The Replication of Type A Influenza Viruses in the Infant Rat: A Marker for Virus Attenuation

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SUMMARY

Twenty recombinant influenza virus strains bearing HSw1N1, H1N1 or H3N2 surface antigens, together with their respective wild-type or laboratory-propagated parent viruses, were inoculated into 2 day-old infant rats and their replication in the turbinates and lungs of these animals observed over a period of 5 days. In addition, the ability of each of the recombinant and parent viruses to enhance a subsequent infection of these infant rats by Haemophilus influenzae type b was determined. The results showed that both parent and recombinant viruses replicated less well in the lungs than in the turbinates of infant rats, but the titres in both tissues were generally lower for the recombinant strains. The capacity of the majority of the recombinant influenza viruses to promote bacterial infection of the infant rats, as determined by the incidence of H. influenzae bacteraemia and meningitis, was also markedly less than that of their parent viruses. A correlation between virulence for man and both the replication in infant rat turbinates and the ability to enhance H. influenzae infection, was established for the virus strains studied. The data are discussed in relationship to the value of the infant rat-H. influenzae system as a laboratory marker for the determination of the virulence of influenza virus strains.

INTRODUCTION

The use of live, attenuated viruses for immunization against influenza may have advantages over the available inactivated vaccines: these include wider acceptability, production in greater volume and possibly the induction of a more solid immunity (Beare et al. 1968; Freestone et al. 1972; Mackenzie, 1977). However, the time required for the production of live virus vaccines is more protracted than for inactivated vaccine since virus strains must first be prepared and then subjected to a sequential series of tests for safety, stability, reactogenicity and transmissibility in volunteers. Any procedures which would lessen the time necessary for these studies would further the development of live influenza virus vaccines. At present, potential live influenza virus vaccine strains are derived by a variety of methods and then inoculated into volunteers to assess their virulence (Tyrrell, 1963; Beare & Hall, 1971; Murphy et al. 1972). Ideally, seronegative volunteers should be used in these tests (Murphy et al. 1972), but such individuals may be few in number (Murphy

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et al. 1972, 1979). Furthermore, the testing of live viruses in volunteers is time-consuming, expensive and potentially dangerous, since some recombinant virus strains are more virulent than their parent viruses (Beare et al. 1975).

To facilitate the development of live virus vaccines, laboratory tests which would measure virulence for man have been sought. Thus, virus infection in ferrets and hamsters and in vitro studies of virus pathogenicity in organ cultures have been studied (Mostow & Tyrrell, 1972, 1973; Hara et al. 1973; Boudreault, 1979); however, none of these systems has proved sufficiently reliable or been fully investigated (Hara et al. 1973). The present studies report on the use of infant rats for the measurement of virulence for man of influenza virus strains and are an extension of previous observations reported from this laboratory (Michaels et al. 1978; Mahmud et al. 1979a, b; Teh et al. 1980). Thus the growth of influenza viruses in rat turbinates and lungs, and the ability of virus infection to potentiate subsequent Haemophilus influenzae infection is presented, together with a correlation of these findings with virulence for man.

**METHODS**

**Virus strains.** The wild-type influenza virus strains A/Swine/1976/30 (HSw1N1), A/Port Chalmers/1/73 (H3N2), A/Victoria/3/75 (H3N2), A/Texas/1/77 (H3N2) and A/FM/1/47 (H1N1) were obtained from Dr J. J. Skehel, National Institute for Medical Research, Mill Hill, London. Strains A/Hong Kong/77 (H1N1), A/USSR/92/77 (H1N1), A/Okuda/57 (H2N2), A/England/42/72 (H3N2) and A/Finland/4/74 (H3N2) were kindly supplied by Dr G. Appleyard, Wellcome Laboratories, Beckenham, Kent. The recombinant influenza virus strains WRL124 (HSw1N1), a recombinant of A/Okuda/57 and A/New Jersey/76, WRL105 (H3N2), a recombinant of A/Okuda/57 and A/Finland/74, and the series of H1N1 recombinants designated WRL206, WRL211, WRL215, WRL216, WRL217 and WRL224, prepared from A/Okuda/57 and A/Hong Kong/77-aa/2, were all obtained from Dr G. Appleyard. The influenza viruses A/New Jersey/8/76 (HSw1N1), A/England/939/69 (H3N2) and A/PR/8/34 (H0N1), together with strain NIB3 (HSw1N1) virus, a recombinant of A/New Jersey/76 and influenza X31 (H3N2) viruses, strain 4a2 (H3N2), a double recombinant of A/Victoria/75 and A/PR/8/34, and virus strains clone 6 (H3N2) and clone 64c (H3N2) viruses, both recombinants of A/England/69 and A/PR8 viruses were kindly supplied by Dr A. S. Beare, Common Cold Research Unit, Salisbury, Wilts. Influenza virus X31 is a recombinant of A/Hong Kong/68 and A/PR8 viruses (Couch et al. 1971).

Influenza virus strains A/Ann Arbor/6/60 (H2N2) and A/Queensland/6/72 (H3N2), the cold-adapted (ca) strain A/Ann Arbor/6/60–7P1 (H2N2), together with recombinant viruses CR-6 (H3N2), CR 19-clone 6 (H3N2), CR22-clone 1 and CR22-clone 5 (H3N2), recombinants of A/Ann Arbor/6/60–7P1 and either A/Queensland/6/72 or A/Victoria/75 viruses, were obtained from Dr H. F. Maassab, School of Public Health, University of Ann Arbor, Mich., U.S.A. The temperature-sensitive (ts) recombinant viruses, ts-1 (E) (H3N2), ts-1 (A) (H3N2) and ts-2(C) (H3N2), and their wild-type parent viruses A/Great Lakes/0389/65 (H2N2) and A/Hong Kong/1/68 (H3N2), were obtained from Dr B. Murphy, National Institute of Infectious and Allergic Diseases, Washington, U.S.A. Influenza viruses RIT4058 (HSw1N1), a recombinant of A/PR/8/34 and A/New Jersey/76, a recombinant of A/PR/8/34 and A/Victoria/75, and RIT4050 (H3N2), were obtained from Dr C. Huygelen, Recherche et Industrie Thérapeutiques, Rixensart; Belgium.

Viruses pools were prepared by the allantoic inoculation of 10 day-old embryonated eggs, as described previously (Mahmud et al. 1979a). The egg infectivity titre (EID<sub>50</sub>) of each virus pool was determined by titration in 10 day-old embryonated eggs, and calculated by the method of Reed & Muench (1938).
**Influenza viruses in the infant rat**

*Animals.* Newborn Wistar strain rats were obtained from a closed, randomly bred colony at the University of Sheffield. For each experiment, several litters of healthy animals were pooled and re-distributed in groups of 10 to 12 to each mother.

*Inoculation of infant rats.* The method used to inoculate infant rats has been described in detail elsewhere (Mahmud et al. 1979a). Briefly, either influenza virus or *H. influenzae* type b (*Hib*), were inoculated into the anterior nares of the infant rat in a vol. of 0.01 ml using a Hamilton syringe fitted to a scalp-vein infusion set. Virus dilutions for inoculation were made in phosphate-buffered saline (PBS), pH 7.4, containing 1% bovine serum albumin and antibiotics; the *Hib* used throughout these studies, strain Pekala, was originally isolated from a patient with meningitis (Michaels et al. 1977), and its storage and preparation for inoculation into infant rats described previously (Michaels et al. 1978).

*Growth of virus in newborn rats.* Each infant rat was inoculated with $10^4$ EID$_{50}$ of influenza virus contained in a vol. of 0.01 ml. At intervals after virus inoculation, groups of four or five rats were killed and the lungs and turbinates removed. The lungs and turbinates were pooled separately and 40% homogenates prepared by grinding with carborundum powder in PBS plus 1% bovine serum albumin. The homogenates were then centrifuged at 450 g for 20 min and the supernatant fluids stored at $-80\,^\circ\text{C}$.

*Titrations of lung and turbinate tissue homogenates* were carried out by the allantois-on-shell (AOS) method (Fazekas de St. Groth et al. 1958), and the egg-bit infectious dose (EBID$_{50}$) calculated by the method of Reed & Muench (1938).

*Determination of *H. influenzae* infection in infant rats.* The method used to determine the bacteraemia and meningitis induced in infant rats by *Hib* has been previously described (Mahmud et al. 1979a). Three days following intranasal inoculation of the normal or virus-infected animals with $10^6$ to $10^7$ viable *Hib* bacteria per 0.01 ml, the rats were decapitated and a blood sample collected into heparinized capillary tubes. Viable counts were carried out on the blood samples using chocolate-agar plates and isolated organisms identified by the capsular-swelling test with standard *Hib* antiserum (Wellcome Laboratories). Meningitis was detected by histological methods following fixation, decalcification and sectioning of the infant rat heads (Moxon et al. 1974).

**RESULTS**

*Replication of naturally-occurring influenza virus strains in infant rat turbinates and lungs*

Table 1 shows the growth characteristics of sixteen naturally-occurring influenza virus strains in the turbinates and lungs of infant rats over a 5 day period. Eight of the strains tested were of the H$_3$N$_2$ serotype, while the remaining eight were H$_2$N$_2$, H$_1$N$_1$, H$_6$N$_1$ or HSw$_1$N$_1$ serotypes. All the viruses tested showed evidence of growth to some extent in infant rat turbinates, and only one strain, A/USSR/77, failed to replicate to detectable levels over 5 days in the lungs of the baby rats. Most of the strains tested grew to higher and more sustained titres in the upper respiratory tract of the infant rat. However, three of the viruses, A/Swine/30, A/New Jersey/76 and A/Texas/77 showed sustained levels of virus growth over 5 days, or virus titres in the lungs of infant rats comparable to those observed in the turbinates of these animals (Table 1).

Maximum titres of virus in both turbinates and lungs were most frequently observed in the first 24 or 48 h following infection and this was most clearly seen for influenza A (H$_3$N$_2$) serotypes. None of the viruses studied showed evidence of a late increase in replication in infant rat turbinates, but three viruses, A/New Jersey/76, A/Great Lakes/65 and A/Texas/77, exhibited peak titre in the lungs on day five p.i.
Table 1. Replication of naturally-occurring influenza viruses in infant rat turbinates and lungs

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Virus strain</th>
<th>Virus titre (log_{10}/ml) in turbinates on day</th>
<th>Virus titre (log_{10}/ml) in lungs on day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>HSw1N1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HoNI</td>
<td>&lt; 1.0</td>
<td>&lt; 1.0</td>
<td>2.4</td>
</tr>
<tr>
<td>H1N1</td>
<td>5.6</td>
<td>4.6</td>
<td>5.0</td>
</tr>
<tr>
<td>H2N2</td>
<td>2.2</td>
<td>4.1</td>
<td>4.4</td>
</tr>
<tr>
<td>H3N2</td>
<td>&lt; 1.0</td>
<td>2.1</td>
<td>3.3</td>
</tr>
<tr>
<td>A/Swine/1976/30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/New Jersey/8/76</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/PR/8/34</td>
<td>3.8</td>
<td>4.3</td>
<td>3.8</td>
</tr>
<tr>
<td>A/Hong Kong/77</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/USSR/92/77</td>
<td>2.2</td>
<td>3.7</td>
<td>4.1</td>
</tr>
<tr>
<td>A/Okuda/57</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/Ann Arbor/6/60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/Great Lakes/65</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/England/939/69</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/England/42/72</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>A/Queensland/6/72</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/Port Chalmers/1/73</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/Victoria/3/75</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Virus titre in pooled homogenates from four or five infant rats.

Replication of recombinant influenza virus strains in infant rat turbinates and lungs

The growth characteristics in infant rat turbinates and lungs of 20 strains of influenza virus derived by recombination from various parental influenza viruses are shown in Table 2. Strain WRL124 is not included here, as the growth characteristics of this virus have not been studied in the infant rat. The strains tested were of three serotypes only, HSw1N1, H1N1, and H3N2, and their growth characteristics were similar in many respects to those of the naturally-occurring influenza virus strains shown in Table 1. Thus, 18 of the 20 recombinant virus strains showed peak titres, in the turbinates of newborn rats, 24 or 48 h p.i. but the titres declined rapidly thereafter. However, the levels of virus growth shown by the recombinant strains in both the lungs and turbinates were generally lower than those of the naturally-occurring strains.

The ability of the recombinant viruses to replicate in the lower respiratory tract of infant rats was considerably less than in the turbinates and this was particularly apparent for the influenza A (H3N2) serotypes. Thus, two of these serotypes WRL215 and WRL217 showed no detectable virus growth in infant rat lungs over the period of observation, and the titres of the other H3N2 recombinant viruses rarely rose above 10^3 EBD_{50}/ml. The ts mutant recombinant, ts-1(A) also showed no detectable virus growth in infant rat lungs. Three recombinant viruses NIB3, WRL216 and CR22-clone 5 grew to their maximum titre in the lungs of infant rats on day 5 p.i. and several viruses, including RIT4058, clone 64c and WRL105, produced relatively high, sustained levels of replication in the lungs throughout the whole period of study.

H. influenzae (HIb) infections in infant rats

Following inoculation of naturally-occurring influenza viruses

Forty-eight h after intranasal infection of infant rats with influenza virus, each animal was inoculated by the same route with 10^6 colony-forming units (c.f.u.) of HIb; the incidence of bacteraemia and meningitis in these rats was measured 72 h later. The results are shown in Fig. 1. At the concentration of HIb used, very few control rats developed bacteraemia and none developed meningitis; in contrast, the incidence of both bacteraemia and menin-
Table 2. Replication of recombinant influenza viruses in infant rat turbinates and lungs

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Recombinant virus</th>
<th>Parent viruses</th>
<th>Virus titre (log$_{10}$/ml) in turbinates on day</th>
<th>Virus titre (log$_{10}$/ml) in lungs on day</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSwiN1</td>
<td>RIT4058</td>
<td>A/PR8 × A/New Jersey/76</td>
<td>4.4* 2.8 2.3 &lt;1.0</td>
<td>5.4 5.1 4.7 4.8</td>
</tr>
<tr>
<td></td>
<td>NIB3</td>
<td>A/PR8 × A/New Jersey/76</td>
<td>&lt;1.0 3.8 &lt;1.0 &lt;1.0</td>
<td>&lt;1.0 &lt;1.0 &lt;2.7 2.9</td>
</tr>
<tr>
<td>H1N1</td>
<td>WRL206</td>
<td>A/Okuda/57 × A/Hong Kong/77</td>
<td>4.3 5.1 2.7 &lt;1.0</td>
<td>2.2 &lt;1.0 &lt;1.0 &lt;1.0</td>
</tr>
<tr>
<td></td>
<td>WRL211</td>
<td>A/Okuda/57 × A/Hong Kong/77</td>
<td>3.3 4.1 2.3 &lt;1.0</td>
<td>&lt;1.0 1.4 1.0 &lt;1.0</td>
</tr>
<tr>
<td></td>
<td>WRL215</td>
<td>A/Okuda/57 × A/Hong Kong/77</td>
<td>2.0 3.6 3.5 1.0</td>
<td>&lt;1.0 &lt;1.0 &lt;1.0 &lt;1.0</td>
</tr>
<tr>
<td></td>
<td>WRL216</td>
<td>A/Okuda/57 × A/Hong Kong/77</td>
<td>&lt;1.0 3.0 4.1 2.7</td>
<td>&lt;1.0 &lt;1.0 &lt;1.0 &lt;1.0</td>
</tr>
<tr>
<td></td>
<td>WRL217</td>
<td>A/Okuda/57 × A/Hong Kong/77</td>
<td>1.6 2.4 2.4 &lt;1.0</td>
<td>&lt;1.0 &lt;1.0 &lt;1.0 &lt;1.0</td>
</tr>
<tr>
<td></td>
<td>WRL218</td>
<td>A/Okuda/57 × A/Hong Kong/77</td>
<td>1.8 3.2 2.8 1.1</td>
<td>1.0 1.3 1.0 &lt;1.0</td>
</tr>
<tr>
<td></td>
<td>WRL219</td>
<td>A/Okuda/57 × A/Hong Kong/77</td>
<td>&lt;1.0 2.5 2.2 &lt;1.0</td>
<td>&lt;1.0 1.0 &lt;1.0 &lt;1.0</td>
</tr>
<tr>
<td></td>
<td>Clone 6</td>
<td>A/PR8 × A/England/69</td>
<td>2.8 5.5 4.5 3.4</td>
<td>&lt;1.0 &lt;1.0 4.3 3.3</td>
</tr>
<tr>
<td></td>
<td>Clone 64c</td>
<td>A/PR8 × A/England/69</td>
<td>3.6 4.2 3.2 &lt;1.0</td>
<td>3.7 3.2 2.9 2.8</td>
</tr>
<tr>
<td></td>
<td>CR-6</td>
<td>A/AA/60-7P1 × A/Queensland/72</td>
<td>3.5 2.3 2.7 &lt;1.0</td>
<td>1.0 1.0 &lt;1.0 &lt;1.0</td>
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<tr>
<td></td>
<td>CR19-clone 6</td>
<td>A/AA/60-7P1 × A/Victoria/75</td>
<td>3.0 2.5 2.3 &lt;1.0</td>
<td>3.7 3.7 2.0 2.4</td>
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<td>CR22-clone 1</td>
<td>A/AA/60-7P1 × A/Victoria/75</td>
<td>3.2 2.9 2.9 2.1</td>
<td>3.1 2.6 2.5 &lt;1.0</td>
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<td></td>
<td>CR22-clone 5</td>
<td>A/AA/60-7P1 × A/Victoria/75</td>
<td>2.3 2.4 2.5 &lt;1.0</td>
<td>&lt;1.0 2.1 1.8 2.3</td>
</tr>
<tr>
<td></td>
<td>ts-2 (C)</td>
<td>A/Great Lakes/65 × A/Hong Kong/68</td>
<td>2.2 2.7 2.0 &lt;1.0</td>
<td>2.7 3.1 2.2 &lt;1.0</td>
</tr>
<tr>
<td></td>
<td>ts-1 (E)</td>
<td>A/Great Lakes/65 × A/Hong Kong/68</td>
<td>&lt;1.0 3.2 2.2 2.0</td>
<td>&lt;1.0 2.3 4.2 3.7</td>
</tr>
<tr>
<td></td>
<td>ts-1 (A)</td>
<td>A/Great Lakes/65 × A/Hong Kong/68</td>
<td>&lt;1.0 4.0 2.3 1.1</td>
<td>&lt;1.0 &lt;1.0 1.0 &lt;1.0</td>
</tr>
<tr>
<td></td>
<td>WRL105</td>
<td>A/Okuda/57 × A/Finland/74</td>
<td>5.7 5.1 5.0 4.8</td>
<td>2.6 3.5 4.1 3.5</td>
</tr>
<tr>
<td></td>
<td>4a2</td>
<td>A/PR8 × A/Victoria/75</td>
<td>2.1 4.8 3.9 2.0</td>
<td>2.0 4.0 3.6 2.0</td>
</tr>
<tr>
<td></td>
<td>RIT4050</td>
<td>A/PR8 × A/Victoria/75</td>
<td>5.3 6.1 3.4 2.1</td>
<td>2.8 3.0 2.1 &lt;1.0</td>
</tr>
</tbody>
</table>

* Virus titre in pooled homogenates from four or five infant rats.
Fig. 1. Incidence of *H. influenzae* type b bacteraemia (□) and meningitis (■) in infant rats previously infected with naturally-occurring influenza virus strains. *Incidence of meningitis not determined.

The extent of this increase varied for the different strains of influenza virus used. Thus, for influenza A (H3N2) viruses and A/PR/8/34 virus, 80 to 100% of animals developed bacteraemia, while for influenza A (Hsw1N1) strains, the incidence was less than 20% (Fig. 1). The incidence of meningitis was also enhanced by prior infection of rats with influenza viruses and again the viruses varied widely in their ability to promote *Hib* meningitis. Thus, for influenza A (H3N2) strains, meningitis was observed in 25 to 90% of infant rats; for influenza A (H1N1) strains the incidence was 10 to 22%, while influenza A (Hsw1N1) viruses rarely predisposed to subsequent meningitis.

Following inoculation of recombinant influenza viruses

The results of pre-infection with various recombinant influenza virus strains on the incidence of bacteraemia and meningitis induced in infant rats by subsequent inoculation with *Hib* is shown in Fig. 2 and it can be seen that with two exceptions, the incidence of bacteraemia was not greater than 36%. Indeed, for the majority of these viruses the incidence of bacteraemia was considerably less than this and for WRL124, CR19-clone 6, CR22-clone 5 and ts-i(A), no enhancement of *Hib* bacteraemia was observed. Similarly, pre-infection of infant rats with the majority of these recombinant viruses elicited little potentiation of meningitis following the intranasal installation of *Hib*. The two exceptions to the above results were WRL105 and RIT4050. Thus, the incidence of *Hib* bacteraemia...
Influenza viruses in the infant rat

Fig. 2. Incidence of *H. influenzae* type b bacteraemia (□) and meningitis (■) in infant rats previously infected with recombinant influenza virus strains.

Fig. 3. Relationship of the degree of bacteraemia to the incidence of meningitis in infant rats receiving $10^6$ colony-forming units of *H. influenzae* type b following influenza virus infection.

* Number of blood samples tested from individual infant rats.
in infant rats pre-infected with WRL105 and RIT4050 recombinant viruses was 77 and 100% while the incidence of meningitis was 62 and 50%, respectively. With the exception of these two strains, the results obtained with the recombinant viruses are in contrast to the considerably greater enhancement of *Hib* bacteraemia and meningitis seen in infant rats pre-infected with the majority of naturally-occurring influenza virus strains (Fig. 1).

**Relationship between *Hib* bacteraemia and meningitis in infant rats**

The relationship between *Hib* bacteraemia, determined by colony counts on chocolate-agar plates with individual blood samples and using an 0.001 ml platinum loop, and the incidence of meningitis, assessed by histological examination, was measured. The results are shown in Fig. 3. A good correlation was found for these two measures of systemic infection by *Hib*. Thus, evidence of meningitis was not found in rats with bacterial counts of $5 \times 10^3$ c.f.u./ml in the blood, but was almost always present when the count was $8 \times 10^4$ c.f.u./ml. Animals with levels of bacteraemia falling between these extremes showed a variable incidence of meningitis that correlated well with the bacterial count.

**DISCUSSION**

The chief limitation to the development and use of live, attenuated influenza virus vaccines is the protracted time required for their production and testing (Michaels *et al.* 1978). Live, attenuated vaccine strains can be readily prepared by recombination (Maassab *et al.* 1969; Beare & Hall, 1971; Mills & Chanock, 1971; Morris *et al.* 1975), but once such strains have been produced, a series of consecutive studies are required to establish virus stability, safety and non-transmissibility, procedures which can delay the development of the vaccine to the point of uselessness.

One requirement in the development of live, vaccine strains is the need to show that the viruses are attenuated in volunteers and a laboratory test which measures attenuation would be of considerable value. Several systems have been investigated for this purpose including the inhibition of ciliary action in ferret or human tracheal organ cultures (Mostow & Tyrrell, 1972, 1973; Boudreault, 1979), virus virulence in ferrets (Lobmann *et al.* 1976; Toms *et al.* 1976; Fenton *et al.* 1977; Campbell *et al.* 1979), virus replication in squirrel monkeys (Berendt & Hall, 1977) and hamsters (Murphy *et al.* 1978). None of these systems has either been investigated sufficiently to establish its value, or has proved unreliable (Hara *et al.* 1973; Abou-Donia *et al.* 1980).

An alternative approach to this problem is to define virulence in terms of the genetic constitution of the virus (Palese, 1977; Oxford *et al.* 1978), but no gene or constellation of genes has been identified as correlating with influenza virus virulence, although an association of virulence with the genes coding for the virus polymerase components has recently been reported (Rott *et al.* 1979). Several other studies indicate that different gene constellations are associated with virulence for a different series of viruses (Beare, 1969; Almond, 1977; Bean & Webster, 1978).

In this study, the behaviour of influenza viruses in an infant rat model has been investigated to establish the value of this system in indicating virus virulence for man. Based on earlier studies, two parameters were selected as possibly discriminating: virus replication in rat turbinates and the ability of prior virus infection to promote systemic infection by *H. influenzae* (Michaels *et al.* 1978; Mahmud *et al.* 1979a, b; Teh *et al.* 1980). The virulence for man of all the naturally-occurring viruses, and all but four, WRL206, WRL215, WRL217 and WRL224, of the recombinant virus strains tested in the infant rat system are known, and these strains can be assigned to one of three categories: (i) fully virulent
viruses, (ii) strains naturally-occurring but of reduced virulence for man and (iii) attenuated virus strains. WRL105 virus was classed as a virulent strain, since recent studies have indicated that this recombinant virus is indeed virulent for man (A. S. Beare, personal communication; Campbell et al. 1979). In Fig. 4, the average titre present in the turbinates of infant rats on days 1, 2 and 3 following virus infection with each of the 32 virus strains has been plotted against the virulence of these strains for man and it can be seen that although there is some correlation of human virus with replication in infant rat turbinates, there is some overlap, and two viruses of low virulence, as well as four attenuated strains, replicated to a higher titre than the virulent A/Hong Kong/68 virus.

A comparison of virus virulence for man and the ability to potentiate \( H1b \) bacteraemia is shown in Fig. 5, and all the viruses virulent for man markedly enhanced subsequent \( H1b \) bacteraemia in infant rats with incidences ranging from 77 to 100%, while all but two of the influenza virus strains of low virulence or attenuated for man showed a relatively reduced ability to induce \( H1b \) bacteraemia. Thus, the ability of influenza virus strains to promote \( H1b \) bacteraemia correlates well with human virulence and this held for 31 of the 33 strains tested.

The exceptions were strains A/PR8 and RIT4050; the former is avirulent for man (Beare & Hall, 1971) and the latter is attenuated (Florent et al. 1977; Jennings et al. 1978; Rocchi et al. 1979). The reasons for these exceptions are not known, but influenza virus A/PR8 has been passaged over many years in a variety of laboratory systems, factors that may contribute to its atypical behaviour, and it is also known to be highly virulent for ferrets.
Fig. 5. Correlation of influenza virus virulence for man with virus enhancement of *H. influenzae* type b bacteraemia in infant rats.

and hamsters (McLaren et al. 1974; Abou-Donia et al. 1980). The atypical behaviour of RIT4050 is less readily explained, but this virus is a recombinant of A/PR8 and possesses four RNA fragments derived from this virus (G. Florent, personal communication). The properties of these genes may explain its anomalous behaviour. However, no such behaviour was apparent for the other A/PR8 recombinant viruses tested in the present study.

The results presented here indicate the potential value of the infant rat as a method of indicating the virulence of live influenza virus strains for man. The model is applicable to recombinant viruses prepared by a number of methods using H5N1, H9N2, H1N1, H2N2 and H3N2 serotypes. Only two of the 33 strains failed to give agreement and in both cases strains attenuated for man were virulent for infant rats; thus, pre-selection of all strains using infant rats would not have resulted in any virulent strains being selected for subsequent volunteer studies. The findings suggest that enhancement of *H1b* bacteraemia in infant rats may provide a valuable marker of human virulence of influenza viruses and its application could facilitate the development of viruses for possible use as live influenza virus vaccines.

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