Appearance of Influenza A Virus Antigenic Variants after Treatment of Infected MDCK Cells with Human Leukocytes

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(Accepted 5 May 1988)

SUMMARY

Exudation of polymorphonuclear leukocytes (PMN) from the infected mucosa is a characteristic feature of influenza virus infection. Since reactive oxygen species generated by PMN can be strong mutagens, the possibility of production of antigenic variants of the virus by virus–PMN interaction was investigated. Cloned influenza A NWS (H1N1) virus multiplying in Madin–Darby canine kidney cells was treated with human peripheral PMN. Assays in the presence and absence of monoclonal antibody to the cloned virus showed a seven- to ten-fold increase in the frequency of variants in the presence of PMN. The mutagenic effect was abolished by addition of superoxide dismutase to the culture.

INTRODUCTION

After intranasal inoculation of ferrets with influenza A virus strong inflammatory cellular responses occur on the infected nasal mucosa (Matsuyama et al., 1980). At the peak of the inflammatory response (48 to 60 h after inoculation), more than 10⁷ cells were found in each nasal wash (10 ml) taken at 4 or 6 h intervals. Polymorphonuclear leukocytes (PMN) predominated (90%) among the exuded cells (Toms et al., 1977). PMN exudation is a characteristic feature of influenza virus infection in humans as well as ferrets (Sweet & Smith, 1980). Indeed, the pyrexia commonly observed during influenza virus infection is probably due to release of a leukocyte pyrogen from the exuded PMN (Sweet et al., 1979).

After encounters with bacteria and some viruses, PMN are known to generate reactive oxygen species, such as superoxide anions, hydrogen peroxide and hydroxyl radicals. These reactive oxygen species induce damage to DNA (Repine et al., 1981; Birnboim, 1982) and hence are potential mutagens or carcinogens. In support of this, an increased mutation rate was observed in Salmonella typhimurium auxotrophic strains by conducting an Ames test in the presence of PMN from human peripheral blood (Weitzman & Stossel, 1981). Also, sister chromatid exchanges in cultured Chinese hamster ovary cells and malignant transformation of C3H mouse fibroblasts were enhanced in the presence of human peripheral PMN (Weitberg et al., 1983; Weitzman et al., 1985).

Hence, it is possible that mutagenic PMN exuded onto an influenza virus-infected mucosa may enhance the production of antigenic variants of the virus. To investigate this possibility, tissue culture monolayers infected with cloned influenza A NWS virus were treated with human PMN. The resulting virus was examined for resistance to neutralizing monoclonal antibody (MAb) raised against the parental NWS cloned virus.

METHODS

Virus, cells and MAbs. Influenza A NWS (H1N1) virus plaque-purified in Madin–Darby canine kidney (MDCK) cells was supplied by Dr H. Ochiai (Toyama Medical and Pharmaceutical University, Toyama, Japan). MDCK cells were cultivated in Eagle’s MEM (Nissui, Tokyo, Japan) containing 0.2% bovine serum albumin and 10 μg/ml trypsin. They were then used for preparation of working stocks of the virus clone, titration of the virus by
the plaque method (Tobita et al., 1975) and cloning of each antigenic variant produced. Mouse ascites fluids containing MAbs B-4 and I-3 (subclasses IgG1 and IgG2a, respectively) directed against NWS virus haemagglutinin were provided by Dr H. Ochiai. The fluids were treated with Vibrio cholerae receptor-destroying enzyme (Takeda Chemical Industries, Osaka, Japan) for haemagglutination inhibiting (HI) antibody titration. Assays were performed by a standard procedure (Dowdle & Coleman, 1974) in microtitre plates with 0.5% chicken erythrocytes and 4 haemagglutinating units of virus.

Preparation of PMN. Human PMN separated by centrifugation on a Ficoll-Hypaque (Mono°Poly Resolving Medium; Flow Laboratories) gradient were treated with 10 ml of 0.83% ammonium chloride solution (two or three times) to disrupt erythrocytes and washed twice with 10 ml of phosphate-buffered saline to remove serum antibodies and erythrocyte superoxide dismutase (SOD); they were then suspended in MEM at a concentration of 2 × 10⁷ cells/ml. The cell population contained more than 97% PMN and these were more than 98% viable as determined by trypan blue dye exclusion.

PMN were disrupted by two cycles of freezing (at −80 °C) and thawing (at 37 °C) or by treatment with distilled water for 2 h.

Treatment of extracellular viruses with PMN. After 24 h cultivation at 37 °C, virus-infected MDCK cells were disrupted by freezing and thawing, and centrifuged at 770 g for 10 min. Virus in the supernatants (1 × 10⁸ p.f.u./ml) was treated with PMN (1 × 10⁶ cells/ml) at 37 °C for 1 h.

Treatment of virus-infected cells with PMN. Monolayers of MDCK cells in a plastic dish (diameter 35 mm) were inoculated with NWS virus (m.o.i. 0.01). After 8 h incubation at 37 °C, the infected MDCK cells were treated with a designated number of PMN suspended in 2 ml of trypsin-containing MEM and incubated for 15 h at 37 °C. In some experiments, bovine erythrocyte SOD (Sigma) and phorbol myristate acetate (PMA; CCR, Edenprairie, Mn., U.S.A.) were used to examine the role of reactive oxygen species in antigenic changes.

Harvested virus was stored at −80 °C until analysis for antigenic variation.

Determination of the frequency of antigenic variants. The supernatant of NWS virus-infected MDCK cells was added to an equal volume of a 1/100 dilution of ascites fluid containing MAb B-4 (HI titre 1/3277). The mixtures were then incubated for 1 h at 37 °C and titrated on MDCK cells for infectious viruses that had escaped neutralization. The frequency of variants was determined by examining the ratio of the virus infective titre (p.f.u.) in the presence and absence of MAb B-4.

Statistics. Differences between means were compared by Student's t-test or the Fishe~Behrens test (Campbell, 1967). Unless otherwise indicated, variations around means were 1 s.e.m.

RESULTS

Increased production of antigenic variants from MDCK cells treated with PMN

Virus-infected MDCK cells were treated with human PMN (5 × 10⁶ cells/culture dish) and incubated for 15 h. Both the cells and the virus grew well under such conditions. The titre of harvested virus was not lower than that of the culture without PMN treatment. The virus population (2 × 10⁸ to 5 × 10⁸ p.f.u./ml) produced in the presence of PMN contained a significantly increased number of variants (Table 1). Since sera of PMN donors showed an HI titre of 60 to the cloned NWS virus, frozen and thawed PMN and osmotically disrupted PMN preparations (HI titres < 16) were examined for activity in variant production. As shown in Table 1, frozen and thawed PMN did not produce a greater number of variants than control cultures. Osmotically disrupted PMN also failed to produce a greater number of variants (data not shown). Treatment of extracellular virus (1 × 10⁸ p.f.u./ml) with PMN (1 × 10⁶ cells/ml) at 37 °C for 1 h resulted in a partial loss of titre (approx. 36% of control), but no increase in the log₁₀ frequency of variants (−6.3 ± 0.2 in the presence and absence of PMN). Thus influenza virus replicating in host cells may develop variants in the presence of living PMN.

The dose-response effect of this PMN-dependent mutagenesis is shown in Fig. 1. After treatment with 10⁷ PMN, there was no further significant change in production of variants. Total virus yields remained the same for all doses of PMN (data not shown).

Effect of PMN in the presence of SOD or PMA

The enhancing effect of PMN on the production of variants was abolished by addition of the antioxidant enzyme SOD to the infected MDCK culture (Table 2). When PMA alone was added to virus-infected MDCK cells, an increased number of variants appeared (Table 2). Because addition of PMA to the mixture of PMN and virus-infected MDCK cells reduced the virus
Influenza A virus mutation by leukocytes

Fig. 1. Frequency of antigenic variants of influenza A virus after treatment of infected MDCK cells with increasing concentrations of PMN. Data are means ± S.E.M. (bars) of more than seven determinations.

Table 1. Production of antigenic variants from influenza A virus-infected MDCK cells in the presence of PMN

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>PMN</th>
<th>Frozen and thawed PMN</th>
<th>None</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-6.1†</td>
<td>-6.4</td>
<td>-6.7</td>
</tr>
<tr>
<td>2</td>
<td>-5.9</td>
<td>-6.8</td>
<td>-6.4</td>
</tr>
<tr>
<td>3</td>
<td>-5.0</td>
<td>-6.6</td>
<td>-6.6</td>
</tr>
<tr>
<td>4</td>
<td>-5.8</td>
<td>-6.5</td>
<td>-6.5</td>
</tr>
<tr>
<td>5</td>
<td>-5.0</td>
<td>ND†</td>
<td>-5.7</td>
</tr>
</tbody>
</table>

-5.6 ± 0.2§ -6.6 ± 0.1 -6.4 ± 0.2

* log_{10} (p.f.u. determined in the presence of MAb/p.f.u. determined in the absence of MAb).
† Calculated as log_{10}(4.0 × 10^2/4.6 × 10^6).
§ Significantly different from the untreated control (P < 0.02).

Table 2. Production of antigenic variants by treatment with PMN in the presence of SOD or PMA

<table>
<thead>
<tr>
<th>Treatment conditions</th>
<th>Frequency of variants*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>-6.3 ± 0.1 (n = 12)</td>
</tr>
<tr>
<td>PMN (1 × 10^6 cells)</td>
<td>-5.3 ± 0.1 (n = 12)†</td>
</tr>
<tr>
<td>PMN (5 × 10^6 cells)</td>
<td>-5.5 ± 0.1 (n = 13)†</td>
</tr>
<tr>
<td>SOD (100 μg/ml)</td>
<td>-6.6 ± 0.1 (n = 7)</td>
</tr>
<tr>
<td>SOD (100 μg/ml) + PMN (5 × 10^6 cells)</td>
<td>-6.4 ± 0.1 (n = 7)‡</td>
</tr>
<tr>
<td>PMA (10 μg/ml)</td>
<td>-5.3 ± 0.1 (n = 14)†</td>
</tr>
<tr>
<td>PMA (10 μg/ml) + PMN (1 × 10^6 cells)</td>
<td>-5.4 ± 0.1 (n = 10)§</td>
</tr>
</tbody>
</table>

* See footnote of Table 1.
† Significantly different from no addition (P < 0.01).
‡ Significantly different from PMN (5 × 10^6 cells) addition (P < 0.001).
§ Not significantly different from addition of PMN alone.
Table 3. HI reactions of selective (B-4) and non-selective (I-3) MAb s with 96 variant clones* of NWS influenza virus

<table>
<thead>
<tr>
<th>No. of clones (%)</th>
<th>HI titre with MAb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B-4 (1024)†</td>
</tr>
<tr>
<td>90 (94)</td>
<td>&lt;32–32</td>
</tr>
<tr>
<td>2 (2)</td>
<td>128</td>
</tr>
<tr>
<td>4 (4)</td>
<td>512–1024</td>
</tr>
</tbody>
</table>

* Derived from virus grown in the presence of PMN and plaque-purified twice in MDCK cells.  
† HI titres with the parental NWS clone.

production to approximately 1/40, before treatment with MAb B-4 the harvested virus was concentrated by centrifugation. No further increase in frequency of variants was observed when both PMA and PMN were present (Table 2).

**HI reactivity of plaque-purified antigenic variants with MAb s**

Plaques were picked from PMN-treated virus that had escaped neutralization by MAb B-4. Virus from 96 arbitrarily selected plaques was plaque-purified twice in MDCK cells and examined for reactivity with the selective B-4 and non-selective I-3 MAbs by using the HI test. As shown in Table 3, most clones showed decreased HI reactivity with MAb B-4 and were therefore considered to be specific antigenic variants.

**DISCUSSION**

Human influenza A viruses are well known for the continuous generation of new antigenic variants. In contrast to most other viral infections, immunity acquired by infection or immunization soon becomes ineffective due mainly to changes in the viral haemagglutinin or neuraminidase antigens. When the appearance of variants from cells infected *in vitro* with influenza A virus, Sendai virus and vesicular stomatitis virus was examined, similar frequencies of variation were observed (Portner *et al.*, 1980). Thus, the mutation frequency of influenza A virus has been considered not to be uniquely high in comparison with other RNA viruses (Buonagurio *et al.*, 1986). Hence other factors must operate in the production of antigenic changes in influenza virus in nature.

In contrast to viruses growing in cultured cells, those infecting animal tissues *in vivo* come under the restrictive influences of the host defence activities. In influenza virus infection in humans and ferrets, exudation of PMN is one important feature of host defence on infected mucous membranes (Sweet & Smith, 1980). Since human peripheral blood PMN show respiratory bursts or chemiluminescence reactions after contact with influenza A viruses (Mills *et al.*, 1981), it is reasonable to suppose that reactive oxygen species would be generated by inflammatory PMN at the infected mucous membrane. If so, replicating influenza virus genomes in nature will be affected by strong destructive and mutagenic agents. In the experiments demonstrated here which possibly simulate the *in vivo* situation, influenza A virus replicating in cultured MDCK cells was exposed to peripheral blood PMN and examined for the appearance of antigenic variants. The results clearly indicate a mutagenic activity of PMN on intracellularly replicating virus. In contrast to the situation for whole virions in an extracellular environment, the availability of numerous copies of the segmented RNA genome to the mutating influence may facilitate the production of infectious mutant virions lacking lethally mutated RNA segments.

Although this paper provides no direct evidence of the mutagenicity of reactive oxygen species, a suppressing effect of exogenous SOD on the PMN-dependent mutagenesis was demonstrated (Table 2). The inhibiting effect of SOD on oxygen-dependent mutagenesis of DNA genomes is well-known (Weitzman & Stossel, 1982; Farr *et al.*, 1986). Thus, it seems that the RNA genome of influenza A virus is also sensitive to oxygen-dependent mutagenesis.
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In the experiments reported here, a single addition of PMN to virus-infected cells produced a significant increase in antigenic variation. In nature, the influence of the PMN may be more profound because the virus replicating in the mucosal epithelium would be under the influence of inflammatory leukocytes continuously recruited for 2 to 4 days.

We thank Dr H. Ochiai for supplying the cloned NWS strain of influenza A virus and monoclonal antibodies, Professor G. Sakaguchi for help in preparation of the manuscript and Professor H. Smith for critical reading of the manuscript and advice.

REFERENCES


(Received 25 January 1988)