Destruction of Lymphocytes by a Virulent Avian Influenza A Virus

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SUMMARY
Infection of chickens by a virulent avian influenza A virus, A/turkey/Ont/7732/66 (H5N9), was associated with a severe lymphopenia. High titres of infectious virus were found in lymphoid tissues early in infection and were accompanied by severe damage to the lymphocyte populations as demonstrated by histopathological examination. Non-lymphoid cell populations in these tissues were unaffected, as were other organs examined. The viral nucleoprotein was localized by immunoperoxidase staining to lymphocytes in affected tissues early in infection.

Virulent influenza A viruses periodically arise in nature and cause devastating losses to the poultry industry. These virulent strains spread systemically and kill their hosts rapidly (Kawaoka et al., 1987; Narayan et al., 1972). A recent example of the advent of a virulent influenza A virus was the 1983 to 1984 outbreak of A/chicken/Pennsylvania/1370/83 in the U.S.A. in which more than 60 million U.S. dollars was expended for eradication (Kawoaka et al., 1984). The magnitude of these losses makes the prevention and control of virulent avian influenza of particular importance to both poultry producers and consumers. Understanding the mechanisms by which these viruses produce severe disease is necessary to the development of appropriate preventative measures.

The virulence of influenza A viruses has been extensively examined at the molecular level (Bosch et al., 1979; Kawaoka et al., 1984), but the mechanisms of virulence in relation to the disease produced in the host have received little attention. The progression of the virus in its host, the target tissues of the virus, and the damage caused by the virus are not clearly defined. Highly virulent avian influenza viruses produce a viraemia and virus is recovered from a large number of organs (Kawaoka et al., 1987; Narayan et al., 1972). However, it is not clear which tissues are actually affected by the viruses since direct examination of the individual tissues for evidence of viral replication and related cytopathology has been limited. To elucidate these aspects, we examined the effect of a highly virulent avian influenza virus, A/turkey/Ont/7732/66 (H5N9) (Ty/Ont), on different tissues in chickens. We found that this virus produces marked effects on the lymphoid tissues.

The virus (Lang et al., 1968) was grown in 10 to 11-day-old embryonated chicken eggs from the first egg passage of virus isolated from the lung of an infected turkey. Virus stock was grown in 10-day-old embryonated chicken eggs and the allantoic fluid with a titre of $10^{8.2}$ EID$_{50}$/ml was frozen at $-70^\circ$ C until use.

White Leghorn chickens (10 to 11 weeks old) were obtained from a closed flock that was serologically negative for influenza and Newcastle disease viruses. Groups of chickens were inoculated intratracheally with $10^2$ EID$_{50}$ of Ty/Ont or of the brain heart infusion broth used to dilute the viruses (referred to as mock-inoculated controls). Inoculated chickens were observed at 12 h intervals for morbidity and mortality. Chickens inoculated with Ty/Ont were bled and killed at 12 h intervals. Total white blood cell (WBC) counts were determined using 0.01% toluidine blue in phosphate-buffered saline (Zinkl, 1986). Blood smears were stained with...
Diff-Quik (American Scientific Products) for differential WBC counts. The total number of lymphocytes per μl of blood was calculated by multiplication of the percentage of lymphocytes determined from the differential WBC count by the total WBC per μl. To confirm infection, tracheal and cloacal swabs were taken, placed into transport medium and inoculated into embryonated chicken eggs. Each chicken was necropsied and examined for gross lesions. Samples of spleen, thymus, bursa, liver, pancreas, caecum, kidney, lung, heart, brain and blood were collected, divided and either frozen in liquid nitrogen for fluorescent antibody studies, frozen on dry ice for virus titration or fixed in 10% buffered formalin for histopathological examination. Tissues placed into 10% buffered formalin were processed and embedded in paraffin within 48 h of collection; sections were stained with haematoxylin and eosin for examination by light microscopy.

To determine the level of infectious virus, tissues were weighed, disrupted with a Stomacher homogenizer (Tekmar, Cincinnati, Ohio, U.S.A.) and titrated for infectious virus in embryonated chicken eggs as previously described (Hinshaw et al., 1978). After 48 h incubation at 35 °C, the presence of virus in allantoic fluid was detected by haemagglutination with chicken erythrocytes.

Frozen sections of tissues from inoculated and control chickens were fixed in methanol: acetone (1:1), then stained by the indirect fluorescent antibody technique using a monoclonal antibody to influenza virus nucleoprotein (NP), 5/1 (van Wyke et al., 1980), supplied by R. G. Webster (St Judes Children’s Research Hospital, Memphis, Tenn., U.S.A.). For immunoperoxidase staining, paraffin-embedded tissues were deparaffinized and viral antigen was detected using the avidin–biotin–immunoperoxidase method (ABC Kit, Vector Laboratories, Burlingame, Ca., U.S.A.). The monoclonal antibody to NP at 1:800 dilution was used as the primary antibody. Sections were counterstained with haematoxylin and examined by light microscopy.

Of the chickens inoculated with 10^3 EID_{50} of Ty/Ont intratracheally the first died 36 and 48 h post-inoculation (p.i.) and by 132 h p.i. all of the chickens were dead. These birds had signs of disease by 12 h p.i., i.e. they were less active than the mock-inoculated controls. By 24 h p.i., most birds had developed watery diarrhoea. This was followed by depression, lethargy and anorexia. Terminally, weakness, ruffled feathers and shivering were observed. Moribund chickens had extreme swelling of the soft tissues of the head and cyanotic discolouration of their combs. Signs of central nervous system involvement such as torticollis or convulsions were not observed.

Titration of tissues determined that high levels of virus early in infection (12 h p.i.) were found in heart, lung, kidney, and lymphoid tissues such as the spleen, thymus and bursa (Table 1) suggesting that the virus involves tissues other than the respiratory tract early in infection. Increased levels of virus in the blood occurred somewhat later. The virus spread rapidly so that by 44 h p.i. it was recovered at levels ranging from 10^5 to 10^8 EID_{50}/g from many other tissues including heart, pancreas, liver, kidney and brain.

The effect of the virus infection on the peripheral blood lymphocytes was examined. Chickens were bled every 12 h and the total number of WBCs and differential cell counts were determined. The total number of WBCs/μl (average of 15 400/μl at 24 h p.i.) of blood of chickens inoculated with Ty/Ont was greater than or equal to that of the controls from 24 to 120 h p.i. (average of 9600/μl 24 h p.i.). In contrast, the differential blood counts revealed marked changes in the number of circulating lymphocytes. Chickens inoculated with Ty/Ont developed a severe lymphopenia compared with the mock-inoculated controls (Fig. 1). This decrease in the total number of circulating lymphocytes was evident 12 h p.i. and was sustained until the death of the birds. The mock-inoculated control chickens had a transient decrease in the number of lymphocytes (Fig. 1) which contrasted with the sustained reduction observed with the chickens infected with Ty/Ont.

To examine the effect of the virus on lymphocytes in lymphoid organs, tissues from chickens killed at 12-h intervals were processed for histopathological examination. Most notable was the severe necrosis of lymphocytes in the spleen, thymus, bursa and the lymphoid tissue of the intestine and lung. As shown in Fig. 2(a), the spleen had multifocal areas of lymphoid necrosis.
Fig. 1. Effect of viral infection on the level of circulating lymphocytes. Birds were inoculated intratracheally with 10^3 EID_{50} of Ty/Ont (●) or brain heart infusion broth (■). Birds were bled at the specified times p.i. and peripheral lymphocytes quantified (10^3/μl) as described in the text.

Table 1. Levels of infectious virus (log_{10} EID_{50}/g) recovered from chickens inoculated with A/Ty/Ont/7732/66

<table>
<thead>
<tr>
<th>Tissue</th>
<th>12</th>
<th>24</th>
<th>38</th>
<th>44</th>
<th>52</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen</td>
<td>3.8</td>
<td>4.6</td>
<td>6.8</td>
<td>8.4</td>
<td>7.9</td>
<td>8.3</td>
</tr>
<tr>
<td>Bursa</td>
<td>3.6</td>
<td>3.3</td>
<td>6.1</td>
<td>5.9</td>
<td>7.0</td>
<td>7.4</td>
</tr>
<tr>
<td>Thymus</td>
<td>2.9</td>
<td>3.0</td>
<td>7.3</td>
<td>7.4</td>
<td>7.8</td>
<td>7.5</td>
</tr>
<tr>
<td>Caecal tonsil</td>
<td>0†</td>
<td>4.9†</td>
<td>6.7†</td>
<td>7.6</td>
<td>7.8†</td>
<td>7.1†</td>
</tr>
<tr>
<td>Lung</td>
<td>5.1</td>
<td>4.4</td>
<td>6.8</td>
<td>7.8</td>
<td>7.3</td>
<td>7.3</td>
</tr>
<tr>
<td>Blood</td>
<td>1.6</td>
<td>2.7</td>
<td>4.4</td>
<td>4.7</td>
<td>3.9†</td>
<td>5.3†</td>
</tr>
<tr>
<td>Brain</td>
<td>1.8</td>
<td>1.1</td>
<td>4.9</td>
<td>5.1</td>
<td>7.6</td>
<td>5.8</td>
</tr>
<tr>
<td>Heart</td>
<td>4.6</td>
<td>4.5</td>
<td>4.7</td>
<td>7.1</td>
<td>7.1</td>
<td>6.4</td>
</tr>
<tr>
<td>Pancreas</td>
<td>2.7</td>
<td>4.6</td>
<td>6.7</td>
<td>7.4</td>
<td>8.1</td>
<td>7.9</td>
</tr>
<tr>
<td>Liver</td>
<td>2.4</td>
<td>4.6</td>
<td>5.2</td>
<td>5.7</td>
<td>6.5</td>
<td>5.9</td>
</tr>
<tr>
<td>Kidney</td>
<td>5.0</td>
<td>4.5</td>
<td>5.9</td>
<td>6.1</td>
<td>6.9</td>
<td>6.1</td>
</tr>
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</table>

* The chickens were inoculated intratracheally with 10^3 EID_{50} of virus. Samples were collected at the times listed and homogenized in a Stomacher blender; virus was assayed as described previously (Hinshaw et al., 1978).
† Value from one sample. All other values are the average of two samples.

The damage to the lymphoid tissues corresponded with the high levels of infectious virus found in these same organs suggesting a detrimental effect of the virus on lymphoid tissue.

Since influenza A virus is often associated with respiratory disease, the lungs of the chickens were examined closely for evidence of damage due to viral infection. Characteristic pathological changes included infiltration of heterophils and mononuclear cells into the peribronchiolar tissue with extension into adjacent air capillaries. A prominent feature was the necrosis of lymphoid nodules present in the lamina propria of the bronchioles as illustrated in Fig. 2(b). In contrast, the respiratory epithelium of the airways was relatively spared. This indicated that, even in the respiratory tract, the predominant change involved damage to the lymphoid tissue present in this organ. Significant lesions in other tissues including the brain, kidney, liver, proventriculus, gizzard, heart and skeletal muscle were not detected by histopathological evaluation. Thus histopathological examination indicated that lymphoid tissues were the most severely damaged as a consequence of viral infection.
Spleens from infected birds were examined for the presence of viral antigen using the monoclonal antibody to the NP for immunofluorescent and immunoperoxidase staining (not shown). There was intense fluorescence of the necrotic lymphoid areas of the spleen. Viral nucleoprotein was detected by immunoperoxidase staining of lymphocytes, identified morphologically by their characteristic nuclear chromatin pattern and scant cytoplasm. In contrast, there was little staining observed in non-lymphoid cells in the spleen and other tissues.
Specific staining of lymphocytes was also found in the peribronchial lymphoid tissue in the lung, and the lymphoid tissue in the caecum. Thus, it seemed that the virus reached lymphoid cell populations in a variety of tissues and was associated with the necrosis of these cells. Whether viral replication in the lymphocytes themselves is responsible for the destruction of these cells is not yet clear. The presence of NP in lymphocytes suggests that viral replication occurs in these cells, but further studies are required to confirm this observation.

Our results are supported by previous experiments with this virus in turkeys by Narayan et al. (1972) who found that high titres of virus developed early in the spleen and other lymphoid tissues and were later followed by virus in the blood and then in other organs. Histopathological examination of tissues from the turkeys also indicated an early and widespread necrosis of the lymphoid tissues in the spleen, lung, thymus, bursa and gastrointestinal tract. Other cells may be involved in viral replication. However, major damage related to viral infection occurs in the lymphoid tissues of the host.

Infection with Ty/Ont also affects lymphocytes in the blood resulting in a severe lymphopenia. Lymphopenia can be produced by infection with many other viruses and by other factors including the stress of being handled (Gross & Siegel, 1984). The latter may explain the transient lymphopenia observed in the mock-inoculated controls. In contrast, the lymphopenia in chickens infected with Ty/Ont was severe and sustained until the death of the bird. It is also known that viral infections can alter lymphocyte distribution so that the number of lymphocytes increases in organs and decreases in the blood. In this study, an infiltration of lymphocytes in organs from birds infected with Ty/Ont was not observed, suggesting that circulating lymphocytes were not redirected. Rather, the necrosis of lymphoid cells suggests that virus-associated killing of lymphocytes is responsible for the lymphopenia produced by this virus.

Although our results indicate that infection with Ty/Ont is related to a profound effect on lymphoid tissues, the role of lymphoid destruction in the pathogenesis of Ty/Ont infection is not clear. Similar effects on lymphocytes have not been described for other highly virulent influenza A viruses. In a recent study of chickens from a field outbreak of A/chicken/Pennsylvania/1370/83 encephalitis, myocarditis, myositis and pancreatitis were reported as the primary lesions (Acland et al., 1984). Lymphoid tissues were not a major target tissue of this virus. It is likely, therefore, that virulent strains of influenza A virus differ in their effect on the lymphoid tissues of their hosts. Currently, we are investigating the basis for lymphocyte destruction with an attenuated variant of Ty/Ont. In addition to reduced mortality in chickens infected with the variant, lymphoid tissues from these birds do not undergo necrosis despite the ability of the variant to spread systemically. Studies with the variant may indicate the viral gene responsible for lymphocyte destruction and elucidate the basis for the lymphoid necrosis.

Our work suggests that the destructive effect of Ty/Ont on lymphoid tissues may be one factor in the pathogenesis of this particular virus. A better understanding of the mechanisms of this effect may enable us to understand better the consequences of influenza A infection in the avian host.

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REFERENCES


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