaluation of candidate vaccines and treatments (11). Primary pneumonic plague in the cynomolgus macaque mimicks that in humans in many important features (5, 9, 18, 22). In both humans and macaques the incubation period is brief with rapid progression to
severe illness, fever, tachypnea, tachycardia, bacteremia in most but not all cases, absence of
hepatosplenomegaly and lymphadenopathy, minimal transaminasemia, often marked blood
leukocytosis, and extensive patchy multilobar pneumonia. Features of human disease not shared
by cynomolgus macaques include cough, hemoptysis, thrombocytopenia, and coagulopathy.

The primary findings of this natural history study indicate that severe primary pneumonic
plague is characterized by two phases, an anti-inflammatory phase and a pro-inflammatory phase
similar to those described in inbred and outbred mouse strains (3, 14). In mice the early phase,
characterized by low concentrations of non-viable bacteria within lung macrophages and a lack
of a pulmonary cytokine and chemokine response, was followed by an explosive growth of *Y.
pestis* in the lung and dissemination to the spleen by 36 h. The apparent lag phase of 24–48 h in
the lung may relate to genetic switches that the *Y. pestis* genome must execute in the 37°C
mammalian environment (14, 15).

*Y. pestis* expresses a number of virulence determinants, particularly the *Yop* virulons (6, 17).
Yop proteins mediate anti-phagocytic effects by dephosphorylating key focal adhesion proteins,
inactivating GTPases that control cytoskeleton dynamics, preventing activation of the master
inflammation regulator NFκB, and inducing macrophage and dendritic cell apoptosis (2). The
effects of this initial paralysis of host phagocytosis mechanisms may have been seen in our
macaques suggested by the lack of cytokine or chemokine response in lung tissue and lack of
neutrophil recruitment at 48 h pe.

Substantial variability in host response is seen among wild-captured macaques to
aerosolized *Y. pestis*. Animals were exposed to approximately 4000–9000 viable aerosolized
bacteria in their breathing zone. This inhaled dose respresents an estimated LD$_{95}$, not an LD$_{100}$,
as we have observed macaques occasionally surviving supralethal doses (7). The gross
pathology typically demonstrated less than 10 distinct inflammatory foci, separated by large areas of normal tissue, in each lung at 72 h pe. Only about 5 to 10% of inhaled particles with particle sizes of 1 to 3 microns are deposited in the alveolar region of the lung of NHP with higher deposition in upper airways including the nasopharynx and tracheal regions (4). If the number of bacteria initiating disease in the alveoli number as few as 10 CFU, a large percentage of bacteria that reach the alveoli are killed by innate defenses. In the study calculating the ED$_{50}$ for cynomolgus macaques (21), an animal exposed to as few as 35 $Y. pestis$ survived while another animal exposed to 270 bacilli died. In this study one macaque (#13436) with elevated serum IL-10 pre-exposure had exhibited mild clinical disease, no elevated temperature, $Y. pestis$ detected in the blood only by qPCR, and low levels of lung tissue cytokines at timed necropsy 72 h pe. These observations suggest that among cynomolgus macaques there are individual variations in critical innate lung defenses which may modulate survival.

An important milestone in the progression of disease is the onset of bacteremia. One animal had a negative blood culture at 72 h pe in spite of fever onset at 60 h pe. The sensitivity of detecting bacteremia is limited by the low plate-inoculum volume of 100 $\mu$L corresponding to a LLOD of 200 CFU per mL of blood. The highly sensitive $Yersinia$ rRNA-specific quantitative real-time PCR (Fig. 2A) detected rRNA in all blood and tissue at 72 h pe. Even qPCR is limited by the sample isolation procedure, since the 48 h macaque blood GAPDH Ct values were at least 3 times less than the other time points, indicating at least 10 times less DNA in the samples. Although our sample size is small, the qPCR assay detecting rRNA may be preferred when onset of bacteremia must be detected with sensitivity.

Fever is the first evidence of systemic disease and is an important milestone useful for initiating post-exposure treatment. In the six macaques observed beyond 48 h pe, the onset of
significant temperature elevation occurred consistently between 55 h and 60 h pe. The onset of fever may have preceded the onset of bacteremia, as bacteremia was detected only by qPCR and not culture in two of three febrile macaques at 72 h pe. Several macaques did not develop a body temperature over 39°C in the current study. Measurements of body temperature by rectal thermometer used in a previous study (21) are subject to bias introduced by anesthesia and are often at variance with simultaneous measurements by implanted chips (19). Intermittent measurement may miss the onset of fever, delaying the onset of treatment. Here, continuous telemetry identified the onset of fever as an abrupt departure from the normal diurnal pattern (Fig. 3) and an absolute temperature of greater than 39°C was not always documented.

Heart rate and respiratory rate appeared to increase above their diurnal rhythm limits at the same time as temperature (Fig. 3), but such increases were often not apparent when inspecting the data in real-time due to minute-to-minute variations. Estimation of respiratory rate by observation alone is difficult in nonhuman primates. Although a recent study found no respiratory distress (21), we observed respiratory distress with labored breathing in most animals after 60 h pe, and tachypnea was confirmed by telemetry in all animals. These observations agree with previous reports of respiratory distress (1, 10). Tachypnea in our animals were signs of successful compensation for modest hypoxemia, as in other studies we have found no evidence of respiratory acidosis and failure (Layton and Koster, unpublished observations). Telemetric measurements also allowed us to identify more precise indicators of impending death, preventing unnecessary suffering while avoiding the error of euthanizing an animal which may survive in spite of illness. Repolarization abnormalities on the ECG, either ST segment depression or T wave inversion, were the most consistent indicators of irreversible disease and have never been seen in survivors (Layton and Koster, unpublished data).
Observations in this study confirm the similar clinical and histopathological features of pneumonic plague in non-human primate and human hosts and identify important parallels with the murine model of pneumonic plague. Future studies will compare the appearance of secondary endpoints of efficacy, including bacteremia and telemetered vital signs, in immunized and non-immunized cynomolgus macaques.

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FIGURE LEGENDS

FIG. 1. Bacterial culture of blood and tissue collected at scheduled necropsy (24, 48, and 72 h post-challenge) and moribund euthanasia following aerosol exposure to *Yersinia pestis*. Data are presented as CFU (log10) per gram of tissue or per mL of whole blood. Samples from three animals were analyzed at each time point, 24 h, 48 h and 72 h. scheduled necropsies, and three moribund animals necropsied at 70 h, 92 h and 94 h p.e. Bars indicate mean values between the three samples; each sample is the mean of three replicate cultures.

FIG. 2. A: Sensitivity of the real-time RT-PCR assay specific for *Y. pestis* rRNA. B: Correlation of quantitative results from bacterial culture and RT-PCR for tissue samples positive for both assays. Results of samples positive by only one assay are presented only in the text. The line is derived from the regression equation relating genome equivalents and CFU for each tissue sample collected from moribund animals.

FIG. 3. Telemetered physiologic data for two macaques moribund at 92 and 94 h p.i. Heart rates (top frame), respiratory rates (middle frame), and body temperatures (bottom frame) are displayed as one-hour averages for each animal. Baseline data are the data from each animal averaged at each hour of the day over 4 days of pre-exposure recordings. Baseline data are graphed as repeated 24-hr periods showing diurnal variation for 24172; the baseline for 13375 is similar to 24172 and not shown for clarity. Data are compared to each animal’s baseline diurnal variation results by two-way ANOVA with repeated measures using Bonferroni post-tests. The hour where the values significantly leave the animal’s baseline are represented by a change in
symbol from open circle or box to closed star with the addition of an arrow marking the location of the star for clarity.

FIG. 4. Cytokine and chemokine levels (mean; standard deviation) of 3 samples each for serum (S), lung tissue with lesion (LL), and normal appearing lung (NL) collected at scheduled necropsies 24 h, 48 h, and 72 h, pe. Three animals in the ‘96 h’ group were moribund at necropsy at 70 h, 92 h, and 94 h, pe respectively.

FIG. 5. Macaque lung images. Uninfected control in upper left frame. Lung 48 h after infection in upper right frame; arrows indicate enlarged tracheobronchial lymph nodes; gray peripheral lung regions due to anesthesia artifact. Lung 72 h pi in lower frames; circles indicate palpably firm nodules in lung parenchyma, denoted as lesion (L) regions.

FIG. 6. Histopathology in lung regions with (lesion, L) and without (non-lesion, NL) gross abnormality. Frame A: normal-appearing lung with focal thickened alveolar septa (example of grade 1) at 48h PE. Frame B: firm nodule (Lesion, L) with hemorrhage and severe alveolitis (example of grade 4) at 72 h pi. Frame C: normal appearing lung (NL) adjacent to tissue in Frame B, with diffuse thickened alveolar septa (example of grade 2) at 72 h PE. Frame D: normal-appearing lung tissue (NL) at 96 h pi, with congestion and alveolitis (example of grade 3). Frame E: table of histopathological scores of lung tissues from 12 macaques. The ‘moribund’ group includes 3 animals euthanized for moribund disease at 70h, 92h and 94 h PE, respectively. Alveolar inflammation refers to predominantly polymorphonuclear leukocyte...
infiltration, while ‘inflammation mono’ refers to mononuclear leukocyte infiltrate without neutrophils.
Blood and Tissue Culture Data as a Function of Time Post-Aerosol Challenge with a Lethal Dose of Yersinia pestis

*Terminal blood samples are presented as CFU/mL*
Correlation between bacterial culture and rRNA RT-PCR derived genome equivalents

Notes: DNA template used in this assay = *Y. pestis* CO92 genomic DNA.
Figure 3

Telemetered Vital Signs Post-exposure

<table>
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<th>Animal</th>
<th>HR (bpm)</th>
<th>RR (breaths/min)</th>
<th>Temp (°C)</th>
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</table>
Serum and lung cytokine and chemokine levels after aerosol exposure to Y. pestis

Figure 4

Serum and lung cytokine and chemokine levels after aerosol exposure to Y. pestis

IL-1 beta

MCP-1

IL-6

MIP-1 alpha

TNF alpha

IL-8

Time After Aerosol Infection

pg/ml

24 hrs. 48 hrs. 72 hrs. 96 hrs.
Figure 5

Gross pathology at necropsy 48h and 72h after aerosol exposure.