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Influenza Virus Infection of Newborn Rats: Virulence of Recombinant Strains Prepared From a Cold-Adapted, Attenuated Parent

By

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With 3 Figures

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Summary

Infant rats were infected with one of a series of influenza A viruses. The growth of viruses in the turbinates or lungs, and the ability of virus infection to potentiate a subsequent bacterial infection by Haemophilus influenzae (HIb), were measured. The three virus strains known to be virulent for man grew to relatively high titres of $10^{5.2}$—$10^{6.8}$ $EBID_{50}$/ml in the turbinates of infant rats at 48 hours post-infection, and virus infection enhanced subsequent systemic infection following intranasal inoculation of rats with HIb. In contrast, influenza virus A/Ann Arbor/6/60—P17 and the three recombinant viruses prepared from this strain, all of which are attenuated for man, replicated to significantly lower titres of $10^{2.6}$—$10^{4.1}$ $EBID_{50}$/ml in infant rats turbinates, and failed to promote systemic infection by HIb to the same degree. The results, together with those of previous studies, suggest that the behaviour of influenza viruses in infant rats may be an indication for virus virulence for man, and thus provide a test which could facilitate the development of live, attenuated virus vaccines.

Introduction

The development of live, attenuated vaccines for immunization against influenza has required studies in human volunteers to measure virus attenuation (1); this is a protracted and potentially dangerous procedure, since recombinant strains may be more virulent for man than the parent viruses from which they are derived (2). To limit the problems of volunteer studies, several laboratory models have been investigated in attempts to develop a reliable and reproducible laboratory method of measuring the virulence of influenza virus strains. Thus, virus growth

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in ferret and human organ cultures (13, 14), virus replication in ferrets (4, 7, 23) and growth in hamsters (16) have been investigated as possible methods of assessing virus virulence for man: the tests using animal models require confirmation and extension to recombinant viruses produced by a variety of methods before an assessment of reliability can be made, whilst the effects of virus growth in organ cultures has been shown to correlate with human virulence for some influenza virus strains, but not for others (6).

The results obtained from studies of influenza virus infection in infant rats suggest that this laboratory model may be of value in the assessment of human virulence of influenza virus strains. Thus, virulent influenza A/England/939/69 (H3N2) virus was found to grow to relatively high titres in infant rat turbinates and to promote significant bacteraemia and meningitis in rats subsequently inoculated with *Haemophilus influenza* type b (HIb); in contrast, attenuated recombinant strains prepared from influenza A/England/939/68 and A/PR/8/34 (H0N1) grew to lower titres in rat turbinates, and rarely enhanced subsequent bacterial infection (11). In addition, several further virulent influenza A virus strains were shown to behave similarly to A/England/939/69 in infant rats, whilst a second series of attenuated recombinants of wild-type and A/PR/8/34 viruses grew to low titres in infant rats and again failed to provoke significant systemic infection by HIb (8). The only contradictory result obtained to date was for A/PR/8/34 virus; this virus is not infective for man (1), but grew to high titres in the turbinates of infant rats, and provoked subsequent HIb bacteraemia and meningitis in all infant rats tested (11). Although these results were encouraging, they were limited to wild-type viruses and attenuated strains derived by recombination of wild-type and A/PR/8/34 viruses; it remained to be determined if similar results could be obtained using recombinant viruses derived by other methods. In the present study, we report the replication of wild-type and cold-adapted viruses, and recombinant viruses derived by recombination of these strains, in infant rat turbinates and lungs. In addition, following infection with these viruses, rats were inoculated intranasally with HIb, and the ability of prior virus infection to promote subsequent bacteraemia and meningitis was determined.

Materials and Methods

Viruses

Influenza A/Victoria/3/75 (H3N2), a wild-type virulent virus, and strain 4A2, a double recombinant of A/Victoria/3/75 and A/PR/8/34 viruses, were obtained from Dr. A. S. Beare, Common Cold Research Unit, Salisbury. Influenza viruses A/Queensland/6/72 (H3N2), A/Ann Arbor/6/60 (H2N2), cold-adapted A/Ann Arbor/6/60—7PI (H2N2), A/Ann Arbor/6/60—CR6 (H3N2) which is a recombinant of A/Ann Arbor/6/60 and A/Queensland/6/72 viruses, and CR19—clone 6, CR22-clone 5, and CR22-clone 1 which are recombinants of A/Ann Arbor/6/60—7PI and A/Victoria/3/75 (H3N2) viruses, were from Dr. H. F. Massaab, The School of Public Health, University of Michigan, Ann Arbor, Michigan, U.S.A.; the development and properties of these viruses have been reported previously (9, 21, 22).

Virus pools were prepared by the allantoic inoculation of 10-day embryonated eggs with 0.2 ml of $10^{-3.0}$ dilution of seed virus. After incubation at 33° C for 72 hours, the allantoic fluids were collected and stored at −80° C. The egg infectivity titre (EID_{50}) of each virus pool was determined by titration in 10-day embryonated eggs, and calculated by the method of Reed and Muench (20).

Virus Replication in Newborn Rats

Wistar strain rats were obtained from a closed randomly-bred colony at the University of Sheffield; litters of 8—12 rats were used at age 48 hours. The influenza virus strain under test was diluted in phosphate buffered saline, pH 7.4 (PBS) containing 2.0 per cent (v/v) bovine serum albumin (BSA) and antibiotics to give a virus suspension containing $10^{4.0}$ EID_{50}/0.01 ml. Using a Hamilton syringe fitted to a 25-gauge scalp vein infusion stylet, 0.01 ml of virus was inoculated into the anterior nares of each rat, as described previously (11). At intervals after virus inoculation, groups of rats were killed, and the lungs and turbinates removed; these tissues were separately suspended at a concentration of 20 per cent (v/v) in PBS containing 2.0 per cent (v/v) BSA and antibiotics, and ground with a pestle and mortar in carborundum powder; the resulting extracts were centrifuged at 3000 rpm for 20 minutes at 4° C, and the supernatants stored at −80° C prior to virus titration.

Virus Titrations

The lung and turbinate extracts from virus-infected infant rats were titrated for virus by the allantoic-on-shell (AOS) method (3). Each virus dilution was inoculated on AOS fragments from four different eggs, since eggs vary in sensitivity to influenza virus infection. After incubation for three days at 33° C with constant shaking, the shell fragments were removed and the culture fluids tested for virus by haemagglutination. The virus titres {egg-bit infectious doses [$(EBID_{50})$]/per 0.05 ml} were calculated by the method of Reed and Muench (20).

Haemophilus Influenza Type b

A heavily-encapsulated strain of *H. influenza type b*, strain Pekala (HIb), isolated from a patient with meningitis and passaged interperitoneally in rats (10), was used throughout; the method for growing, storing and reconstituting HIb for inoculation into infant rats has been described previously (11). A suspension of 3.7×10^6 viable HIb organisms/0.01 ml of isotonic saline was used to inoculate rats two days after virus infection; the method of intranasal inoculation was identical to that used for influenza virus inoculation. At the time of rat inoculation a viable count was carried out on each HIb suspension to ensure that the correct number of HIb were given, since this has been found to be important (8).

Three days following intranasal inoculation of rats with HIb, the animals were decapitated and a sample of free-flowing blood collected in a capillary tube. A 1:10 dilution of blood was prepared in isotonic saline, and the concentration of organisms measured by viable counting on chocolate agar, as described previously (11). At the time of examination rat heads were removed, skinned and fixed in buffered formalin for 7—10 days. After fixation, the specimens were treated with decalcifying solution for two hours (RDO, Bethlehem Instruments Ltd., Paradise, Hemmel Hempstead, Herts), trimmed to produce coronal and sagittal blocks, further decalcified for 1:5 hours and then processed and sectioned. Sections were stained with haemotoxylin and eosin, and examined histologically for evidence of meningitis (15).

Results

Control Viruses in Infant Rats

Virus Replication

In each set of experiments to study the behaviour of recombinant viruses in newborn rats, a parallel investigation of control virulent A/Victoria/3/75 and attenuated 4A2 influenza viruses was carried out; the behaviour of these two strains was found to be reproducible from experiment to experiment. The mean titres of virus found in four rat turbinates and lungs at various times after intra-

nasal inoculation with $10^{4.0}$ EID_{50} of influenza A/Victoria/3/75 virus is shown in Figure 1. A peak titre of $10^{6.8}$ $EBID_{50}$/ml was detected in turbinate extracts at 48 hours following virus inoculation; after this time the titres fell, and on day six the titre was $10^{2.3}$ $EBID_{50}$/ml. The titres of virus present in lung extracts were lower than those in the turbinates in the early period of observation: thus, peak titres of $10^{4.3}$ $EBID_{50}$/ml were found at 48 hours post-inoculation; these levels subsequently fell, and on day three and four, virus titres were similar to those seen in turbinate extracts (Fig. 1). In contrast, the titres of virus detected in the turbinates of infant rats infected with influenza virus 4A2 were significantly lower than found in influenza A/Victoria/3/75 virus-infected animals: the greatest concentration of virus was detected at 48 hours post inoculation, when the turbinate extracts contained $10^{4.8}$ $EBID_{50}$/ml and lung extracts contained $10^{4.0}$ $EBID_{50}$/ml of virus. The titres of virus fell progressively after this time (Fig. 1).

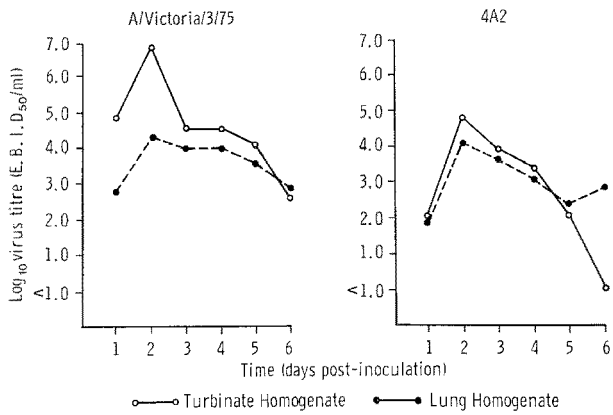

Fig. 1. Growth of control influenza viruses in infant rat turbinate and lung tissue

Table 1. *Incidence of bacteraemia and meningitis in rats following influenza virus infection, and subsequent inoculation with* H. influenzae type b

		No. rats with bacterial infection[b]			
		Bacteraemia		Meningitis	
	Infecting virus[a]	No.	%	No.	%
Control viruses	A/Victoria/3/75	9/12	75	5/12	42
	4A2	3/11	27	1/11	9
	Nil	0/12	—	0/12	—
Parental viruses	A/Ann Arbor/6/60	6/12	50	5/12	42
	A/Ann Arbor/6/60—7PI	2/12	17	0/12	—
	A/Queensland/6/72	10/10	100	10/12	83
Recombinant viruses	A/AA/6/60—CR6	1/12	8.3	0/12	—
	CR19, Clone 6	0/11	—	0/12	—
	CR22, Clone 5	0/12	—	0/12	—
	CR22, Clone 1	1/12	8.3	0/12	—

[a] Each rat inoculated i/n with $10^{4.0}$ EID_{50} of virus in an 0.01 ml volume

[b] Each rat inoculated with 3.7×10^6 colony forming units of *H. influenzae type b* at 48 hours after virus infection

Incidence of Bacterial Infection

Forty-eight hours following infection with $10^{4.0}$ EID_{50} of influenza virus, each rat was inoculated with $3.7 \times 10^{6.0}$ CFU of HIb; the incidence of bacteraemia and meningitis in these animals was determined 48 hours after bacterial infection. The results are shown in Table 1. For rats previously inoculated with A/Victoria/3/75 virus, nine of 12 (75 per cent) developed bacteraemia five of 12 (42 per cent) showed evidence of bacterial meningitis. In contrast, for animals inoculated with 4A2 virus only three showed developed bacteraemia (27 per cent) and one (9.1 per cent) developed meningitis. None of the control animals given bacteria only developed either bacteraemia or meningitis (Table 1). Thus, the incidence of bacteraemia and meningitis following HIb inoculation was significantly higher for animals given virulent A/Victoria/3/75 virus than for those given attenuated 4A2 virus ($P = <0.025$).

Parental Influenza Viruses in Infant Rats

Virus Replication

The replication of wild-type A/Ann Arbor/6/60 virus, cold-adapted A/Ann Arbor/6/60—P17 virus, derived from the wild-type strain, and A/Queensland/76/2 viruses, the latter two being the parental strains used for the production of recombinant virus strains, was studied in infant rats. The results are shown in Figure 2. For influenza virus A/Ann Arbor/6/60, peak mean virus titres were detected in the turbinates at 48 hours post-inoculation, when the titre was $10^{4.6}$ $EBID_{50}$/ml; the virus titres declined sharply after this time, and no detectable virus was found in the turbinate extracts made on days five and six. In contrast, virus titres in rat lung extracts remained fairly constant with titres of $10^{2.8}$ to $10^{4.6}$ $EBID_{50}$/ml detected during the observation period. The results of similar experiments carried out with cold-adapted strain A/Ann Arbor/6/60—PI7 showed a distinct growth pattern in rats to the wild-type parent. Thus, virus was not detected in turbinate extracts on days one or two following intranasal inoculation with $10^{4.0}$ of A/Ann Arbor/6/60—7PI; subsequently titres rose to a maximum of $10^{3.6}$ $EBID_{50}$/ml on day 6. Titres in lung extracts were maximal on day 2 to 4 post-inoculation but virus was not detected in extracts collected on days one, five and six following virus inoculation (Fig. 2). The titres of virus detected in the turbinates and lungs of infant rats inoculated with A/Queensland/6/72 virus showed peak titres in both lungs and turbinates at 24 hours, post-inoculation; the titres declined rapidly after this time, and virus was not detected in the lungs on days four and five following virus infection (Fig. 2).

Incidence of Bacterial Infection

The incidence of bacteraemia and meningitis in infant rats inoculated with $10^{4.0}$ EID_{50} of the parental strains of influenza virus and subsequently challenged with 3.7×10^{6} CFU of HIb is shown in Table 1. The incidence of bacteraemia was 50—100 per cent for rats inoculated with wild-type A/Ann Arbor/6/60 and A/Queensland/6/72, and the incidence of meningitis was 42 and 83 per cent, respectively. In contrast, only 2 of the 12 rats (17 per cent) developed HIb bacteraemia following prior inoculation with cold-adapted A/Ann Arbor/6/60—PI7

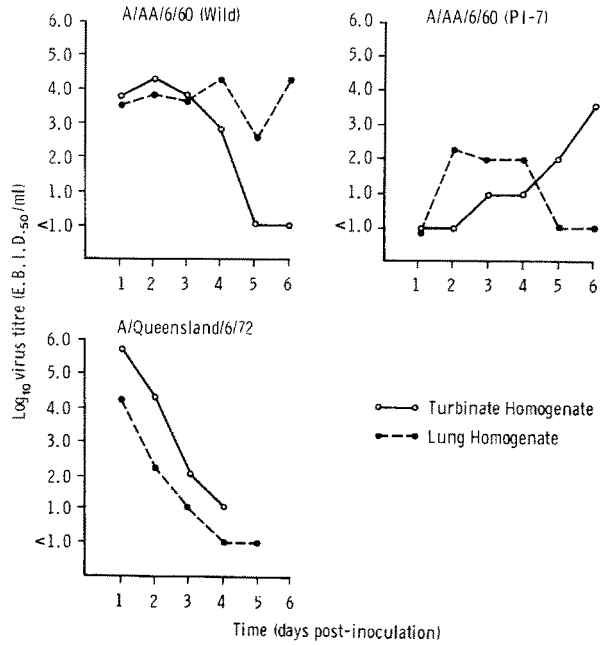

Fig. 2. Growth of parent influenza viruses in infant rat turbinate and lung tissue

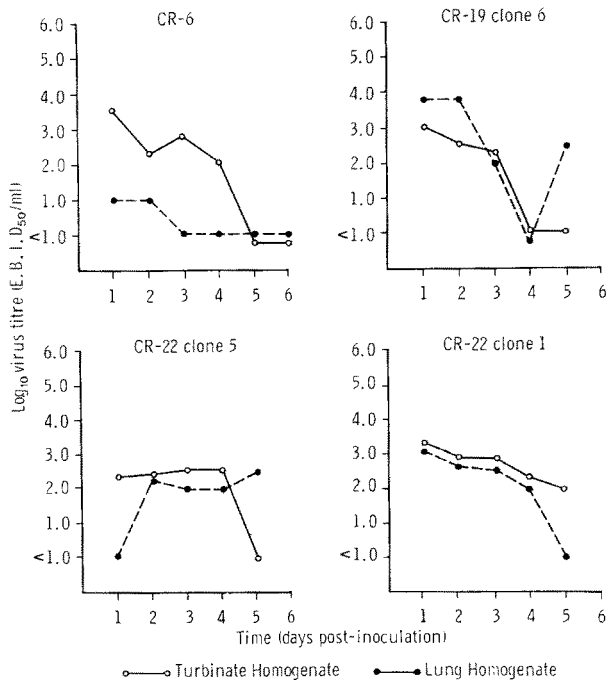

Fig. 3. Growth of recombinant viruses in infant rat turbinate and lung tissue

virus, and none of the animals showed evidence of meningitis. The results indicate that two wild-type strains of virus predisposed infant rats to a high level of subsequent bacterial infection, whilst the attenuated strain did not.

Recombinant Viruses in Infant Rats

Virus Replication

Following inoculation of infant rats with recombinant viruses A/Ann Arbor/6/60—CR6, CR19-clone 6 and CR22-clone 1 or CR22-clone 5, virus titres present in turbinate extracts were relatively low; thus, following intranasal inoculation with $10^{4.0}$ EID_{50} of virus, peak virus titres were detected at day 1—2 post-inoculation (Fig. 3); these titres varied from $10^{3.6}$ $EBID_{50}$/ml for A/Ann Arbor/6/60—CR6 to $10^{2.3}$ $EBID_{50}$/ml for CR22-clone 5 (Fig. 3). The titres of virus in lung extracts showed highly individual behaviour. For A/Ann Arbor/6/60—CR6 virus, low titres were found on day one and two post-inoculation, and virus could not be detected after this time. In contrast, for viruses CR19-clone 6 and CR22-clone 1, peak titres were found in the lungs early after infection, and subsequently fell, whilst for the CR22-clone 5 strain no virus was found on day one, post-inoculation, but virus at titres of $10^{2.0}$ $EBID_{50}$/ml and $10^{2.6}$ $EBID_{50}$/ml found on days two and five, respectively, following virus inoculation (Fig. 3).

Incidence of Bacterial Infection

The incidence of bacteraemia and meningitis in infant rats inoculated with HIb 48 hours after infection with recombinant influenza viruses is shown in Table 1. Only one animal previously inoculated with A/Ann Arbor/6/60—CR6 and one animal inoculated with CR22-clone 1 developed bacteraemia; none of these animals given CR19-clone 6 or CR22-clone 5 developed systemic bacterial infection. In addition, bacterial meningitis was not detected histologically in any of the animals inoculated with these recombinant viruses (Table 1).

Correlation of Results

From the results of previous studies, the parameters of influenza virus infection of infant rats which discriminated best virulent and attenuated strains are the virus titres present in turbinate extracts at 48 hours after intranasal inoculation of rats aged 48 hours with $10^{4.0}$ EID_{50} of virus; the virus titres in the lung show no discrimination (8, 11). In addition, bacterial infection with 10^5—10^6 HIb organisms gave the most discriminating results for bacterial infection (8). The results obtained for the present series of viruses using these criteria are shown in Table 2. The three virulent strains of influenza virus A/Victoria/3/75, A/Ann Arbor/6/60 and A/Queensland/6/72 each grew to relatively high titres in the turbinates of infant rats; the titres at 48 hours post-inoculation varied from $10^{5.2}$ to $10^{6.8}$ $EBID_{50}$/ml of tissue extract. In contrast, the five attenuated virus strains tested grew to lower titres; at 48 hours post-infection, the titres of these viruses present in turbinate extracts varied from $10^{2.6}$ $EBID_{50}$/ml for CR19-clone 6 to $10^{4.1}$ $EBID_{50}$/ml A/Ann Arbor/6/60—7PI. The ability of the influenza virus strains to enhance subsequent HIb infection also differed for virulent and attenuat-

ed strains (Table 2). Thus, for the three virulent strains the incidence of HIb bacteraemia varied from 50—100 per cent, whilst the incidence of meningitis was 42 and 83 per cent for the two strains tested. In contrast the ability of the attenuated virus strains to enhance bacterial infection was markedly lower; the incidence of bacteraemia varied from 17 per cent for A/Ann Arbor/6/60—7 PI to nil for CR 19-clone 6 and CR 22-clone 5 virus strains. None of the attenuated viruses enhanced HIb meningitis in infant rats.

Table 2. *Correlation between virus virulence for man, growth in rat turbinates and ability to enhance HIb infection for various influenza strains*

Virus pathogenicity for man	Virus strain	Titre of virus ($EBID_{50}$/ml) in turbinates[a]	Incidence of HIb infection[b]			
			Bacteraemia	(%)	Meningitis	(%)
Virulent	A/Victoria/3/75	$10^{6.8}$	9/12	(75)	5/12	(42)
	A/Ann Arbor/6/60	$10^{5.2}$	6/12	(50)	NT	
	A/Queensland/6/72	$10^{6.2}$	10/10	(100)	10/12	(83)
Attenuated	A/AA/6/60—7 PI	$10^{4.1}$	2/12	(17)	0/12	(—)
	A/AA/60—CR 6	$10^{4.0}$	1/12	(8.3)	0/12	(—)
	CR 19 clone 6	$10^{2.6}$	0/11	(—)	0/12	(—)
	CR 22 clone 1	$10^{3.3}$	1/12	(8.3)	0/12	(—)
	CR 22 clone 5	$10^{2.8}$	0/12	(—)	0/12	(—)
Control	Nil	—	0/12	(—)	0/12	(—)

[a] At 48 hours p.i. following inoculation with 10^4 EID_{50}/0.01 ml
[b] 10^5—10^6 HIb/0.01 ml inoculated 48 hours after virus infection

Discussion

The suggested laboratory methods for measuring the virulence of influenza viruses for man have included the effects of virus replication in ferrets (4, 7, 19, 23), virus replication in hamster lung (16), and growth in organ cultures of human and ferret tracheal tissue (13, 14); however, either the results have failed to give a complete correlation between virulence for man (6), or the studies are not yet sufficiently extensive to allow judgement. The more recently developed techniques of specifically identifying RNA segments of the influenza virus genome could theoretically allow an identification of those genetic elements which singly or collectively express the property of human virulence (18), but again data supporting this theory is lacking at the present time. Studies from this laboratory have indicated that the response of infant rats to influenza virus infection may be distinct for virulent and attenuated virus strains; thus, strains virulent for man grew to relatively high titre in infant rat turbinates and enhanced subsequent bacterial infection by HIb, whilst attenuated strains grew to significantly lower titres in rat turbinates and failed to enhance subsequent bacterial infection to the same degree (8, 10, 11). A total of 11 influenza virus strains have been investigated in this system to date, and for ten of these a direct correlation was found between virulence for man and the effects on infant rats. The exception was

influenza virus A/PR/8/34 which is non-infectious for man (1) but grew to high titres in rat turbinates and enhanced markedly subsequent HIb infection (11). All the strains examined in these studies were either wild-type viruses or recombinants of wild-type and A/PR/8/34 viruses; it remained unknown if the infant rat model would be of value in assessing human virulence in studies of recombinant strains produced from other parent viruses.

In the present study, we have examined influenza virus strains produced by recombination of virulent and cold-adapted A/AA/6/60—7PI viruses. The results indicated that for three wild-type viruses and five attenuated recombinant viruses complete correlation was found for human virulence and the effects of virus infection on infant rats. These results, together with those of previous studies (8, 11) support the suggestion that the infant rat may be a valuable model for assessing virus virulence for man. It is necessary, however, that studies of this model should be extended to include further wild-type viruses and recombinant viruses produced by other methods, such as recombination with the attenuated strain A/Okuda/78 (H3N2) (5, 12) and mutagen-induced *ts* strains (16, 17). These studies are now in progress.

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