Pathophysiologic correlates of acute porcine pleuropneumonia

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Objective—To develop and evaluate an in vivo model to study early events in the pathogenesis of acute porcine pleuropneumonia.

Animals—Thirty-six 6- to 8-week-old pigs.

Procedure—Pigs were inoculated intranasally or endotracheally with Actinobacillus pleuropneumoniae; inoculation routes were compared by evaluation of clinical signs, gross and microscopic lung lesions, hematologic changes, serum zinc, iron, and haptoglobin concentrations, and inflammatory cytokines.

Results—The 2 inoculation routes resulted in similar findings, although intranasal inoculation caused unilateral gross lung lesions, whereas endotracheal inoculation caused bilateral gross lesions. Clinical signs of disease were observed < 2 hours after endotracheal inoculation and 6 to 8 hours after intranasal inoculation. Total WBC counts did not differ significantly after inoculation, although band and neutrophils increased significantly. Inoculation induced rapid influx of macrophages into the lung and local induction of proinflammatory cytokines. Northern blot analysis of total RNA from lung tissue indicated that inoculated pigs had increased concentrations of interleukin (IL)-1ß, IL-1α, and IL-8; tumor necrosis factor messenger RNA concentration was not increased.

Conclusions—Endotracheal inoculation with A. pleuropneumoniae rapidly and consistently induced diffuse bilateral pneumonia; thus, this method may be useful for the study of acute pathophysiologic changes associated with bacterial pneumonia and may provide an experimental model for testing modalities for prevention and treatment of this and other respiratory tract diseases of pigs. (Am J Vet Res 2000;61:684–690)

Respiratory tract disease is a major health concern for the swine industry worldwide. Although changes in swine management, such as segregated early weaning and 3-site production, have contributed to improved herd health, porcine pleuropneumonia continues to be an important cause of economic losses on farms. Porcine pleuropneumonia is caused by Actinobacillus pleuropneumoniae and characterized by necrotizing fibrinous pneumonia with high morbidity and mortality, especially among young pigs.1 The gram-negative organism causes lung lesions without influence of other bacteria or viruses but is often associated with physical and environmental stress. Considerable information is available regarding potential virulence factors of A. pleuropneumoniae2-4; however, the pathogenesis of this disease is still poorly understood, and efficacious vaccines are lacking.

Inflammatory cytokines, including tumor necrosis factor (TNF), interleukin (IL)-1, and IL-8, have been examined in various models of septic lung injury and adult respiratory distress syndrome. These soluble mediators appear to be pivotal in the development of lung inflammation and cell infiltration, which inevitably lead to tissue destruction.5-7 In vitro experiments have established that porcine alveolar macrophages respond to A. pleuropneumoniae bacterial lysates and purified Escherichia coli lipopolysaccharide (LPS) by producing exaggerated concentrations of TNF, IL-1, and IL-8 messenger RNA (mRNA).8-10 These experiments suggest that inflammatory cytokines may also have a role in the pathogenesis of acute porcine pleuropneumonia.

Several swine models of A. pleuropneumoniae–induced respiratory tract disease have been described.11-15 These models used aerosol, intranasal, endotracheal, or endobronchial inoculation routes, various serotypes of A. pleuropneumoniae, and differed in the quantity of inoculum delivered; various degrees of severity of infection resulted. Cytokine expression studies require a well-characterized, reproducible, and reliable disease model, particularly if experiments are restricted to small numbers of animals per treatment group. Such a model may aid the study of acute pathophysiologic changes associated with pleuropneumonia and help in developing better strategies for treatment and prevention of pleuropneumonia and other respiratory tract diseases of swine. Therefore, the objectives of this study were to characterize an in vivo model for the study of early events in porcine respiratory tract disease, including route of administration, associated pathophysiologic and clinical changes, and expression of inflammatory cytokines.

Materials and Methods

Pigs—Thirty-six conventional crossbred pigs, 6- to 8-weeks-old, were housed in isolation facilities according to University of Minnesota Institutional Animal Care and Use Committee guidelines. Pigs were obtained from 2 source herds and determined to be seronegative for A. pleuropneumoniae by the Iowa State University Diagnostic Laboratory, Ames, Iowa. After necropsy, pigs were determined to be free of Mycoplasma hyopneumoniae by use of immunohistochem-
A *pleuropneumoniae* growth inoculum—Actinobacillus *pleuropneumoniae* serotype 1, strain L91-2, a field strain obtained from a disease outbreak in Sioux City, Iowa, in log-arithmetic growth phase was used to prepare inocula for pigs. Cultures of *A. pleuropneumoniae* were grown in 3% tryptic soy broth that contained 1% casein hydrolysate, 0.1% yeast extract, and 0.001% nicotinamide dinucleotide. Bacterial cells were washed once with PBS solution (PBSS), pH 7.4, and diluted to approximately 5 × 10^5 colony-forming units (CFU)/ml. The washed cells were diluted in PBSS that contained 5% bovine serum albumin (BSA) to approximately 5 × 10^3 CFU/ml for intranasal inoculation or 1 × 10^4 CFU/ml for endotracheal inoculation. Sham inoculation fluid consisted of 5% BSA in PBSS.

Inoculations—For intranasal inoculations, 7 nonanesthetized pigs were secured in a vertical position with head fully extended upward, to open the larynx. Two milliliters of bacterial culture fluid were administered by placing drops of fluid into the nostrils while the pig's mouth was held closed, alternating between right and left nostrils during a 2-minute period, to obtain a dose of approximately 1 × 10^5 CFU/pig. Two control pigs were inoculated intranasally with sham inoculation fluid in a similar manner. Pigs were euthanized 18 to 24 hours after inoculation, and lung specimens were obtained for bacteriologic culture.

For endotracheal inoculation, 16 pigs were anesthetized by IM administration of xylazine (2.5 mg/kg of body weight) and ketamine hydrochloride (12 mg/kg). Pigs were positioned in an upright position, supported in a sling, and raised approximately 3 feet above the floor. The head was raised to approximately 45° for intubation. A lighted laryngoscope was used to enable proper placement of an uncuffed endotracheal tube (inner diameter, 4.5 mm) containing a CO₂ port. Five milliliters of bacterial culture fluid in a 12-ml syringe were administered through the CO₂ port to yield an inoculum of approximately 5 × 10^5 CFU/pig. Bacterial plate counts of the suspensions were performed after inoculations to determine viable counts. Five control pigs were inoculated endotracheally with sham inoculation fluid in a similar manner. Pigs were euthanized 18 to 24 hours after inoculation, and lung specimens were obtained for bacteriologic culture.

Clinical signs and blood collection—Pigs were monitored for signs of depression, nasal discharge, inappetence, temperature were taken prior to and at various times throughout study. Venous blood samples were collected before bacterial inoculation and immediately prior to euthanasia, unless otherwise specified.

Inflammatory cytokine expression—To test the hypothesis that *A. pleuropneumoniae* inoculation induced the expression of inflammatory cytokines in the lung, 4 pigs were inoculated intranasally with *A. pleuropneumoniae*, and 2 pigs were sham-inoculated intranasally with PBSS/BSA. Approximately 48 hours later, pigs were euthanized, and lung tissue was collected from regions containing lesions (inoculated pigs) or from the middle lobes (control pigs) and processed to obtain total RNA for northern blotting to detect TNF, IL-1α, IL-1β, and IL-8.

RNA isolation from lung tissue—At necropsy, 1-g pieces of lung tissue were placed in 10 ml of solution (3M guanidine thiocyanate, 50 mM Tris HCl, 25 mM EDTA, 0.5% Sarkosyl, 1% β-mercaptoethanol, pH 7.4) in a 50-ml sterile centrifuge tube and immediately homogenized for 30 seconds. Homogenized tissues were kept on ice until phenol and chloroform extraction. One milliliter of each homogenate was placed in a 14-ml polypropylene tube and acidified with 100 μl of 2M sodium acetate, pH 4.0. Samples were extracted twice with 3 ml of phenol and 2 ml of chloroform. The upper aqueous phase containing RNA was transferred to a clean 14-ml tube, precipitated with an equal volume of cold isopropanol, and pelleted by centrifugation at 10,000 × g for 20 minutes. Pellets were resolubilized in 300 μl of guanidine solution, precipitated as before with isopropanol, washed with 500 μl of 70% ethanol, dried at 20 C, and resolubilized in RNase-free water. Concentration of RNA was determined by measuring optical density at 260 nm.

Northern blotting—Denaturation and electrophoresis of RNA in 0.7% agarose-2.2% formaldehyde gels were performed as described. Equivalency of RNA lane loadings was monitored by the addition of 0.1% ethidium bromide (1 μg/lane); RNA was detected in gels by transillumination with UV light. The RNA was transferred onto nylon membranes' in 20X salt-sodium-citrate (SSC) buffer (1X SSC contains 150 mM NaCl and 15 mM sodium citrate, pH 7.0). The RNA was fixed to membranes for 4 minutes by use of UV irradiation, and prehybridization was performed for 30 minutes at 42 C. Prehybridization buffer containing 0.25M phosphate buffer containing 1 mM EDTA, 7% *sodium dodecyl sulfate* (SDS), and 50% formamide. Hybridization was performed overnight at 42 C with complimentary DNA (cDNA) probes for TNF, IL-1α, IL-1β, and IL-8. Membranes were washed with a solution containing 0.1X SSC and 0.1% SDS at 60 C and autoradiographed overnight at ~70 C by use of radiographic film and 2 intensifier screens.

Northern blot probes—The following porcine cytokine cDNAs were cloned from a Agt10 cDNA library and used to derive the probes used for northern blotting: TNF (Genbank accession No. X57321), IL-1α (Genbank accession No. AAI0273), IL-1β (Genbank accession No. X57321), and IL-8. A porcine β-actin probe (Genbank accession No. U70786) of 706-base-pair size was cloned by reverse transcription-polymerase chain reaction from a porcine fibroblast cell line and used to monitor lane loadings and transfer efficiency. All probes used in northern blotting were random-primed with [32P]dCTP to a specific activity of 5 to 10 X 10^6 cpm/μg of DNA. After labeling, unincorporated nucleotides were removed by passing the mixture over a cross-linked dextran-matrix column. Final probe concentration was adjusted to 3 X 10^5 cpm/ml of hybridization buffer for hybridization overnight at 42 C.

Gross and microscopic examinations—Lung weight (including heart) at necropsy was recorded and expressed as a percentage of body weight. Lesions (areas of hemorrhage and consolidation) were scored as described, using the following scale: 0 = no lesions; 1 = 1 or 2 lesions (each < 15 mm in diameter); 2 = 1 or 2 lesions (each < 50 mm in diameter) or a few scattered petechiae; 3 = more than 2 lesions (each > 50 mm diameter) or numerous scattered petechiae, ≤ one third of lung parenchyma affected; 4 = lesions more severe than those scored as 3. Lesion distributions were recorded on lung tracings, and lungs were photographed. Lung tissue specimens for microscopic examination were obtained from within lesions, at the edge of lesions, and from a location 3 cm beyond the periphery of lesions. For lungs that did not have gross lesions, specimens were obtained from cranial, middle, and caudal lobes. Blocks of tissue (volume, approx 0.5 cm ) were fixed immediately in paraformaldehyde-lysine-periodate (PLP) for 18 to 20 hours and embedded in paraffin.
Immunohistochemistry—Immunohistochemical staining was performed on 6-µm-thick sections of PLP-fixed lung tissue by use of a mouse monoclonal antihuman histiocyte antibody, rabbit polyclonal antilysozyme antibody, or mouse monoclonal antihuman neurofilament antibody. Briefly, tissue sections on slides treated with 1% N-(β-aminoethyl)-γ-amino propyltrimethoxysilane were deparaffinized in a 60°C oven for 20 minutes and dipped (3 minutes) twice in xylene. Tissues were hydrated in graded ethanol dips (initial concentration, 100%) and immersed in PBS. Tissue sections were enzymatically treated with trypsin (1g/L in 0.01 M Tris HCl, pH 7.4) containing 0.002 M CaCl₂ for 10 minutes, rinsed in cold tap water for 10 minutes, blocked with 5% horse serum (HS) diluted in PBSS for 30 minutes, and incubated with primary antibody for 1 hour at 37 C. Antibody dilutions in PBSS containing 0.5% HS were 1:50 for antihuman histiocyte antibody, 1:200 for antilysozyme antibody, and 1:100 for antineurofilament antibody. After 2 washes in PBSS, endogenous peroxidase activity was blocked by incubating slides in methanol containing 3% H₂O₂ for 10 minutes, followed by 2 PBSS washes. Peroxidase-conjugated antimouse or antirabbit secondary antibody was added at a 1:200 dilution in PBSS containing 0.5% HS for 30 min at 37 C. Tissue sections were washed twice in PBSS, diaminobenzidine substrate was applied for 10 minutes, and slides were counterstained with Harris hematoxylin stain. Slides were dehydrated through graded ethanol rinses, dipped in xylene, and mounted. Procedures were performed at 20 C unless otherwise specified.

Haptoglobin, iron, and zinc determinations—Because haptoglobin, an acute-phase protein, is induced by IL-6 and may be a predictor of infection, serum haptoglobin concentration was measured by use of cross-reactive antihuman haptoglobin polyclonal antisera in rate nephelometry, as described by the manufacturer. Values were obtained by interpolation of a standard curve developed from human haptoglobin standards.

Because serum zinc and iron concentrations have been correlated with the production of IL-1 and are commonly detected early in the course of disease, we examined serum zinc and iron concentrations to determine their usefulness as indicators of A. pleuropneumoniae infection. To evaluate the effect of bacterial inoculation on serum concentrations of zinc and iron, rates of concentration change were examined. At 0, 2, 4, 8, and 24 hours after inoculation, blood samples were obtained from 3 endotracheally inoculated pigs and 1 control pig for atomic absorption spectral analysis. In addition, WBC counts, differential leukocyte counts, and nonspecific esterase staining were performed on samples collected before inoculation and at 24 hours after inoculation.

Statistical analyses—Repeated-measures analysis of variance was performed to determine whether experimental data varied significantly, and P values < 0.05 were considered significant. Individual differences between groups and interactions among groups were determined by the Fisher protected test of least-significant differences. Kruskal-Wallis nonparametric analysis was used to identify interactions between route and treatment for the effects of inoculation on lung weights. Changes in serum zinc and iron concentrations were compared over time.

Figure 1—Photographs of gross lung lesions detected 18 to 24 hours after intranasal (A) or endotracheal (B) inoculation of Actinobacillus pleuropneumoniae in pigs. Notice unilateral distribution of lesions in (A) and bilateral distribution of lesions in (B).

Figure 2—Band neutrophil cell counts (mean ± SEM) in control pigs (sham-inoculated) and A. pleuropneumoniae-inoculated pigs, determined 18 to 24 hours after inoculation. A—Preinoculation and postinoculation values for control and A. pleuropneumoniae-inoculated pigs (APP), irrespective of route of inoculation. Numerals above bars indicate No. of pigs per group. B—Preinoculation and postinoculation values for control and A. pleuropneumoniae-inoculated pigs that were inoculated endotracheally (ET) or intranasally (IN). Numerals above bars indicate No. of pigs per group.
Results

Clinical signs—The most commonly observed clinical signs of disease in A pleuropneumoniae-inoculated pigs, regardless of inoculation route, were increased respiratory rate and dyspnea. These signs were apparent as early as 2 hours after endotracheal inoculation or within 6 to 8 hours of intranasal inoculation. Vomiting was the earliest observed sign of illness in pigs inoculated intranasally and developed within 2 hours of inoculation. Vomiting was not observed in pigs inoculated endotracheally. Signs of depression and inappetence were also commonly detected in pigs inoculated by either route. Sham-inoculated pigs appeared clinically normal.

Gross pathologic findings and bacterial cultures—The distribution of lung lesions in intranasally versus endotracheally inoculated animals varied, but the characteristics of the lesions remained the same. A pleuropneumoniae-infected lungs were consolidated, edematous, and hemorrhagic, irrespective of inoculation route. The pleural cavity contained fluid and fibrin. Intranasal inoculation consistently resulted in unilateral gross lesions (Fig 1A), whereas endotracheal inoculation induced bilateral lesions (Fig 1B). All A pleuropneumoniae-inoculated pigs had gross lung lesions, and lung involvement ranged from grade 1 to grade 4. One sham-inoculated pig had grade-1 lesions. However, results of bacteriologic culture were negative for specimens from this lung, suggesting that the changes developed postmortem, and the lung was normal in appearance otherwise. Actinobacillus pleuropneumoniae serotype 1 was cultured from all bacteria-inoculated pigs. No bacteria were cultured from pigs inoculated with PBS/BSA.

Lung weight expressed as a percentage of total body weight was significantly higher for A pleuropneumoniae-inoculated pigs, compared with sham-inoculated pigs (P < 0.001). Lung weights of endotracheally inoculated pigs were not significantly different (P = 0.12) from those of intranasally inoculated pigs.

WBC counts—Except for band neutrophils, significant differences were not observed in total or differential leukocyte counts between samples obtained before bacterial inoculation and those obtained 18 to 24 hours after inoculation. Band neutrophil cell counts were significantly (P < 0.001) increased 18 to 24 hours after inoculation with A pleuropneumoniae, compared with preinoculation values and those of control pigs (Fig 2A). However, when endotracheally and intranasally inoculated pigs were considered separately, the increase in band neutrophils was not significant in pigs inoculated intranasally with A pleuropneumoniae, compared with control pigs (Fig 2B).

Haptoglobin, iron, and zinc concentrations—Haptoglobin concentrations in A pleuropneumoniae-
inoculated pigs were significantly \((P = 0.01)\) increased 18 to 24 hours after inoculation, irrespective of route of administration (Fig 3), although endotracheal inoculation more consistently induced increased haptoglobin concentration than did intranasal inoculation (data not shown). Sham inoculation did not affect serum haptoglobin concentration.

Serum zinc and iron concentrations were profoundly \((P < 0.001)\) decreased in pigs 18 to 24 hours after inoculation with \textit{A pleuropneumoniae}, irrespective of route of inoculation, compared with preinoculation values. Serum iron and zinc concentrations were not affected by sham inoculation. A significant linear relationship between time and decreased serum zinc \((P = 0.005)\) and iron \((P = 0.029)\) concentrations was detected (Fig 4). Iron concentrations decreased rapidly at 4 to 8 hours and remained low for the duration of the 24-hour study. Zinc concentrations decreased steadily during the study.

Histologic and immunohistochemical findings—Microscopic examination of lung tissue sections from \textit{A pleuropneumoniae}-inoculated pigs revealed microscopic hemorrhage, fibrinous pleural exudate, phlebothrombosis, and alveoli filled with fibrin and necrotic cellular debris (Fig 5). Interlobular septa were thickened and contained macrophages and neutrophils that stained with antihuman histiocyte and antilysozyme antibodies (Fig 6); areas of necrosis also contained numerous degenerating cells that were difficult to identify. Preferential staining of macrophages by antihuman histiocyte antibody was apparent. Antineurofilament control antibody did not react with tissues from inoculated pigs. Lung tissues from control pigs were microscopically normal and contained few cells that reacted with antihuman histiocyte or antilysozyme antibodies.

Inflammatory cytokine expression—Messenger RNA of cytokines IL-1\(\alpha\), IL-1\(\beta\), and IL-8 was detected 48 hours after inoculation with \textit{A pleuropneumoniae} in 2 of 4 pigs (Fig 7); these cytokines were not detected in tissues from control pigs, and TNF mRNA was not detected in any pigs.

Discussion

Increased respiratory rate and dyspnea were the most commonly observed clinical signs of disease with both routes of infection. Signs of disease were apparent earlier in pigs inoculated endotracheally, developing within 2 hours, compared with 6 to 8 hours for pigs inoculated intranasally. Endotracheal administration directly instilled bacteria into the lung, which bypassed the mechanical barriers of the nasopharynx. Furthermore, intranasal administration may result in large portions of the inoculum being swallowed, consistent with the vomiting observed in these pigs. These factors could account for the earlier appearance of clin-
ical signs in pigs inoculated endotracheally. Acute measures of infection may be less variable between animals following endotracheal administration because of the short lag period between administration and the observed effects.

That this infection model bypasses the upper portions of the respiratory tract is an important consideration in its use for measuring protective immunity induced by vaccination. Intratracheal challenge negates any confounding contribution of local innate immunity in the upper portions of the respiratory tract to protective immunity. However, a comparison of endotracheal to aerosol challenge could be used as a tool to characterize specific components of innate and protective immunity.

Endotracheal administration was superior to intranasal inoculation for several reasons. First, endotracheal administration consistently induced signs of disease in all inoculated pigs and resulted in uniform bilateral lung lesions. By comparison, in 1 study that used intranasal administration, only 25% of inoculated pigs had clinical signs of disease 20 hours after inoculation; intranasal administration also often results in unilateral lesions. Bilateral lung lesions permit the option of using 1 lung for tissue collection and bacteriologic culture and the other lung for collection of lavage fluid and cells. If lung lesions are not distributed equally in the lungs, it is not possible to assume that pathophysiologic conditions are equivalent in each lung. In addition, inflammatory cytokines were detected in only 2 of 4 pigs inoculated intranasally in the study reported here, which further indicates the variability of results obtained after intranasal inoculation. Results of another study indicated that endotracheal inoculation resulted in a reproducible and predictable inflammatory cytokine response, with rapid increases in concentrations of IL-1 and IL-8, but not TNF, after bacterial inoculation. The second advantage of endotracheal administration is decreased stress, compared with pigs that were inoculated intranasally; endotracheally inoculated pigs were lightly anesthetized, whereas intranasally inoculated pigs were not anesthetized. Blood samples were obtained from endotracheally inoculated pigs without excessive restraint, which resulted in less tissue injury and RBC hemolysis. This is especially important if the hemoglobin-binding method is used to determine haptoglobin concentration. In addition, the endotracheal tube was easily positioned and the inoculum consistently delivered as a dispersed mist via the tube’s CO₂ port.

Acute A pleuropneumoniae infection did not cause increased total WBC counts at 24 hours after inoculation, perhaps because this period was too brief for a WBC response. Alternatively, an acute severe infection may deplete leukocytes at the same rate at which they are replaced in the blood, resulting in total WBC count that remains unchanged. Our findings were in disagreement with reports indicating substantial increases of total WBC counts after intratracheal inoculation with sonicated A pleuropneumoniae serotype 5 in germ-free pigs or intrabronchial administration of live A pleuropneumoniae. These differences may be attributed to variation between sonicated bacteria and intact live organisms or the difference between germ-free and conventional pigs. We observed increased percentages of band neutrophils at 24 hours in pigs inoculated endotracheally, which represents a left shift (release of immature cells into the circulation) and is consistent with bacterial infection and experimentally induced pleuropneumonia. Thus, band neutrophil counts provide information regarding A pleuropneumonia infection that is not provided by total WBC count.

Profound reductions in serum zinc and iron concentrations (50 and 64%, respectively) were observed within 8 hours of inoculation and were not dependent on route of inoculation. Van Leengoed and Kamp described similar reductions in an endobronchial model of pleuropneumonia, with 54 and 80% reduction in serum zinc and iron concentration, respectively, compared with preinoculation values. It has been suggested that such reductions are inhibitory to bacterial growth in plasma and are a component of the protective mechanisms of the acute-phase response. Several inflammatory cytokines, including TNF, IL-1, and IL-6, are involved in regulation of the acute-phase response; TNF and IL-1, in particular, cause reductions in plasma concentrations of these metals in rabbits. The mechanism appears to operate via cytokine induction of metallothionein, which binds the metals, effectively removing them from the circulation. Acute-phase responses may also alter iron concentrations via the induction of ferritin and the resulting cellular sequestration of iron.

Concentrations of haptoglobin, an acute-phase protein, were increased in response to A pleuropneumoniae infection, although differences from preinoculation and control pig values were not as substantial as those of serum zinc and iron concentrations. Hall et al also reported substantial increases in serum haptoglobin concentration in pigs with experimentally induced and naturally developing pleuropneumonia. In the study reported here, antibodies directed against porcine haptoglobin were not available, so human reagents that cross-react with pig haptoglobin were used; lack of porcine-specific reagents may have resulted in lowered assay sensitivity.

Infiltrating macrophages and neutrophils are associated with acute porcine pleuropneumonia. The unambiguous identification of these cell types is complicated by a lack of specific antibodies that react in paraformaldehyde-fixed tissues. In our study, an antibody directed against human histiocytes was used to identify macrophage-like cells in lung lesions from pigs inoculated with A pleuropneumoniae; positive staining was also observed with antilysozyme antibodies. The antihuman histiocyte antibody preferentially stained mononuclear cells, whereas the antilysozyme antibody reacted with segmented and mononuclear cells.


AJVR, Vol 61, No. 6, June 2000 689
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