Severity of Fever in Influenza: Differential Pyrogenicity in Ferrets Exhibited by H1N1 and H3N2 Strains of Differing Virulence

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SUMMARY

Intracardial inoculation of large quantities (200 μg viral protein/kg body weight) of infectious or u.v.-inactivated purified influenza viruses into ferrets resulted in a rapid febrile response which was significantly lower for two recently isolated H1N1 viruses, A/USSR/90/77 and A/Fiji/15899/83, than for two virulent clones, 7a and 64c, of the A/Puerto Rico/8/34-A/England/939/69 (H3N2) reassortant virus system. These results, which are in accord with the severity of fever produced by these strains in intranasally infected ferrets, show that influenza virus strains can differ in their capacity to induce fever (probably reflecting a differential capacity to induce endogenous pyrogen from phagocytes) and indicate, since u.v.-inactivated strains are pyrogenic, that this may be due to differences between strains in the nature or amount of certain virion components.

INTRODUCTION

Fever and the other constitutional effects of human influenza (headache, myalgia, listlessness, nausea, shivering, anorexia and depression) appear to result from the production of a pyrogen [probably endogenous pyrogen (EP), but possibly interferon] by phagocytes in response to infection (Rawlins & Cranston, 1973; Scott et al., 1981; Dinarello & Wolff, 1982; Gander, 1982). This is supported by studies of influenza in the ferret using virulent (clones 7a and 64c) and attenuated [clone 64d and A/Puerto Rico/8/34 (PR/8)] strains of the A/Puerto Rico/8/34-A/England/939/69 reassortant virus system (Toms et al., 1977; Sweet et al., 1979; Matsuyama et al., 1980). In this animal, fever has been shown to result from production of a pyrogen with the properties of EP by inflammatory phagocytes following interaction with influenza virus in the upper respiratory tract (URT) (Sweet et al., 1979).

Clinical studies of influenza during the recent H1N1 epidemics suggest that influenza viruses might vary in their ability to produce fever and the other constitutional effects. The recent H1N1 isolates produce relatively mild illness in man (Kung et al., 1978; Wright et al., 1981) and in a study of primary H3N2 and H1N1 influenza virus infections in children between 1977 and 1982, infection with the H1N1 strains resulted in a smaller proportion of cases developing fever, and lower mean febrile temperatures, than infection with H3N2 strains (Frank et al., 1984). Influenza viruses also produce differing febrile responses in ferrets. The virulent clones 7a (H3N2) and 64c (H3N2) produced a more severe fever than the attenuated clone 64d (H3N2) and strain PR/8 (H1N1) (Matsuyama et al., 1980) (Table 1). The lower febrile responses produced by clone 64d and PR/8 were probably due, respectively, to the lower titre of clone 64d found in the URT compared with the virulent strains, and the lower inflammatory response (and, therefore, fewer phagocytes available for interaction with virus) elicited by PR/8 (Toms et al., 1977; Matsuyama et al., 1980; Smith & Sweet, 1984) (Table 1). The virulent clone 64c produced a level of fever and inflammatory response similar to that produced by the virulent clone 7a, although the titre of clone 64c in the URT was slightly lower than that of clone 7a (Matsuyama et al., 1980); this suggested that it had a greater capacity than 7a to stimulate the
Table 1. Maximum levels of upper respiratory tract virus and inflammatory response and fever in ferrets inoculated intranasally with influenza strains from the A/Puerto Rico/8/34-A/England/939/69 reassortant system and recent H1N1 isolates

<table>
<thead>
<tr>
<th>Virus (serotype)</th>
<th>Virus titre (log_{10} EID_{50}/ml)</th>
<th>Inflammatory cell count (log_{10})</th>
<th>Rise (°C)</th>
<th>Level*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR/8 (H1N1)†</td>
<td>7.1</td>
<td>6.4</td>
<td>1.5</td>
<td>22</td>
</tr>
<tr>
<td>Clone 64c (H3N2)‡</td>
<td>6.1</td>
<td>7.1</td>
<td>2.5</td>
<td>37</td>
</tr>
<tr>
<td>Clone 64d (H3N2)§</td>
<td>5.7</td>
<td>7.0</td>
<td>1.8</td>
<td>14</td>
</tr>
<tr>
<td>Clone 7a (H3N2)‡</td>
<td>6.8</td>
<td>7.1</td>
<td>2.8</td>
<td>43</td>
</tr>
<tr>
<td>A/USSR (H1N1)§</td>
<td>6.2</td>
<td>7.0</td>
<td>1.7</td>
<td>10</td>
</tr>
<tr>
<td>A/Fiji (H1N1)§</td>
<td>6.5</td>
<td>7.1</td>
<td>1.3</td>
<td>6</td>
</tr>
</tbody>
</table>

* In arbitrary units.
† Matsuyama et al. (1980).
‡ Toms et al. (1977).
§ Coates et al. (1985).

release of EP (Smith & Sweet, 1984) (Table 1). Further evidence for the differential ability of influenza strains to stimulate EP release was provided by the demonstration that the two recent H1N1 isolates A/USSR/90/77 (A/USSR) and A/Fiji/15899/83 (A/Fiji) elicited much lower febrile responses than the H3N2 clones 7a and 64c, despite similar nasal virus levels and inflammatory responses for all four strains (Coates et al., 1985) (Table 1). This finding is also consistent with the human epidemiological results which suggest that recent H1N1 strains are less pyrogenic than H3N2 strains.

In this study, we have compared the capacity of clones 7a, 64c and 64d and strains PR/8, A/USSR and A/Fiji to induce rapid fever in ferrets following intracardial inoculation of large quantities of purified virus. We have used infectious and U.V.-inactivated virus preparations to determine whether infectivity is essential for pyrogenicity or if virion components might themselves be pyrogenic.

**METHODS**

_Viruses and their assay._ The H3N2 clones 7a, 64c and 64d of the A/Puerto Rico/8/34-A/England/939/69 reassortant virus system and the parental virus A/Puerto Rico/8/34 (H1N1) have been described previously (Sweet et al., 1974b; Matsuyama et al., 1980) as have the H1N1 viruses A/USSR/90/77 and A/Fiji/15899/83 (Coates et al., 1985). Seed stocks were prepared as previously described (Sweet et al., 1974a) and titrated in eggs or allantois-on-shell cultures (egg-bits) as described previously (Coates et al., 1985); titres are expressed as 50% egg infectious dose (EID_{50}) or 50% egg-bit infectious dose (EBID_{50}).

_Preparation of purified influenza viruses._ Twelve-day-old hens' eggs were inoculated intra-allantoically with 10^{2.4} EBID_{50} per egg of seed stock. After 24 h incubation at 35 °C, the allantoic fluid was harvested and debris removed from the fluid by low-speed centrifugation (1000 g at 4 °C for 20 min). The allantoic fluid was then centrifuged at 100000 g for 2.5 h at 4 °C and the sedimented virus was resuspended in Dubbeco's phosphate-buffered saline A (PBS-A) containing 0.2% (w/v) bovine serum albumin (BSA). The virus was re-sedimented, resuspended in PBS-A + 0.2% BSA and loaded onto a 20 to 60% (w/w) sucrose gradient prepared in TSE buffer [0.85% (w/v) NaCl, 0.01 M-Tris-HCl pH 7.4, 1 mM-EDTA], which was centrifuged at 110000 g for 18 h at 4 °C. The turbid virus band was harvested from the gradient, diluted with PBS-A + 0.2% BSA and sedimented by centrifugation as above. The purified virus pellet was resuspended in 1 ml PBS-A + 0.2% BSA and the concentration of viral protein was determined by the method of Lowry et al. (1951). The concentration of viral protein equaled the total concentration of protein in the resuspended virus minus the concentration of protein in the PBS-A + 0.2% BSA used to resuspend the virus; in general, BSA accounted for approximately one-third of the total protein in such virus preparations. For infectious virus stocks, the virus suspension was diluted with PBS-A + 0.2% BSA to give 200 μg viral protein/ml and stored at -70 °C (BSA was included to protect and prevent aggregation of the virus during freezing and thawing). For inactivated virus stocks, the virus suspension was diluted with PBS-A to give 200 μg viral protein/ml and then irradiated with 254 nm wavelength (u.v.) light (Camlab, Cambridge, U.K.) at a distance of 7.5 cm for 5 min (intensity at 7.5 cm = 120 μW/cm²). The virus suspension, in an open glass Petri dish on ice, was stirred throughout the irradiation process. After irradiation, BSA was added to 0.2% and the virus stored at -70 °C. The effectiveness of the irradiation procedure was checked by double passage in eggs. Virus
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Preparations of 200 μg protein/ml routinely gave haemagglutination (HA) titres of 2048 to 4096 HA units/0.2 ml (51 to 102 HA units/μg protein) which are equivalent to the levels of purity achieved by other methods of influenza virus purification published previously (Arora et al., 1973; Sweet et al., 1974a). Preliminary PAGE analysis of virus purified in the above manner showed no evidence of host protein contamination.

Inoculation of ferrets. Adult male ferrets, of approximately 1 kg body weight (obtained from A. S. Roe, Little Fakenham, Norfolk, U.K.) were inoculated by cardiac puncture under ether anaesthesia with virus suspension (in PBS-A + 0.2% BSA) at a dose of 200 μg viral protein/kg body weight. Following inoculation, rectal temperatures were measured (without anaesthesia) at hourly intervals using a digital thermometer (R.S. Components Ltd, Birmingham, U.K.) with an accuracy of 0.1 °C, attached to a type K thermocouple input. Fever was considered significant if the rectal temperature increased by >0.7 °C above the mean pre-inoculation temperature (determined as described by Toms et al., 1977), a rise of 0.7 °C being twice the standard deviation of the average pre-inoculation mean (Campbell et al., 1979). The rise was plotted against time post-inoculation and the level of fever (fever index) was assessed by determining mathematically the area under the curve >0.7 °C (expressed as °Ch). The significance of the difference between the mean fever levels for different virus strains was tested using the t-test for independent samples. Control experiments (in which rectal temperatures were monitored at hourly intervals up to 9 h post-inoculation) showed that intracardial inoculation of ferrets with PBS-A + 0.2% BSA did not produce a febrile response.

Sterilization of materials. To prevent endotoxin contamination, glassware was sterilized by heating in an oven at 160 °C for 2 h, and solutions and centrifuge tubes were autoclaved at 15 p.s.i. for 2 h (BSA and sucrose solutions were filter-sterilized). Non-pyrogenic sterile plasticware, syringes and hypodermic needles were used.

RESULTS

Infectious influenza virus strains

Infectious influenza virus strains were inoculated intracardially into ferrets at a dose of 200 μg viral protein/kg body weight. The resultant mean febrile response for each strain is shown in Fig. 1. Clones 64c, 64d and 7a (all H3N2) produced similar peak temperature rises of +2.9, +3.2 and +3.0 °C, respectively, above the pre-inoculation mean at 5 h post-inoculation. Strain PR/8 (H1N1) produced two mean peaks of +2.2 and +1.9 °C at 3 and 9 h post-inoculation, respectively. The two recent H1N1 isolates also produced biphasic febrile responses in inoculated ferrets, although such responses were generally much lower than those for clones 64d, 7a and 64c and strain PR/8. The mean response to A/USSR showed two peaks of +1.3 °C at 3 h post-inoculation and +1.6 °C at 6 to 7 h post-inoculation; the mean response to A/Fiji showed three peaks (although only two occurred in each animal; the timing of the second peak varied considerably) of +1.0 °C at 4 h, +1.2 °C at 7 h and +1.3 °C at 9 h post-inoculation.

To compare the results statistically, the fever index for individual animals was calculated (Table 2). The mean fever indices of clones 64d and 7a and strain PR/8 were not significantly different, and although the mean fever index for clone 64d was significantly greater than that for clone 64c (P < 0.05), the latter was not significantly different from those for clone 7a and strain PR/8. However, the mean fever indices for all four of these strains were significantly greater (P < 0.01) than those for A/USSR and A/Fiji.

U.v.-inactivated influenza virus strains

The u.v.-inactivated influenza virus stocks also induced febrile responses on intracardial inoculation into ferrets, although these were lower than those induced by the equivalent infectious strains and monophasic in all cases (Fig. 2). Strain PR/8 and clones 64c and 64d all produced a mean maximum rectal temperature rise of +1.9 °C above the pre-inoculation mean at 4, 5 and 3 h post-inoculation, respectively. Although clone 7a produced a slightly lower mean peak febrile response of +1.5 °C at 4 h post-inoculation, the mean maximum temperature rises for the recent H1N1 isolates, A/USSR and A/Fiji, were again very much lower: +0.3 and +0.9 °C, respectively, at 3 h post-inoculation.

There was no significant difference between the mean fever indices of clone 64c, strain PR/8 and clone 64d (Table 3), or between those of clone 7a, strain PR/8 and clone 64d, although the mean fever index for clone 7a was significantly lower than that for clone 64c (P < 0.001). The mean fever indices for A/USSR and A/Fiji were not significantly different, but both were
Fig. 1. Mean change in rectal temperature, in relation to pre-inoculation mean, in ferrets inoculated intracardially with infectious influenza virus strains (a) PR/8, (b) clone 64c, (c) clone 64d, (d) clone 7a, (e) A/USSR and (f) A/Fiji, at a dose of 200 μg viral protein/kg body weight. For numbers of animals tested, see Table 2. Bars represent the standard error of the mean.

Table 2. Mean areas under fever curves for ferrets inoculated intracardially with infectious influenza virus strains

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>No. animals tested</th>
<th>Mean area under fever curve (°C)</th>
<th>Standard error of the mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR/8</td>
<td>14</td>
<td>9.78</td>
<td>1.37</td>
</tr>
<tr>
<td>Clone 64c</td>
<td>5</td>
<td>9.02</td>
<td>1.06</td>
</tr>
<tr>
<td>Clone 64d</td>
<td>7</td>
<td>14.34</td>
<td>1.62</td>
</tr>
<tr>
<td>Clone 7a</td>
<td>5</td>
<td>12.30</td>
<td>1.71</td>
</tr>
<tr>
<td>A/USSR</td>
<td>6</td>
<td>4.66</td>
<td>0.71</td>
</tr>
<tr>
<td>A/Fiji</td>
<td>6</td>
<td>2.04</td>
<td>0.71</td>
</tr>
</tbody>
</table>

*Area under the fever curve greater than +0.7 °C (see Methods).
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Fig. 2. Mean change in rectal temperature, in relation to pre-inoculation mean, in ferrets inoculated intracardially with u.v.-inactivated influenza virus strains (a) PR/8, (b) clone 64c, (c) clone 64d, (d) clone 7a, (e) A/USSR and (f) A/Fiji, at a dose of 200 μg viral protein/kg body weight. For numbers of animals tested, see Table 3. Bars represent the standard error of the mean.

Table 3. Mean areas under fever curves for ferrets inoculated intracardially with u.v.-inactivated influenza virus strains

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>No. animals tested</th>
<th>Mean area under fever curve (°C)</th>
<th>Standard error of the mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR/8</td>
<td>10</td>
<td>3.62</td>
<td>0.72</td>
</tr>
<tr>
<td>Clone 64c</td>
<td>11</td>
<td>4.74</td>
<td>0.69</td>
</tr>
<tr>
<td>Clone 64d</td>
<td>7</td>
<td>3.60</td>
<td>0.70</td>
</tr>
<tr>
<td>Clone 7a</td>
<td>21</td>
<td>2.70</td>
<td>0.29</td>
</tr>
<tr>
<td>A/USSR</td>
<td>7</td>
<td>0.19</td>
<td>0.09</td>
</tr>
<tr>
<td>A/Fiji</td>
<td>7</td>
<td>0.96</td>
<td>0.47</td>
</tr>
</tbody>
</table>

* Area under the fever curve greater than +0.7°C (see Methods).

significantly lower than those for PR/8 (P < 0.001 and P < 0.02, respectively) and all three H3N2 clones (P < 0.001 and P < 0.01, respectively).

DISCUSSION

Several previous studies (Wagner et al., 1949; Atkins & Huang, 1958; Kosel & Kohlhage, 1970) have shown that relatively large quantities of infectious and inactivated influenza viruses
are pyrogenic when inoculated intravenously into animals. The results presented here, however, demonstrate for the first time that influenza viruses can differ in their pyrogenicity and that these differences are in accord with the relative severity of disease produced by them in man and experimental animals. The rapid fevers produced in ferrets by intracardial inoculation of large quantities of infectious and u.v.-inactivated A/USSR and A/Fiji were markedly and significantly lower than those produced by the same amounts of the four strains of the A/Puerto Rico/8/34-A/England/939/69 reassortant virus system. This result is consistent with previous observations that although these recent H1N1 isolates grew as well, and stimulated as high an inflammatory response, in the URT of intranasally inoculated ferrets as the virulent clones of this reassortant system, they induced much lower levels of pyrexial response (Toms et al., 1977; Matsuyama et al., 1980; Coates et al., 1985). In addition, this result is in accord with their reported less severe illness in man (Kung et al., 1978; Wright et al., 1981; Frank et al., 1985).

Low pyrogenicity is not necessarily a property of all H1N1 strains, since PR/8 (H1N1) produced significantly more fever than the modern H1N1 viruses. PR/8 has been passaged many times in vitro and in experimental animals since isolation and, therefore, may not be typical of either old or recent H1N1 isolates. Nevertheless, it is possible that recent H1N1 strains are less pyrogenic than H1N1 strains isolated prior to 1957, as many exhibit a temperature-sensitive phenotype (Kung et al., 1978; Oxford et al., 1980).

The results for the recent H1N1 strains suggest that they have a lower ability than the four strains of the reassortant system to stimulate the release of EP from phagocytes. The results for the u.v.-inactivated viruses support the previous suggestion that clone 64c has a greater capacity to stimulate the release of EP than clone 7a, although this is not evident in the infectious virus results. The fact that the u.v.-inactivated viruses produced fever suggests that one or more virion components may act as exogenous pyrogens, although the greater pyrexial responses to the infectious virus imply either that u.v. inactivation has a detrimental effect on these pyrogens or that limited replication of the virus in the phagocytes may result in greater production of EP. The results for the infectious strains (the H1N1 strains) stimulated biphasic pyrexial responses which were not produced by the equivalent u.v.-inactivated viruses; it is possible that the second peak represents the action of another pyrogen other than EP, e.g. interferon (Dinarello et al., 1984) or prostaglandin (Milton, 1982), the production of which is not stimulated by the u.v.-inactivated strains. This second peak may also be present, but masked, within the overall much greater pyrexial responses to the three H3N2 infectious clones (see Fig. 1).

With regard to virion components acting as exogenous pyrogens, both lipid (Siegert & Braune, 1964) and other host-derived components of the virion (Brand & Liew, 1983) have been suggested as possible mediators of pyrogenicity. However, all strains used in the present study (which exhibit differential pyrogenicity) were grown in embryonated hens' eggs under the same conditions. Hence, it is possible that components coded for by the virus genome are involved and that they differ from strain to strain in their capacity to induce EP from phagocytes. The abilities of different influenza virus strains to induce the release of EP from human and ferret phagocytes in vitro are at present being compared, both with infectious and u.v.-inactivated virus. Any significant differences between u.v.-inactivated strains could lead to an examination of isolated virion components.

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REFERENCES
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