TITLE: Pseudomonas Pneumonia of Mink: Pathogenesis, Vaccination, and Serologic Studies

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SUMMARY

Fulminating pneumonia was produced in mink by the intratracheal administration of Pseudomonas aeruginosa. The sequence of pulmonary lesions was focal inflammation, focal necrosis, and widespread inflammation and necrosis. Secondary lesions of peracute hemorrhage and necrosis were the result of bacterial spread via the airways. Invasion of vessel walls by P aeruginosa was a terminal event and was secondary to bacillary invasion and necrosis of adjacent tissues. Regional (lymphatic) and systemic spread of bacteria followed the development of pulmonary lesions, but there was little morphologic evidence of tissue damage in other organs. Immunofluorescence studies showed that P aeruginosa antigen was dispersed within pulmonary cells and was free in the lung parenchyma. Mink surviving beyond post-infection hour 60 had a macrophage infiltration into limited pulmonary lesions.

A vaccine trial was conducted with P aeruginosa lipopolysaccharides (LPS) used as antigen, and an enzyme-linked immunosorbent assay was used to detect antibody. Antibody was detected in mink after vaccination with LPS or natural exposure. Mink with antibody to LPS, from vaccination or naturally acquired, were resistant to experimental infection.

Pseudomonas pneumonia (hemorrhagic pneumonia) is an acute infectious disease of ranch mink caused by Pseudomonas aeruginosa. It occurs sporadically, and mortality has varied from less than 1% to 50% of the mink on affected farms.

Primary lesions are a consolidation of l or more lobes of the lungs and generalized pulmonary hyperemia and hemorrhage.[1-3,5,7,8] Hemorrhagic pleural

exudate is present in some cases. Microscopic lesions in the lungs vary with the degree of gross consolidation.[5,8] In hyperemic lungs, there are severe inflammatory hypermia, necrosis, and hemorrhage in the alveoli and bronchioles. Numerous bacteria, but few inflammatory cells, are present. In consolidated lobes, extensive proteinaceous exudate, erythrocytes, and variable numbers of neutrophils are within alveoli. Areas of necrosis contain many neutrophils. Invasion of vascular walls by the bacteria has been noticed. Pseudomonas aeruginosa has consistently been isolated in pure culture from the lungs and other organs of diseased mink.

Previous experimental infections of mink with P aeruginosa were de- signed mainly to establish P aeruginosa as the primary cause of the disease[3,6] or to test various vaccine preparations.[9,10] The pathogenesis of the disease has not been reported.

The purpose of the present investigation was to characterize the pathogenesis of the pulmonary lesions and consequent sepsis. In addition, the response of mink to vaccination with P aeruginosa lipopolysaccharides (LPS) was measured by the enzyme-linked immunosorbent assay (ELISA).[11] The relationship of detectable antibody to challenge-exposure susceptibility was investigated.

Materials and Methods

Bacteria - Isolates of P aeruginosa (Fisher[12] serotypes I and 7) were obtained from natural occurrences of pseudomonas pneumonia in commercial mink herds. Bacteria were grown overnight in tryptic-soy broth, washed, and suspended to a standard optical density (OD). Serial 10-fold dilutions were made in phosphate-buffered saline solution (PBSS) and numbers of viable organisms were determined by quantitative culture.

Isolates of P aeruginosa from experimental mink were identified by production of fluorescent pigment, characteristic reaction on triple sugar iron agar, growth in broth at 41 C, and cytochrome oxidase production. All isolates were serotyped by plate agglutination, according to the Fisher serotype scheme.[12]

Vaccine - Heptavalent P aeruginosa LPS vaccine (Fisher serotypes 1-7)[a] was used. The vaccine dose was 0.3 ml (0.25 mg of LPS).

Serologic Procedures - Mink sera were tested for antibodies to P aeruginosa LPS by passive hemagglutination (PHA)[13] and ELISA. The ELISA was conducted with slight modifications of standard indirect microplate procedures.[14] Wells were coated with antigen (LPS) at a concentration of 50 ug/ml. Sera were diluted 1:8 for testing. The conjugate was rabbit anti-mink immunoglobulin (Ig) G conjugated to alkaline phosphatase.[b] Results were expressed as the mean OD of test wells minus the mean of diluent controls. Results of ELISA were compared among groups by Student's t test. Where there were significant differences in variances, means were compared by the establishment of confidence intervals.

Animals - Pastel female mink kits (12 to 14 weeks old) were obtained from a commercial mink ranch that had no history of pseudomonas pneumonia. Mink were negative for antibody to Aleutian disease virus, as determined by immune counterelectrophoresis[15] and had no phenotypic manifestations of the Chediak-Higashi syndrome.[16] The mink were housed in individual wire pens in an unheated building and were given water and a standard wet, commercially available diet free choice.

Experimental Design – Pathogenesis - Mink (n = 41) lacking antibodies to LPS type 7 were randomly assigned to groups to be killed at postinoculation hours (pin) 4, 8, 16, 24, 36, 48, 60, or 84. Each group contained 4 infected and I control mink, except for the PIH-84 group which contained 5 infected and I control mink. Principals were anesthetized with ketamine hydrochloride[c] and acepromazine maleate.[d] Mink were inoculated with I ml (1,600 viable bacteria) of serotype 7 P aeruginosa intratracheally with a rigid metal catheter per os. The mink were then held in a vertical position for 30 s and were placed in their cages in ventral recumbency. Control mink were given PBSS in the same manner.

At each sampling time, mink to be killed were anesthetized with ketamineacepromazine and the pharynx and rectum were swabbed, and swab samples were plated on pseudomonas isolation agar.[e] The mink were exsanguinated and immediately necropsied. A total of I g each of liver, kidney,

spleen, and right cranial lobe of the lungs and an entire bronchial lymph node were aseptically removed and were ground with 3 ml of PBSS. The supernatant (I ml) was cultured on isolation agar. The remainder of the lungs were perfused via the trachea with 10% formalin-l% glutaraldehyde in phosphate buffer at a column height of 12 cm. Other tissues were fixed in the same fixative. Mink that died were necropsied at the time they were found. Bacterial cultures were made from tissue directly onto isolation agar.

Microscopy -Tissues were processed for routine light microscopy with paraffin embedding. Sections were stained with hematoxylin and eosin and May-Grunwald Giemsa. Selected lung sections were stained with Martius scarlet blue for fibrin and Verhoeff's stain for elastic fibers. For electron microscopy, representative sections of lungs were rinsed in buffer, fixed in osmium, and embedded in Epon-aral-dite. Plastic sections (I um) were stained with basic fuchsin and methylene blue and were examined by light microscopy. Thin plastic sections were stained with uranyl acetate and lead citrate and were examined with an electron microscope.[f]

Experimental Design -Vaccine Trial - Mink (n = 40) were assigned at random to vaccinated and nonvaccinated groups of 20 each. Vaccinated mink were given 2 subcutaneous doses of LPS vaccine at post-vaccination days (PVD) 0 and 14. Control mink were given PBSS in a similar manner. Serum samples were taken immediately before the 1st vaccination (PVD 0) and on PVD 30. The challenge exposure was given on PVD 30.

For challenge exposure, mink were assigned to I of 5 dosage groups with 4 vaccinated and 4 nonvaccinated mink in each group. The challenge-exposure doses were 10-fold dilutions from $10^{2,8}$ to $10^{6,8}$ serotype I organisms/ml. The bacterial suspension (I ml) was administered intratracheally. Mink that died were necropsied within 12 hours. Mink that survived experimental challenge exposure were killed 14 days after challenge exposure (PVD 44). Tissues were processed for light microscopy and pseudomonas isolation. Sera were assayed for anti-LPS antibody by PHA and ELISA, and correlations between antibody response and survival of challenge exposure were sought.

Results

Pathogenesis - Gross Lesions - Gross lesions were first visible in 2 of 4 infected mink at PIH 16. Scattered throughout the lungs were I to 3 mm, red-to-tan foci. At PIH 24, the foci were 3 to 5 mm in 2 of the mink and 5 to 10 mm in another. At 36 hours, all mink had irregular areas of red-to-brown discoloration in all lobes. The largest areas of involvement were 10 by 20 mm. In I mink, the accessory lobe was diffusely discolored and failed to collapse.

At PIH 48 and thereafter, lesions varied. In 2 mink killed at PIH 48 and 60, the left cranial lobe was red-brown, somewhat firm, and failed to collapse. Irregular red-tan foci were scattered in the other lobes. Other mink killed at PIH 48 and 60 had focal lesions similar to those in mink killed at PIH 36. At PIH 84, 1 mink had no gross lesions, whereas another had a few 5 to 10 mm foci scattered through the lungs.

Bronchial lymph nodes were large and red in mink with well-developed pulmonary lesions; the thymus was shrunken and hemorrhagic. The spleen was large in some mink, but this change was not consistently related to pulmonary lesions or bacterial cultural results.

Five mink died of the infection between PIH 36 and 72. A thin san- guinous exudate was present around the nose and mouth. One or 2 lobes of the lungs were dark-red to red-brown and were consolidated; the cranial lobes and the right cranial lobes were most often affected. Other lobes were generally congested, contained patchy hemorrhages, and did not collapse completely.

Light Microscopy - Early lesions were detected in lungs at PIH 8 and 16. Lesions consisted of neutrophils and macrophages within alveoli and adjacent terminal bronchioles. Characteristic foci of exudation and necrosis were present at PIH 24 and later. Dense proteinaceous exudate partially filled I to 3 alveoli and the involved alveolar septa were necrotic. Neutrophils and a few macrophages with pale, foamy cytoplasm were within the surrounding alveoli and small bronchioles. Hemorrhage and an occasional bacterium were in a few foci. Perivascular connective tissues were edematous and contained an infiltrate of neutrophils and a few macrophages.

The characteristic inflammatory reaction spread in the lungs up to PIH 60. Necrotic foci involved a number of alveoli. Neutrophils and pale eosinophilic exudate filled alveoli in large portions of the lobe. Macrophages became more numerous in the infiltrate after PIH 36. Mucosal degeneration and leukocytic infiltrate extended into the larger bronchioles. In grossly consolidated lungs, necrotic foci were often confluent and contained many bacteria. Bacteria were aligned along necrotic septa and were occasionally in edematous perivascular tissues. In the nonconsolidated lobes, foci of necrosis and hemorrhage were associated with bacteria in bronchioles and adjacent alveoli. Leukocytic infiltration was often mild. Lesions were similar in mink that died of the inflection, except that necrosis was more extensive. Bacteria were more numerous and occasionally were around and within the wall of small vessels. Lobes ipsilateral to the consolidated lobe(s) contained more bacteria and necrosis than did contralateral lobes.

In some of the mink surviving to PIH 48 and beyond, the inflammatory lesions were less intense, and macrophages were a prominent part of the infiltrate. At PIH 60 to 84, macrophages were often numerous in perivascular cuffs. Macrophages and a few neutrophils also were within the adjacent alveoli. Larger bronchioles had an infiltration of macrophages and neutrophils, and there was variable loss or proliferation of mucosal epithelium.

Electron Microscopy-In the early lesions (PIH 8 to 16), alveolar capillary spaces were widened, and neutrophils were scattered through the capillaries and alveoli. Neutrophils were often migrating through the alveolar septa, but alveolar lining cells were generally intact. At PIH 24, necrotic foci were characterized by degeneration and loss of alveolar epithelial and endothelial cells, and fibrin was within the alveolar spaces. Macrophages became more numerous by PIH 36 and were variable in appearance. Many contained large amounts of membranous debris within cytoplasmic vacuoles. Many of the leukocytes showed degenerative changes, but bacteria were rarely identified.

In the severely affected lungs at PIH 48 and 60, bacteria were numerous. Some bacteria were associated with neutrophils or macrophages, but most of

these leukocytes were degenerated. Free bacteria and fibrin were common and alveolar septa often could not be discerned. Type 11 alveolar epithelial cells were condensed and contained numerous cytoplasmic vacuoles.

Mink surviving to PIH 60 to 84 had only occasional small foci of necrosis of alveolar septal cells. Type 11 epithelial cells were prominent, as were other large cuboidal cells. Macrophages ranged in appearance from those with simple cytoplasmic borders to complex cells with numerous intracytoplasmic inclusions of membranous debris.

Bacterial Isolation - Bacterial cultures of lung from mink at PIH 4 to 84 were positive for P aeruginosa. Pseudomonas aeruginosa was grown from bronchial lymph nodes of mink killed at PIH 16 to 60, livers of mink killed at PIH 24 to 60, and spleens of mink killed at PIH 16 to 48 (Table 1). Cultures from the lungs and bronchial nodes had a moderate-to-heavy growth (10 to 100 and > 100 colonies/ g of tissue, respectively). Cultures from other sites varied markedly in density of bacterial growth. Rectal cultures from only 2 mink with consolidated lobes (PIH 48 to 60 hours) were positive. Renal cultures were also positive in these 2 and in an additional mink at PIH 60.

In mink that died of the infection, cultures from all sites yielded a heavy growth of P aeruginosa. All cultures from control mink were negative, except for a few colonies from the spleen of the PIH-24 control. All isolates had the same agglutinating reaction as the isolate used for experimental infection.

Challenge - Exposure Infection of Vaccinated Mink – Challenge exposure infection resulted in the death of 13 nonvaccinated mink; no vaccinated mink died. Deaths occurred between 18 and 84 hours after challenge exposure (PVD 31 to 34), most within 24 hours. Total mortality in the nonvaccinated mink was spread throughout all dosage groups, with 2 or 3 of 4 mink dying in each group.

In mink that died within 24 hours, lesions differed from those in the pathogenesis study in that entire lungs were variably hemorrhagic and there was no consolidation. Microscopically, there were diffuse hyperemia, multifocal hemorrhage, and a few inflammatory cells. In the 3 mink that died after 24 hours after challenge exposure, a mild diffuse infiltration of neutrophils and macrophages

was in some lobes. All sites that were cultured yielded a heavy growth of P aeruginosa, serotype I.

Surviving mink were killed 14 days after challenge exposure (PVD 44). Pseudomonas aeruginosa (serotype 1) was cultured from the pharynx of only I mink. Gross lesions were not present. In 3 mink, there were single 1-mm foci containing mononuclear vascular cuffs and numerous macrophages and cuboidal epithelial cells in the adjacent alveoli.

Serologic Findings - Mink that survived challenge exposure had higher ELISA OD values for LPS I in prechallenge-exposure sera than did mink that died (Table 2). Of the non-vaccinated mink, those with ELISA OD readings < 0.1 died. Three of 9 nonvaccinated mink with OD values 0.1- 0.5 survived, independent of challenge-exposure dose. The 4 other surviving nonvaccinated mink had ELISA OD values > 2.0. All vaccinated mink had ELISA OD values > 0.5, and 16 of 20 had values > 2.0.

All mink with antibody detectable by PHA before challenge exposure survived. The numbers of mink with antibody detectable by PHA and with antibody detectable by ELISA are compared in Table 3. A positive ELISA was considered to be an OD value > 0.5, the value associated with protection from challenge exposure. Thirteen mink without detectable PHA antibody (7 vaccinated and 6 nonvaccinated) survived challenge exposure. Of the vaccinated mink, 4 had anti-LPS IgG (2-mercaptoethanol-resistant antibody) detectable by PHA.

Three of 4 nonvaccinated mink with preinfection ELISA OD Values > 2.0 had no detectable antibody by PHA. After infection, these mink had low or nondetectable PHA titer compared with the titers of other nonvaccinated survivors (1:16 compared with 1:1,024).

Discussion

The present study showed that mink are susceptible to pulmonary infection with P aeruginosa, with an intratracheal LD50 < 10^3. Previous studies have reported an intranasal LD50 Of 10^3-10^4 P aeruginosa for mink.[9] However, these

mink most likely had the Chediak-Higashi syndrome[16] and direct comparisons of susceptibility among mink in these studies may not be valid. The LD^{50} in mink contrasts to that reported in other species. The number of organisms reported necessary to induce lethal pulmonary infection was 10^6 or 10^8 in rodents with burn trauma or immunosuppressive therapy.[18,19] Various isolates of P aeruginosa have been tested in rabbits, including an isolate from mink with pneumonia.[g] The intratracheal LD50 was 10^8-10^9.

The relationship of extracellular metabolites to virulence in P aeruginosa has recently been reviewed.[20] The relationship of P aeruginosa toxins to the pathogenesis of pseudomonas pneumonia in mink has been indirectly demonstrated in vaccine trials with toxoids of pseudomonas protease and elastase.[9] Mink vaccinated with toxoids were subsequently resistant to challenge exposure with P aeruginosa. Some of the early changes in pulmonary epithelial and endothelial cells in infected mink in the present study were similar to those caused by purified protease in rabbit lungs.[21]

It has been suggested that pulmonary lesions in mink are due to direct toxic effects of P aeruginosa, but that the pneumonia also may be secondary to septicemia and secondary changes in septal capillaries.[5] In the current study, lymphatic and systemic spread of bacteria appeared to follow the development of pulmonary lesions and was probably secondary to necrosis of pulmonary tissues. Changes in the pulmonary hematoaerial barrier have previously been suggested as the basis for penetration of bacteria into the vascular space of experimentally infected mice with bum trauma.[19]

Although bacteria were apparently sequestered in the reticuloendothelial organs (liver and spleen) of infected mink, there was no definite morphologic evidence of tissue damage in these organs. Changes seen in the thymus were secondary to stress, toxemia, or both. Whether systemic toxicity is involved in the ultimate demise of mink with pseudomonas pneumonia is unimportant, considering the extent of pulmonary damage.

Pseudomonas aeruginosa is cleared from the lung by alveolar macrophages, and specific antibody enhances phagocytosis.[22] This may explain

the lack of marked lesions in vaccinated mink after challenge exposure, because bacteria were probably phagocytized before large amounts of toxins could be produced. In the present pathogenesis study, degenerative morphologic changes were evident in leukocytes when necrosis occurred in the lung. These changes would surely have impaired further clearance of bacteria.

Two variations of the lesions of pseudomonas pneumonia have been described in mink.[5] One lesion consisted of peracute hemorrhage and necrosis with little leukocyte infiltration. The other lesion contained more leukocytes and dense exudate within the alveoli. The difference in lesions was associated with infection by different strains. This difference also was observed in the current experiments. Mink in the present vaccine trial, even in the low-dosage group, generally died earlier than did mink in the pathogenesis experiment. Mink in our pathogenesis experiment had less pulmonary hemorrhage and more inflammatory infiltrate within the lung. Lesions induced by experimental infection were similar to those of the field cases[h] from which the bacteria were isolated. Hence, this difference in tissue response appears to be a property of the bacterial strain. Serotype I organisms were used for the vaccine trial challenge exposure because this is the serotype that we have most often isolated in natural cases of mink pneumonia. Serotype 7 organisms were used for the pathogenesis study because approximately 20% of our experimental mink had naturally occurring antibodies to serotype 1.

A distinctive lesion, selective vasculitis or pseudomonas vasculitis, has been associated with necrotic lesions due to P aeruginosa in the lungs of man[23,24] and mink.[5,9] The lesion consisted of a large number of bacteria within the perivascular tissues and media of the involved vessels. In some studies, bacteria were within perivascular spaces but not necessarily within the adjacent vessel wall; the tunica intima of involved vessels was often spared.[5,23,25] It has been suggested that bacillary invasion of vessel walls in the mink lung may be a secondary change[5] or that the lesion may lead to necrosis of the surrounding tissue.[9] In the current study, vascular invasion by bacteria was a terminal or postmortem change and was secondary to necrosis and bacillary invasion of the

surrounding tissue. Bacteria appeared to spread to unaffected areas of the lung by the airways and to cause peracute necrosis and hemorrhage in these sites.

The Fisher serotype schema for P aeruginosa was based on challenge-exposure protection in vaccinated mice.[12] Antibodies to the LPS antigens have been detected by PHA and are considered to be type-specific.[13,26] We have previously detected antibodies to LPS in mink by PHA, and these mink were resistant to experimental infection with P aeruginosa of the same serotype.[h] However, the relative susceptibility of mink without antibody detectable by PHA has been variable. Many of the vaccinated mink and a few of the nonvaccinated mink in the current study had antibody that was nonagglutinating but was detectable by ELISA. This antibody was protective, as judged by the results of experimental challenge exposure. Mink responded to vaccination with LPS with a strong antibody response.

The ELISA was used in this experiment as a screening procedure, ie, to detect antibody at a single dilution of serum and to assign a qualitative value to the reaction. Sera were tested at 1:8 because this dilution was determined to be optimum for detecting positive reactions.[h] The ELISA reactions with an OD > 2.0 appeared to have surpassed the saturation limit of the enzyme system, and sera would have to be tested at higher dilutions to obtain comparative results. Results of ELISA are not a direct measure of antibody concentration, but depend upon the affinity of the antibody.[27,28] However, the affinity of antibody may be important in its in vivo protective capacity in certain bacterial infections.[27]

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TABLE 1—Isolation of *Pseudomonas aeruginosa* from Intratracheally Inoculated Mink

PIH	Pharynx	Lung	Bronchial node	Liver	Spleen
4	0/4	4/4	0/4	0/4	0/4
8	0/4	3/4	1/4	1/4	0/4
16	0/4	4/4	4/4	0/4	2/4
24	1/4	4/4	3/4	3/4	4/4
36	2/4	4/4	4/4	2/4	4/4
48	1/3	3/3	3/3	2/3	2/3
60	2/3	3/3	3/3	2/3	1/3
84	1/2	1/2	0/2	0/2	0/2

* Results expressed as No. of mink from which *P aeruginosa* was isolated/No. of mink examined.

TABLE 2—Influence of Prechallenge Exposure Specific Antibody Value* on Response to Challenge-Exposure Inoculation with *P aeruginosa*

Challenge exposure	No. of mink	Prechallenge ELISA OD reading†
Survival	27‡	1.999 ± 0.785
Death	13§	0.133 ± 0.126

* ELISA. † Mean ± SD of OD readings of ELISA reactions. All sera were diluted 1:8 before testing. Mean ELISA OD of mink that survived > mean of mink that died, ($P < 0.01$). ‡ 20 vaccinated and 7 nonvaccinated mink. § Nonvaccinated mink.

TABLE 3—Antibody Response to *P aeruginosa* Immunotype 1 Lipopolysaccharide in Mink as Measured by PHA and ELISA

Sampling time	Treatment group	Serotest PHA*	PHA (+2ME)†	ELISA‡
Initial	Nonvaccinated	1/20	1/20	3/20
(Prevaccination)	Vaccinated	4/20	1/20	2/20
Prechallenge exposure	Nonvaccinated	1/20	0/20	4/20
(Postvaccination)	Vaccinated	13/20	4/20	20/20
Postchallenge exposure	Nonvaccinated	6/7	5/7	6/7
	Vaccinated	14/20	8/20	20/20

* Passive hemagglutination titers were judged to be significant if sensitized cells agglutinated at least 1 dilution higher than a negative control (unsensitized cells). † Sera treated with 2 mercaptoethanol. ‡ Corrected ELISA OD reading > 0.5.

Results are expressed as No. of mink with detectable antibody/No. of mink in the group.