Inflammation during Acute Influenza in Pigs: A Possible Model for Humans?

Human influenza viruses. Typical outbreaks involve an abrupt onset of fever, anorexia, tachypnea, dyspnea, and coughing. Considerable economic losses result from growth retardation or weight loss. Experimental viral infections of pigs have documented massive viral replication in lung epithelial cells, accompanied by polymorphonuclear leukocyte infiltration and epithelial degeneration [1]. However, the exact mechanisms by which SI virus produces lung pathology and disease have not been studied.

During respiratory virus infections, interferon-α (IFN-α), tumor necrosis factor-α (TNF-α), and interleukin-1 (IL-1) are among the first cytokines to be produced. These three cytokines have been demonstrated in bronchoalveolar washings from influenza virus–infected mice [2–4]. TNF-α and IL-1 play preeminent roles in neutrophil migration to sites of infection and in neutrophil activation. Both cytokines can induce fever, an-

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Bronchoalveolar Interferon-α, Tumor Necrosis Factor-α, Interleukin-1, and Inflammation during Acute Influenza in Pigs: A Possible Model for Humans?

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Biologically active interferon-α, tumor necrosis factor-α (TNF-α), and interleukin-1 (IL-1) were detected in bronchoalveolar lavage (BAL) fluids of 3-week-old cesarian-derived colostrum-deprived pigs inoculated with H1N1 influenza virus. Cytokine titers and lung virus titers were significantly higher 18–24 h after inoculation than at 48–72 h after inoculation in all 4 litters of pigs examined. All three cytokines were positively correlated with a 3- to 4-fold increase in BAL cell numbers (P < .036) and with a drastic neutrophil infiltration (24%–77% of BAL cells vs. 0–1.5% in controls) (P < .001). In addition, cytokine production coincided with the onset of general and respiratory symptoms of influenza and with the development of a necrotizing bronchopneumonia. This study is the first demonstration of TNF-α and IL-1 in BAL fluids of a natural influenza virus host. It documents that pigs may be a highly valuable experimental model in human influenza virus pneumonia.
orexia, and weight loss, particularly if produced at higher levels [5]. Evidence for a role of TNF-α and IL-1 in influenza virus pathogenesis and disease is growing [4, 6].

The pathogenesis of human influenza has been studied almost exclusively in volunteers and in small laboratory animals. Pigs, unlike mice and guinea pigs, are natural influenza virus hosts, and their lungs resemble those of humans in many physiologic aspects. They may thus represent a highly valuable experimental model.

Here we studied the production of IFN-α, TNF-α, and IL-1 in the lungs of pigs and its relation with disease or inflammation during the acute stage of influenza virus infection. Furthermore, we assessed the usefulness of pigs as a biomedical model in human influenza virus research.

Materials and Methods

Experimental design, virologic examinations, and lung inflammatory parameters. Four litters (18 total) of 3-week-old cesarian-derived colostrum-deprived CDCD pigs were used. They were inoculated intratracheally with 10^7 EID_{50} of influenza virus (H1N1 A/Sw/Belgium/1/83), third passage in embryonated eggs. Control pigs were left either uninoculated or inoculated with PBS or with sterile allantoic fluid (table 1). Inocula contained <1.25 endotoxin units/mL in the limulus assay. Pigs were euthanized at 18, 24, 48, or 72 h after inoculation with virus or at 24 h after inoculation with PBS or allantoic fluid. Gross lung lesions were scored by visual inspection. Samples from the left apical and diaphragmatic lung were collected for standard histopathology, influenza virus titrations, and fluorescent antibody stainings [7]. The right lung was lavaged with 60 mL of PBS. Bronchoalveolar lavage (BAL) cells were separated by centrifugation at 400 g and counted, and cytospin preparations were stained with DiffQuik (Baxter, Düdingen, Switzerland) to determine percentage of neutrophils. Cytokine bioassays. Cell-free BAL fluids were concentrated 10 times by dialysis against 20% polyethylene glycol (molecular weight, 20,000) and cleared of residual virus by ultracentrifugation at 100,000 g. Bioassays were done with 2-fold dilutions of BAL fluid samples in 96-well microtiter plates. IFN-α activity was determined in a cytopathic effect reduction assay by use of MDBK cells and vesicular stomatitis virus [8]. Percentage of cytopathic effect was determined spectrophotometrically after staining with neutral red. The number of units of IFN-α activity per milliliter was defined as the reciprocal of the dilution producing 50% inhibition of cytopathic effect. IFN-α specificity was demonstrated by neutralization of samples with rabbit antiserum against recombinant porcine IFN-α (gift from C. La Bonnardière, Jouy en Josas, France). TNF-α was assayed as cytotoxic activity in PK(15) subclone 15 cells (gift from G. Bertoni, Bern, Switzerland) in the presence of actinomycin D [9]. The plates were stained with crystal violet and read spectrophotometrically. The number of units of TNF-α per milliliter was defined as the reciprocal of the dilution producing 50% cytotoxicity. TNF-α specificity was established by neutralization with rabbit anti-human TNF-α (Innogenetics, Zwijndrecht, Belgium). IL-1 was measured as proliferative activity in D10(N4)M cells in the presence of concanavalin A and recombinant human IL-2 [10]. Percentage of proliferation was quantitated by the MTT method, and optical densities were measured. The

| Group | Inoculation | Euthanasia (h after inoculation) | Clinical symptoms (h after inoculation)* | Virus titer (log 10 EID_{50}/g of lung) | BAL cells (×10^6) | BAL cytokine titers (biologic units/mL) |
|-------|-------------|---------------------------------|--------------------------------------|------------------------------------------|-------------------|--------------------------------------|
|       |             |                                 | Apical | Diaphragmatic | Total | Neutrophils (%) | IFN-α | TNF-α | IL-1 |
| 1     | None        | 24                              | None   | Neg           | Neg   | 60            | 0.5   | —     | —     |
|       | H1N1        | 24                              | 17–    | 8.2           | 8.7   | 160           | 77    | 176,600 | 370 | 535 |
|       | H1N1        | 48                              | 24–    | 7.7           | 6.4   | 90            | 11    | 13,700  | 225 | —     |
|       | H1N1        | 72                              | 24–    | 6.4           | 7.7   | 33            | 15    | 9100    | 95  | 75   |
| 2     | PBS         | 24                              | None   | Neg           | Neg   | 35            | 1.5   | —      | —   | —    |
|       | H1N1        | 18                              | 18–    | 9.0           | 9.0   | 132           | 60    | 140,400  | 225 | 245  |
|       | H1N1        | 24                              | 22–    | 7.2           | 8.4   | 132           | 38    | 13,300   | 30  | 145  |
|       | H1N1        | 48                              | 24–    | 5.5           | 7.7   | 80            | 5     | 400     | —   | —    |
|       | H1N1        | 72                              | None   | 6.0           | 7.0   | 76            | 2     | 1000    | —   | —    |
| 3     | Allantoic fluid | 24                          | None   | Neg           | Neg   | 54            | 0     | —       | —   | —    |
|       | H1N1        | 18                              | None   | 10.2          | 9.7   | 112           | 46    | 27,800   | 95  | 520  |
|       | H1N1        | 24                              | 20–    | 6.4           | 8.7   | 147           | 56    | 337,400  | 175 | 170  |
|       | H1N1        | 48                              | 22–    | 7.5           | 8.2   | 35            | 7     | 1100    | —   | —    |
|       | H1N1        | 72                              | 22–36  | 6.7           | 6.5   | 45            | 6     | 34,200   | —   | —    |
| 4     | Allantoic fluid | 24                          | None   | Neg           | Neg   | 60            | 1     | —       | —   | —    |
|       | H1N1        | 18                              | None   | 8.4           | 7.7   | 40            | 24    | 27,800   | 25  | 150  |
|       | H1N1        | 24                              | None   | 7.4           | 8.0   | 43            | 8     | 11,000   | —   | —    |
|       | H1N1        | 48                              | 18–    | 5.7           | 8.2   | 64            | 0.5   | 2900    | —   | —    |

NOTE. Individual data from pigs belonging to 4 different litters (groups) are given. —, no cytokine response detected; neg, negative.
* Where range has no ending value, symptoms persisted until euthanasia.
Results

Clinical responses, influenza virus titers, BAL cell numbers, percentage of neutrophils, and cytokine titers of individual pigs are summarized in table 1.

Clinical symptoms. Control pigs remained healthy. In influenza virus–inoculated pigs, lethargy, shivering, anorexia, tachypnea, and labored abdominal respiration developed between 18 and 24 h after inoculation. Recovery started between 48 and 72 h after inoculation. Pigs in group 1 were most severely affected.

Influenza virus replication. Control pigs tested negative for virus. Virus titers and immunofluorescence scores were similar in the 4 influenza virus–inoculated groups (P > .577). Titers in apical and diaphragmatic lung lobes were significantly higher 18–24 h after inoculation than at 48–72 h after inoculation (P < .016 and .008, respectively). Fluorescence was evident in all sections examined. Eighteen and 24 h after inoculation, bronchi/bronchioli and alveoli had, respectively, 90% and 30% of their epithelial cells fluorescing. By 48–72 h, more of the alveolar tissue became involved.

Lung inflammatory changes. Control pigs did not have macroscopic or microscopic lung pathology. BAL cell numbers were between 35 and 60 × 10^6. Fewer than 1.5% of cells were neutrophils; >95% had macrophage morphology.

After influenza virus inoculation, gross lung lesions appeared between 48 and 72 h after inoculation. At that time, ~85%, 18%, 18%, and 8% of lung tissue was affected in groups 1, 2, 3, and 4, respectively. On histopathology, bronchi/bronchioli and, to a lesser degree, alveoli showed epithelial necrosis and massive neutrophil infiltration at 18–24 h after inoculation. Forty-eight and 72 h after inoculation, bronchioli and alveoli were filled with exudate containing necrotic debris and macrophages and only few neutrophils. Histologic changes were most dramatic in group 1. BAL cell numbers were between 112 and 160 × 10^6 at 18–24 h after inoculation and consisted of a maximum of 56%–77% neutrophils in groups 1, 2, and 3. In group 4, cell numbers did not exceed 43 × 10^6 and neutrophil infiltration was only 24%.

Cytokine production. While controls were negative, IFN-α, TNF-α, and IL-1 were detected in the 4 virus-infected groups. IFN-α was detected from 18 through 72 h after inoculation, with maximal levels between 18 and 24 h. Peak IFN titers were 176,600, 140,400, and 337,400 U/mL in groups 1, 2, and 3, respectively. In group 4, IFN titers peaked at only 27,800 U. TNF-α was detected at 18 or 24 h (or both) after inoculation in all 4 groups. By 48 and 72 h after inoculation, TNF-α was found only in pigs from group 1. Peak TNF titers were 370, 225, and 175 U/mL in groups 1, 2, and 3, respectively, but only 25 U/mL in group 4.

IL-1 activity was found 18 or 24 h (or both) after inoculation in all groups. Maximum IL-1 titers were 553, 245, 520, and 150 U/mL in the 4 respective groups. Only in group 1 pigs was IL-1 also found at 72 h after inoculation. Levels of the three cytokines were significantly higher 18–24 h after inoculation than at 48–72 h after inoculation (P < .016 for all three cytokines). Significant correlations were noted between production of all three cytokines and BAL cell numbers (P < .036 for each) and percentage of neutrophils (P < .001 for each). Cytokine production coincided with the onset of clinical disease and development of bronchopneumonia.

Discussion

At the start of this study, it was unknown whether the lungs of 3-week-old CDCD pigs are fully capable of TNF-α and IL-1 production. Thus far, TNF-α and IL-1 have only been demonstrated in the lungs of pigs 6–8 weeks old and older, either conventional [11] or gnotobiotic [12]. In our study, the use of CDCD pigs was required, since we regularly detect TNF-α and/or IL-1 in BAL fluids from conventional pigs in the absence of experimental viral infections. The age of 3 weeks was selected for practical reasons. Because sanitary status and age may influence cytokine production, we performed a preliminary experiment in 3-week-old CDCD pigs. Two pigs were inoculated intratracheally with 17 mg of Escherichia coli O111: B4 lipopolysaccharide, a known potent TNF-α and IL-1 inducer. BAL fluids collected 6 and 12 h after inoculation revealed TNF titers of 184 and 128 U/mL and IL-1 titers of 564 and 596 U/mL in the respective bioassays. Consequently, 3-week-old CDCD pigs were found suitable for further influenza virus–cytokine studies.

To our knowledge, this is the first demonstration of influenza virus–induced TNF-α and IL-1 in BAL fluids of a natural virus host. Interestingly, influenza virus was an equally effective inducer of TNF-α and IL-1 as was E. coli endotoxin. TNF-α and IL-1 have remarkably overlapping and synergistic effects, several of which are consistent with clinicopathologic manifestations of influenza [5]. Our findings in pigs are in agreement with previous reports in mice [3, 4] and further substantiate the
hypothesis that TNF-α and IL-1 contribute to clinicopathologic effects of influenza. First of all, both cytokines were positively correlated (ρ > .879) with neutrophil recruitment to the lungs, and peak cytokine production coincided with the onset of clinical disease. Furthermore, there was a clear association between individual TNF levels on the one hand and the extent of neutrophil infiltration and severity of lung pathology on the other. Group 1 pigs, which had consistently higher values of TNF than the other groups, showed most severe disease and lesions. Group 4 pigs, with barely detectable TNF levels, also had lower neutrophil infiltration than the other groups.

Interferon-α was detected at extremely high titers in some pigs. As for TNF-α and IL-1, IFN-α production was tightly correlated (ρ > .879) with neutrophil infiltration to the lungs, and peak IFN production coincided with the appearance of illness. These findings support the idea that interferons contribute to constitutional and other effects during influenza. Indeed, IFN-α is intrinsically pyrogenic [13] and can mediate neutrophil migration and stimulation. Besides, it has been shown in vitro that IFN-α may enhance the neutrophil respiratory burst to influenza virus [14]. Given the excessive amounts of IFN-α in pig BAL fluids, IFN could considerably add to the pyrogenic and inflammatory effects of TNF-α and IL-1.

In group 4, overall cytokine production was lower than in the other groups. Most striking was the virtual lack of TNF production. Differences in cytokine production between this and previous groups cannot be attributed to differences in viral multiplication. One possibility is that genetic components contribute to decreased cytokine production, since group 4 pigs belonged to a genetically different line. It would be worthwhile to examine a larger number of genetically different pigs for their cytokine responses on influenza infection and clinical outcomes.

Although influenza viruses in humans primarily infect the upper respiratory tract, influenza pneumonia yearly causes high mortality in patients with cardiopulmonary disease and in the elderly. Experimental research thus remains of high priority, and commonly used animal models have some limitations. In ferrets, for example, upper respiratory tract infection predominates, and alveolar infection and pneumonitis are less pronounced [15]. Mice and guinea pigs, on the other hand, are not natural hosts, and influenza virus strains have to be adapted to them. Also, mice show a fall in body temperature instead of fever and, with more virulent strains, the infection is invariably lethal. In this study, pigs—as natural hosts—have shown to be valuable animal models for research on human influenza.

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