

Master donor viruses A/Leningrad/134/17/57 (H2N2) and B/USSR/60/69 and derived reassortants used in live attenuated influenza vaccine (LAIV) do not display neurovirulent properties in a mouse model

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Abstract Demonstration of the absence of neurovirulent properties of reassortant viruses contained in live attenuated influenza vaccine (LAIV) is a regulatory requirement. A mouse model was used to detect neurovirulent properties of the cold-adapted, temperature-sensitive and attenuated influenza master donor viruses (MDVs) A/Leningrad/134/17/57 (H2N2) and B/USSR/60/69 and derived reassortant influenza viruses. A/NWS/33 (H1N1), which is known to be neurovirulent in mice, was used as a positive control. Under conditions where the positive control virus induced symptoms of disease and showed viral replication in the upper respiratory tract as well as in the brain, replication of the influenza master donor viruses and reassortant influenza A and B viruses was limited to the upper respiratory tract where they were administered. None of the mice inoculated with MDVs or reassortant influenza viruses suffered from disease, and no virus or viral replication was observed in the brains of these mice. The results demonstrate the absence of neurovirulent properties of the MDVs and reassortant influenza viruses derived therefrom used in LAIV.

Introduction

Influenza virus infection has been associated with several neurological disorders, including febrile seizures, influenza encephalopathy, post-influenza encephalitis, post-encephalitic Parkinson's disease, Guillain Barré syndrome, Kleine–Levin syndrome and Reye's syndrome [6, 20]. Clinical outcomes of these diseases are highly variable and may range from complete recovery without sequelae to severe neurological dysfunction or death. These diseases, however, are rare, and their aetiology and pathogenesis are still largely unknown [6, 11, 20].

Though neurological disease is rarely seen upon influenza virus infection, neurovirulence is a concern with live virus vaccines. It is for this reason that regulatory authorities require that the absence of neurovirulent properties of viruses used in live attenuated influenza vaccine (LAIV) be demonstrated [2]. Neurovirulence of influenza virus, which has been defined as the ability to undergo multicycle replication in the brain, has been studied in mice, using the neurovirulent A/NWS/33 (H1N1) virus [18, 22]. The availability of this virus as a positive control virus makes mice an attractive animal model to study neurovirulent properties of other influenza viruses, in particular those used in LAIV.

The master donor viruses A/Leningrad/134/17/57 (H2N2) and B/USSR/60/69, which are the basis of LAIV, were obtained by repeated passages in embryonated chicken eggs at low temperature (25–26°C), which rendered them cold-adapted (ca), temperature-sensitive (ts) and attenuated (att) [1, 8, 10]. These master donor viruses are used to generate reassortant influenza viruses that contain the surface glycoproteins haemagglutinin and neuraminidase of currently circulating influenza viruses, while the ca/ts/att phenotype is inherited from the master

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donor virus. A combination of influenza A H1N1 and H3N2 reassortant viruses and an influenza B reassortant virus are used in LAIV. The ca phenotype enables these viruses to replicate relatively efficiently at low temperatures, while the ts phenotype limits replication at high temperatures. Following intranasal administration of LAIV, viral replication will primarily take place in the nasopharynx of the upper respiratory tract, where lower temperatures prevail, while replication becomes restricted in the relatively warmer lower respiratory tract.

In the present paper, we confirmed that A/NWS/33 (H1N1) is a suitable positive control virus to detect neurovirulent properties in mice. Using the mouse model, neurovirulent properties of the influenza master donor viruses A/Leningrad/134/17/57 (H2N2) and B/USSR/60/69 as well as reassortant influenza A and B viruses derived therefrom were studied.

Materials and methods

Animals, cells and viruses

In all studies, female Swiss mice at the age of 4–6 weeks were used. Madin Darby canine kidney (MDCK) cells and A/NWS/33 (H1N1) were obtained from the American type culture collection (ATCC). Master donor viruses A/Leningrad/134/17/57 (H2N2) and B/USSR/60/69 were originally obtained from the Institute of Experimental Medicine, St. Petersburg, Russia. Wild-type viruses A/Brisbane/59/07 (H1N1), A/Brisbane/10/07 (H3N2), B/Brisbane/3/07 and B/Brisbane/60/08 were obtained from the National Institute for Biological Standards and Control (NIBSC), UK. 6:2 reassortant influenza viruses were obtained by co-infection of MDCK cells or embryonated chicken eggs with master donor virus and wild-type virus. Viruses were amplified either in MDCK cells or embryonated chicken eggs.

Neurovirulence studies

Prior to conducting animal studies, approval was obtained from the animal experimentation committee. Anesthetized mice (ten per group) were inoculated either intracerebrally with 30 μ l or intranasally with 50 μ l of A/NWS/33 (H1N1), A/Leningrad/134/17/57 (H2N2) (referred to as MDV A), or B/USSR/60/69 (referred to as MDV B) at titers varying from 3 to 8 \log_{10} TCID₅₀/ml or non-infected allantoic fluid as a negative control. Five days after inoculation, five mice per group were euthanized by intraperitoneal injection of 150 μ l dolethal, and their brains were collected. Brains were split into two portions: one half was collected in shredder tubes with ceramic beads in 500 μ l

PBS containing antibiotics, antimycotics and 4% (w/v) sucrose, ground and kept at -70°C until used for virus detection by PCR and titration; the other half was collected in 4% (v/v) formaldehyde solution for histopathology. At day 14, the remaining surviving mice were euthanized, and their brains were analyzed as above. If mice were found dead during the study, their brains were collected on that day, if possible. To avoid unnecessary suffering, some mice were euthanized, and their brains were collected on non-projected days. Non-projected days are days before day 5 or days between days 5 and 14.

In another study, ten mice per group were inoculated intranasally with 50 μ l live A/NWS/33, live MDV A or MDV B at a titer of $6.7 \pm 0.5 \log_{10}$ TCID₅₀/ml, or with 50 μ l beta-propiolactone (BPL)-inactivated MDV A or MDV B or PBS as a negative control. Virus inactivation was confirmed by titration. Three days post-inoculation, nasal turbinates and brains were collected for virus detection by PCR and titration.

In three good laboratory practice (GLP) studies, groups of 40 mice were used, and 50 μ l of virus at a titer of $7.0 \pm 0.5 \log_{10}$ TCID₅₀/ml was administered intranasally. Ten mice per group were sacrificed 3 days post-inoculation, and nasal lavages and turbinates were collected for virus titration. These mice served as controls to detect whether virus replication in the upper respiratory tract had taken place. The remaining 30 mice per group were euthanized 5 days post-inoculation, and their brains were collected for virus titration.

Clinical symptoms

Mice were observed daily for clinical symptoms. General symptoms of distress (piloerection, hunched posture, weight loss and reduced physical activity) as well as neurological symptoms (paresis and paralysis) were recorded. Symptoms were classified as mild if only one general symptom of distress was observed, moderate if more than one of these symptoms was observed and severe if mice either suffered from neurological symptoms, were found dead or were euthanized because of the seriousness of the symptoms.

RNA isolation, cDNA synthesis and PCR

RNA of 70 μ l tissue homogenate was isolated according to the manufacturer's instructions (QiaAmp Viral RNA Kit, Qiagen). Seven microliter of RNA was used to synthesize cDNA according to the manufacturer's instructions (Quantitect Reverse Transcription Kit, Qiagen) using the forward primers described below. For PCR, 6 μ l cDNA template was used in a total reaction volume of 50 μ l PCR buffer containing 2 μ l of 100 pmol solutions of forward

and reverse influenza nucleoprotein-specific primers (Influenza A: 5'-CAAAACAGCCAAGTATACAGCC [forward] and 5'-AGTAGAAACAAGGGTATTTTCCTT AAT [reverse], Influenza B: 5'-CCCAGAAGATCAGGT GCAACTG [forward] and 5'-TGCTTGCTTAGAGCAAT AGGTC [reverse]), 5 μ l of a 2 mM solution of dNTPs and 1 μ l of a 1 unit/ μ l solution of SuperTaq polymerase. PCR conditions were as follows: 4 min at 94°C followed by 30 cycles of 30 s at 94°C, 1 min at 50°C and 2 min at 68°C and a final elongation step of 7 min at 68°C. RNA isolated from A/NWS/33 (H1N1), MDV A and MDV B was used to prepare cDNA, which served as positive controls in the PCR. Negative PCR controls consisted of PCR reaction mixtures without template cDNA.

Titration

Tissue homogenates were titrated on MDCK cells. Briefly, serial dilutions of tissue homogenates were inoculated on MDCK cells and incubated for 6 days at 32°C and 5% CO₂, followed by measurement of haemagglutination of 0.5% (v/v) chicken or 1% (v/v) guinea pig red blood cells added to tissue culture supernatants. The titer was calculated according Reed and Muench [16] and expressed as log₁₀ TCID₅₀/ml.

Histopathology

Tissue specimens of the brains were used to detect histopathological changes. Histopathological examination of the brains was classified as showing meningeal lymphocytic infiltration, perivascular mononuclear cuffing, gliosis and necrotic foci associated with Gitter cells and were graded as absent (0), minimal (1), slight (2) or moderate (3).

Results

A/NWS/33 (H1N1) has been demonstrated in the past to be neurovirulent in mice and therefore could be a suitable positive control virus to detect neurovirulent properties of other influenza viruses [22]. To confirm the neurovirulent properties of A/NWS/33 (H1N1) and to determine the dose to be used as a positive control, mice were inoculated intracerebrally or intranasally with different doses of A/NWS/33 (H1N1). It was shown that, following intracerebral inoculation at a dose of 5.5 log₁₀ TCID₅₀ or higher, virus could be detected in the brain and induced symptoms of disease as well as histopathological changes within 5 days (Table 1). No virus could be detected by PCR in the brain of mice euthanized at day 14, demonstrating that the virus was cleared in 14 days. Between days 5 and 14, one mouse in the 1.5 log₁₀ TCID₅₀/dose group and one mouse

in the 5.5 log₁₀ TCID₅₀/dose group were euthanized because of the seriousness of the symptoms and only the brains of these mice, like those euthanized at day 5, were found to be positive by PCR. Following intranasal inoculation, virus could be detected in the brains at day 5 in a minority of mice, and no virus was detected at day 14. Between days 5 and 14, four mice in the 3.7 log₁₀ TCID₅₀/dose group, 2 mice in the 5.7 log₁₀ TCID₅₀/dose group and one mouse in the in the 6.7 log₁₀ TCID₅₀/dose group died or were euthanized because of the seriousness of the symptoms, and the brains of these mice were found to be positive by PCR. Histopathological changes in the brain were on average less clear for mice inoculated intranasally than those inoculated intracerebrally. In contrast, symptoms of disease seemed more pronounced but appeared at a later stage in mice inoculated intranasally than those inoculated intracerebrally, and macroscopy of the lungs of mice inoculated intranasally (3.7 TCID₅₀/dose and higher) revealed significant lung lesions, whereas virtually no changes in the lungs were observed for mice inoculated intracerebrally (data not shown). Together, these results show that A/NWS/33 (H1N1) is both a pneumo- and neurotropic virus that can be detected in the brains of mice inoculated either via the intracerebral or intranasal route, confirming the usefulness of this virus as a positive control to detect neurovirulent properties.

To demonstrate that both MDV A and MDV B can replicate in mice, and to demonstrate that live virus is needed to obtain positive PCR and titration results, mice were inoculated intranasally with either live or inactivated MDV A or MDV B as well as with A/NWS/33 (H1N1) and PBS as positive and negative controls, respectively. Since the optimum day of replication of influenza viruses after intranasal administration is typically day 3, nasal turbinates were collected at that day to detect whether viral replication had taken place. To determine whether viral replication could already be detected in the brain of the positive control on that same day (in the previous study this was only measured at day 5), brains were also isolated and subjected to PCR and titration. The results show that inactivated viruses did not yield positive PCRs in the brain or in the upper respiratory tract (URT), whereas live viruses were positive by PCR in the URT (Table 2). With the exception of one mouse, which showed a low titer for inactivated MDV B in the URT, none of the mice that were inoculated with inactivated virus had detectable virus titers in their brains or URT, whereas the majority of mice that were inoculated with live virus did show detectable virus titers in the URT. The sample from the mouse that was found to be weakly positive in titration most likely was contaminated with live MDV B during titration, since the inactivated MDV B used for inoculation was confirmed to be negative in titration and because PCR conducted on the same sample

Table 1 Neurovirulent properties of A/NWS/33 (H1N1)

Titer (log ₁₀ TCID ₅₀ /dose) and route of inoculation ^a	Days post-inoculation	PCR-positive brains	Clinical symptoms ^b				Histopathology ^c			
			None	Mild	Moderate	Severe	A	B	C	D
0 i.c. (neg. control)	0–5	–	5/5	–	–	–	0	0	0	0
	6–14	–	5/5	–	–	–	0	0	0	0
1.5 i.c.	0–5	–	5/5	–	–	–	0	0	0	0
	6–14	1/5 ^d	2/5	1/5	1/5	1/5	1	0	2	3
3.5 i.c.	0–5	3/5	3/5	2/5	–	–	3	3	1	2
	6–14	–	5/5	–	–	–	0	1	2	0
5.5 i.c.	0–5	5/5	–	–	2/5	3/5 (2)	3	2	2	2
	6–14	1/5 ^d	4/5	–	–	1/5	2	0	2	3
6.5 i.c.	0–5	8/8	–	1/8	4/8	3/8 (3)	2	2	2	2
	6–14	–	–	1/2	1/2	–	1	0	1	3
0 i.n. (neg. control)	0–5	–	5/5	–	–	–	0	0	0	0
	6–14	–	5/5	–	–	–	0	0	0	0
1.7 i.n.	0–5	–	5/5	–	–	–	0	0	0	0
	6–14	–	5/5	–	–	–	0	0	0	0
3.7 i.n.	0–5	2/5	5/5	–	–	–	1	1	0	0
	6–14	4/5 ^d	–	1/5	–	4/5 (2)	2	0	2	0
5.7 i.n.	0–5	–	–	–	4/5	1/5 (1)	0	0	0	0
	6–14	2/5 ^d	–	–	1/5	4/5 (2)	1	1	2	0
6.7 i.n.	0–5	1/6	–	–	3/6	3/6 (3)	1	2	2	0
	6–14	1/4 ^d	–	1/4	1/4	2/4 (1)	3	2	2	0

–, negative or none

^a i.c., intracerebral; i.n., intranasal

^b Symptoms are classified as None, no clinical symptoms; Mild, one of the following general symptoms of distress: piloerection, hunched posture, weight loss, reduced physical activity, Moderate, more than one of the previous symptoms; Severe, neurological symptoms (paresis or paralysis), death or euthanization because of seriousness of symptoms. The number of dead mice is shown in brackets

^c Histopathology is classified as A. Meningal lymphocytic infiltration, B. Perivascular mononuclear cuffing, C. Gliosis, D. Necrotic foci associated with gitter cells with the following grades: 0: absent, 1: minimal, 2: slight and 3: moderate

^d Mice that died or were euthanized because of symptoms on non-projected dates

Table 2 Replication of influenza A and B viruses in mice

Inoculum ^a	Virus detection					
	URT day 3 p.i. ^b			Brains day 3 p.i.		
	PCR no. pos.	Titration no. pos.	Mean titer ^c	PCR no. pos.	Titration no. pos.	Mean titer ^c
A/NWS/33 (H1N1) live	5/5	5/5	6.0	–	–	na
MDV A live	5/5	3/5	3.5	–	–	na
MDV A inactivated	–	–	na	–	–	na
MDV B live	4/5	5/5	4.7	–	–	na
MDV B inactivated	–	1/5	2.1	–	–	na
Negative control	–	–	na	–	–	na

–, negative; na, not applicable

^a Inocula administered intranasally at an infectious titer of $5.4 \pm 0.5 \log_{10}$ TCID₅₀/dose; infectious titers of inactivated viruses were confirmed to be negative

^b URT, upper respiratory tract; p.i., post-inoculation

^c Mean titer of positive samples expressed as log₁₀ TCID₅₀/ml

was also negative. Thus, both MDV A and MDV B do replicate in the URT of mice, which is needed to obtain positive PCR and titration results. From this experiment, it is also clear that day 3 is too early to detect viral replication in the brain following intranasal administration. None of the mice inoculated with A/NWS/33 (H1N1) showed any positive PCR or titration results in their brains at day 3.

After having demonstrated that both master donor viruses were able to replicate in mice, neurovirulent properties of the MDVs were studied using A/NWS/33 (H1N1) as a positive control. Mice inoculated intracerebrally with A/NWS/33 (H1N1) showed symptoms and histopathological changes in their brains (Table 3). Virus could be detected by PCR in the brains of 4 out of 5 mice, and infectious titers were measured in the brains of all mice at day 5. An example of PCR analysis is shown in Fig. 1. Like in the first study, no virus could be detected by PCR or titration at day 14. The only mouse that was PCR and titration-positive beyond day 5 was one that was found dead on day 6. Mice inoculated intracerebrally with MDV A or MDV B showed only mild symptoms and minimal histopathological changes, if any at all. Moreover, no virus could be detected in the brains by PCR or titration, demonstrating that the master donor viruses were cleared within 5 days of inoculation and were not able to reside or replicate in the brain. Following intranasal inoculation with A/NWS/33 (H1N1), the same pattern was observed as in the first study: clinical symptoms were more pronounced and appeared later in time than those of mice inoculated

intracerebrally. Histopathological changes of the brain were virtually absent in mice inoculated intranasally, but macroscopy of the lungs revealed significant lesions in this group (data not shown). In contrast to the first study, this time, virus could be detected by PCR at day 5 in the brains of all mice that were inoculated intranasally. With the exception of one mouse showing mild symptoms and minimal histopathological changes following intranasal inoculation with MDV A, none of the mice inoculated intranasally with MDV A or B showed clinical symptoms or histopathological changes. No virus could be detected in the brains of any of these mice, either by PCR or by titration, demonstrating that these viruses do not reach the brain after intranasal administration.

From the above studies, it is clear that it is not useful to analyze brains at day 14, as no virus can be detected anymore by that time. While day 14 is far too late, day 3 is too early to detect virus in the brain. Day 5 has been shown to be a good time point for measurement, as live virus can be detected in the brain by that time and, to prevent unnecessary suffering, is the most suitable day for euthanasia. Since live virus could be detected in the brain of control mice after intranasal inoculation, and because the route of vaccination of LAIV is intranasal, subsequent studies only used the intranasal route and included subgroups of mice that were sacrificed at day 3 to detect viral replication in the upper respiratory tract.

In the first of three studies conducted in accordance with GLP, it was shown that both MDV A and MDV B did

Table 3 Neurovirulent properties of influenza A and B master donor viruses

Inoculum and route of inoculation ^a	Days post-inoculation	PCR-positive brains	Titration-positive brains	Clinical symptoms ^b				Histopathology ^c			
				None	Mild	Moderate	Severe	A	B	C	D
A/NWS/33 (H1N1) i.c.	0–5	4/5	5/5	–	2/5	2/5	1/5 (1)	2	2	3	3
	6–14	1/5 ^d	1/5 ^d	–	1/5	3/5	1/5 (1)	1	2	1	1
MDV A i.c.	0–5	–	–	5/5	–	–	–	1	1	0	0
	6–14	–	–	3/5	2/5	–	–	0	0	0	0
MDV B i.c.	0–5	–	–	5/5	–	–	–	0	1	0	0
	6–14	–	–	5/5	–	–	–	0	0	0	0
A/NWS/33 (H1N1) i.n.	0–5	5/5	4/5	5/5	–	–	–	1	0	0	0
	6–14	2/5 ^d	1/5 ^d	–	–	1/5	4/5 (3)	0	0	0	0
MDV A i.n.	0–5	–	–	5/5	–	–	–	0	0	0	0
	6–14	–	–	4/5	1/5	–	–	1	0	0	0
MDV B i.n.	0–5	–	–	5/5	–	–	–	0	0	0	0
	6–14	–	–	5/5	–	–	–	0	0	0	0

–, negative or none

^a Inocula administered intracerebrally (i.c.) or intranasally (i.n.) at infectious titers of 5.5 log₁₀ TCID₅₀/dose or 5.7 log₁₀ TCID₅₀/dose, respectively

^b See Table 1 for classification of symptoms. The number of dead mice is shown in brackets

^c See Table 1 for classification of histopathology

^d Mice that died or were euthanized because of symptoms on non-projected dates

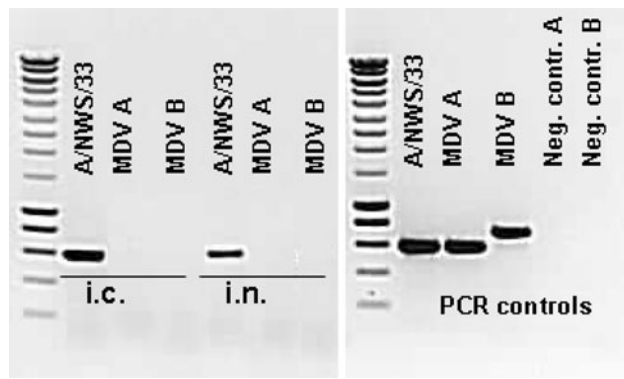


Fig. 1 Example of PCR analysis of brain homogenates. *Left panel* mice inoculated intracerebrally (i.c.) or intranasally (i.n.) with A/NWS/33, MDV A or MDV B. *Right panel* positive (A/NWS/33, MDV A and MDV B) and negative PCR controls

replicate in the upper respiratory tract but were unable to spread to the brain (Table 4). In contrast, A/NWS/33 (H1N1) spread to and replicated in the brains of the majority of the mice after intranasal administration. While A/NWS/33 (H1N1) induced symptoms of disease, none of the master donor viruses did. In the second GLP study, 6:2

reassortant influenza viruses of the flu season 2008–2009 were generated (A44/Brisbane/59/07 (H1N1), A44/Brisbane/10/07 (H3N2) and B56/Brisbane/3/07). It was demonstrated that these reassortant viruses are able to replicate in the upper respiratory tract. In contrast to mice inoculated with A/NWS/33 (H1N1), mice inoculated with 6:2 reassortant influenza viruses showed virtually no symptoms of disease and did not show infectious titers in their brains. In this experiment, the number of mice that showed replication and the virus titers that were obtained for the influenza A reassortant viruses were low, as were the number of positives and titers obtained in the brains of mice inoculated with A/NWS/33 (H1N1). In the third GLP study, a new 6:2 reassortant virus (B56/Brisbane/60/08) based on MDV B was studied. Again, virus replication was demonstrated in the upper respiratory tract, with virtually no symptoms, and again, no virus was measured in the brain, whereas A/NWS/33 (H1N1) replicated both in the upper respiratory tract and in the brain and also induced symptoms of disease. These three studies clearly demonstrate that both MDV A and MDV B as well as reassortants derived therefrom are unable to reach and replicate in the brain following intranasal administration.

Table 4 Good laboratory practice (GLP) studies to detect neurovirulent properties of master donor viruses and reassortant viruses

Inoculum ^a	Virus replication				Clinical symptoms ^c			
	URT day 3 p.i. ^b		Brains day 5 p.i.		None	Mild	Moderate	Severe
	No pos.	Mean titer ^d	No. pos.	Mean titer ^d				
GLP study 1								
A/NWS/33 (H1N1)	10/10	5.8	25/29	3.4	–	16/30	7/30	7/30 (7)
MDV A	4/10	3.0	–	–	30/30	–	–	–
MDV B	10/10	5.8	–	–	29/29	–	–	–
Negative control	–	–	–	–	29/29	–	–	–
GLP study 2								
A/NWS/33 (H1N1)	10/10	>5.0	13/30	2.5	1/30	8/30	14/30	7/30 (7)
A44/Brisbane/59/07 (H1N1) ^e	4/10	2.4	–	–	29/30	1/30	–	–
A44/Brisbane/10/07 (H3N2) ^e	2/10	2.2	–	–	30/30	–	–	–
B56/Brisbane/3/07 ^e	10/10	3.5	–	–	30/30	–	–	–
Negative control	–	–	nd	na	30/30	–	–	–
GLP study 3								
A/NWS/33 (H1N1)	10/10	5.9	25/30	3.2	–	6/30	18/30	6/30 (6)
B56/Brisbane/60/08 ^e	9/10	4.6	–	–	29/30	1/30	–	–
Negative control	–	–	nd	na	30/30	–	–	–

–, negative or none; na, not applicable; nd, not done

^a Inocula administered intranasally at an infectious titer of $5.7 \pm 0.5 \log_{10}$ TCID₅₀/dose

^b URT, upper respiratory tract; p.i., post-inoculation

^c See Table 1 for classification of symptoms. The number of dead mice is shown in brackets

^d Mean titer of positive samples expressed as \log_{10} TCID₅₀/ml

^e Reassortant virus based on master donor virus

Discussion

Demonstration of the absence of neurovirulent properties of viruses contained in LAIV is a requirement by regulatory authorities [2]. While mice, ferrets and monkeys are the most frequently used models in influenza virology, historically, monkeys have been used to study neurovirulence. According to the European Pharmacopoeia the brains of monkeys are to be inoculated with virus to be tested, followed by observation of the animals for neurological symptoms and histopathological analysis of the brain at the end of the observation period. This model lacks a positive control virus, and there is no need to demonstrate the presence of virus or viral replication in the brain following inoculation. The mouse model offers several advantages in studying neurovirulent properties of influenza viruses. In contrast to ferrets and monkeys, mice are easy to handle and accommodate and allow for big group sizes. Ferrets and monkeys are not easily available in sufficient numbers, and there are ethical objections against using monkeys when other animal models are available. The most important advantage of mice, however, is the availability of a positive control virus such as A/NWS/33 (H1N1) that replicates in the brain of mice [22]. For ferrets and monkeys, a positive control would need to be sought that is potentially highly pathogenic to humans. Indeed, highly pathogenic H5N1 strains have been shown to be neurovirulent in ferrets [4, 5, 12, 23], whereas the recent novel, and less pathogenic, H1N1 virus is not [15]. To the best of our knowledge, there are no reports available of influenza viruses invading and replicating in the brain of monkeys, but if such a virus was available, it most likely would be highly pathogenic to humans. Thus, the mouse model is preferable from a safety, ethical and practical point of view. In addition, the use of small-animal models is also encouraged by regulatory authorities [2].

We studied neurovirulent properties of the *ca*, *ts* and *att* master donor viruses A/Leningrad/134/17/57 (H2N2) (MDV A) and B/USSR/60/69 (MDV B) as well as *ca/ts/att* reassortant influenza A and B viruses derived from them in a mouse model using A/NWS/33 (H1N1) as a positive control virus. Summarizing all of the studies, in none of the mice inoculated intranasally with MDV A, MDV B or reassortant influenza A and B viruses, could virus be detected in the brain 5 days post-inoculation, whereas in the majority of mice inoculated with A/NWS/33 (H1N1), the presence of virus and viral replication in the brain was demonstrated. We did not isolate brains of mice inoculated intranasally with master donor viruses or derived reassortants between days 5 and 14. Although, based on the data, it cannot be excluded that master donor viruses or reassortants may reach the brain between days 5 and 14, it is highly unlikely that they replicate in the brain. In support

of this, master donor viruses inoculated directly into the brain could not be detected anymore at day 5. Furthermore, if master donor viruses or reassortants were able to replicate in the brain (i.e., were neurovirulent), this most likely would be noticed due to the appearance of clinical symptoms. None of the mice inoculated either intracerebrally or intranasally suffered from disease, whereas mice inoculated with A/NWS/33 (H1N1) clearly did. Thus, neither the master donor viruses nor the reassortants derived from them displayed neurovirulent properties in this mouse model.

For mice inoculated intranasally, there seemed to be no relationship between the dose of A/NWS/33 (H1N1) administered and the number of mice that were found virus-positive in the brain in the first experiment. In mice receiving the highest doses (including $5.7 \log_{10}$ TCID₅₀/dose), fewer virus-positive brains were found compared to mice receiving a lower dose. It is not clear where the variation seen in the first experiment comes from. However, in subsequent experiments, virus could be detected and viral replication demonstrated in the brains of the majority of mice inoculated with approximately $5.7 \log_{10}$ TCID₅₀/dose.

While for MDV B and reassortant influenza B viruses, viral replication in the upper respiratory tract could be clearly demonstrated in the vast majority of mice, for MDV A and reassortant influenza A viruses, both the number of mice that showed viral replication in the upper respiratory tract and the viral titers obtained were relatively low, most notably in GLP study 2. While procedural imperfections cannot be ruled out for this particular study (the number of positive brains and the titers of control virus were also relatively low), it appears that the mice used are less susceptible to MDV A and influenza A reassortants compared to MDV B and influenza B reassortants. This is explained by the fact that mice are not natural hosts for influenza virus, and seasonal influenza viruses usually need prior adaptation to yield high titers [21]. Results may improve when another breed of mice is used or when the doses administered are increased. Nevertheless, viral replication in the upper respiratory tract, in the absence of clinical signs, has been demonstrated for the master donor viruses and reassortant viruses derived from them, which supports the usefulness of this animal model.

The influenza viruses contained in LAIV have a *ca/ts/att* phenotype. The *ca/ts/att* influenza viruses A/Leningrad/134/17/57 (H2N2) and B/USSR/60/69 were generated previously in Russia, and these strains are currently in use as master donor viruses to generate reassortant influenza A and B viruses containing the surface glycoproteins haemagglutinin and neuraminidase of currently circulating strains, whilst the gene segments conferring the *ca/ts/att* phenotype are inherited from the master donor virus

(so-called 6:2 reassortants). In light of this phenotype, the need for neurovirulence studies could be questioned for the influenza viruses contained in LAIV. Indeed, these viruses will be restricted in their replication outside the upper respiratory tract. The *ca/ts/att* phenotype results from multiple mutations, and loss of attenuation would require reversion of multiple mutations, which is unlikely to occur *in vivo* [9] or *in vitro*. Indeed, we have demonstrated that these mutations are conserved after 15 successive passages of both master donor viruses (unpublished data). The genome composition, the presence of mutations and temperature sensitivity are confirmed for each virus strain prior to their use in LAIV. In addition, while the surface glycoproteins haemagglutinin (HA) and neuraminidase (NA) have been shown to be involved in neurovirulence of mouse-adapted strains, other genes may play a role as well [22]. The master donor virus A/Leningrad/134/17/57 (H2N2) was isolated during the 1957 pandemic. In contrast to the pandemic of 1918, which has been associated with encephalitis lethargica, the pandemics of 1957 and 1968 have not been associated with neurological disease [13]. Furthermore, vaccine strains are always 6:2 reassortants, and therefore any additional role of the genes coding for internal viral proteins is excluded. Finally, ample experience with LAIV in Russia ($>30 \times 10^6$ doses) using numerous different 6:2 reassortant strains has not revealed any vaccine-related neurological disease.

Despite the observed temporal and/or geographical association of influenza epidemics with neurological disease, it remains to be demonstrated that influenza virus is a direct cause. For example, influenza virus or viral antigens are rarely demonstrated in the cerebrospinal fluid or brain of patients suffering from influenza encephalopathy [7, 14, 19]. In addition, Guillain Barré, Kleine–Levin and Reye's syndrome all are associated with infections not necessarily involving influenza virus, and, in the case of Reye's syndrome, the role of viral infection, if there is any, may be limited [3, 17]. It appears that invasion and replication of influenza virus in the central nervous system (CNS) is not the primary cause of influenza-associated neurological disease but that infection with viruses may occasionally induce excessive immunological and physiological responses leading to neurological disorders. Indeed, hypercytokinemia has been suggested to be involved in the pathogenesis of influenza encephalopathy [11, 14, 19]. The fact that influenza-associated encephalopathy was observed in Japan, but not elsewhere, during an H3N2 epidemic may be explained by a genetic component that predisposes an individual for hypercytokinemia [14]. This supports the idea that not neuroinvasion, but the response to infection plays a primary role in influenza-associated encephalopathy. Hypercytokinemia has not been reported for LAIV.

Thus, for strains contained in LAIV to be neurovirulent and cause neurological disease, a combination of properties and events is required. First, the wild-type influenza strain used in reassortment must have neurovirulent properties, allowing the virus to reach and replicate in the CNS, which is rare for seasonal human influenza. Second, NA and/or HA alone must play a decisive role in neurovirulence. Third, the resulting neurovirulent 6:2 reassortant, which has been checked phenotypically and genotypically, must reverse multiple mutations *in vivo* to lose its temperature-sensitive and attenuated phenotype to allow for spread and replication outside the upper respiratory tract, which is highly unlikely. The concurrence of these properties and events during seasonal influenza vaccination with LAIV is negligible.

In conclusion, the mouse model has been demonstrated to be useful for studying neurovirulence. Although the chances on neuroinvasion and subsequent neurological disease may be considered negligible for viruses contained in LAIV, the absence of neurovirulent properties can be demonstrated using the mouse model.

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