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H1N1 influenza A virus neuraminidase modulates infectivity in mice

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ABSTRACT

In the 2 years since the onset of the H1N1 2009 pandemic virus (H1N1pdm09), sporadic cases of oseltamivir-resistant viruses have been reported. We investigated the impact of oseltamivir-resistant neuraminidase from H1N1 Brisbane-like (seasonal) and H1N1pdm09 viruses on viral pathogenicity in mice. Reassortant viruses with the neuraminidase from seasonal H1N1 virus were obtained by co-infection of a H1N1pdm09 virus and an oseltamivir-resistant H1N1 Brisbane-like virus. Oseltamivir-resistant H1N1pdm09 viruses were also isolated from patients. After biochemical characterization, the pathogenicity of these viruses was assessed in a murine model. We confirmed a higher infectivity, in mice, of the H1N1pdm09 virus compared to seasonal viruses. Surprisingly, the oseltamivir-resistant H1N1pdm09 virus was more infectious than its sensitive counterpart. Moreover, the association of H1N1pdm09 hemagglutinin and an oseltamivir-resistant neuraminidase improved the infectivity of reassortant viruses in mice, regardless of the NA origin: seasonal (Brisbane-like) or pandemic strain. This study highlights the need to closely monitor the emergence of oseltamivir-resistant viruses.

1. Introduction

By the end of the winter season 2007–2008, the frequency of H1N1 viruses related to A/Brisbane/59/2007 reference strain (seasonal strain named sH1N1) containing the oseltamivir-resistance mutation H275Y in neuraminidase (NA) was 25% (Meijer et al., 2009). A year later during the 2008–2009 winter season, almost all circulating sH1N1 viruses carried the H275Y mutation and continued to spread without drug selective pressure (Dharan et al., 2009; Kramarz et al., 2009).

However, following the emergence of the H1N1 pandemic 2009 virus (H1N1pdm09, pandemic strain abbreviated pH1N1) in April 2009 (Garten et al., 2009; Neumann et al., 2009), oseltamivir-resistant pH1N1 viruses were only sporadically identified around the world (in August 2011, the WHO reported 570 cumulative cases) (WHO, 2011b). Identified cases were mainly associated with antiviral treatment or post exposure prophylaxis (Ferraris et al., 2010). Human-to-human transmission of oseltamivir-resistant pH1N1 virus remained limited (Lackenby et al., 2011) despite sustained viral fitness and the observed transmissibility of both reverse genetics generated (Brookes et al., 2011; Octaviani et al., 2011) and naturally occurring (Baz et al., 2009; Hamelin et al., 2010; Kiso et al., 2010; Memoli et al., 2010) H275Y NA pH1N1 viruses in ferrets.

Neuraminidase mutations conferring resistance to oseltamivir or zanamivir, induce a dramatic decrease of NA activity, an impairment of growth *in vitro* (Yen et al., 2006), and a reduction of infectivity, pathogenicity, and transmissibility *in vivo*. However an oseltamivir resistant A/Brisbane/59/2007-like H1N1 (sH1N1) emerged with H275Y NA and replaced the sensitive one. It remains unclear why sH1N1 viruses better tolerate the functional defect caused by the H275Y mutation compared to other H1N1 viruses. Several studies have shown that this functional defect could be counterbalanced by permissive secondary mutations in NA, such as R222Q, V234M (Bloom et al., 2010) or D344N (Bloom et al., 2011; Rameix-Welti et al., 2006). Interestingly such mutations were not found in the H275Y NA of pH1N1 viruses that were also characterized by a small decrease in enzyme activity and viral fitness (Brookes et al., 2011). Moreover, contrary to oseltamivir-resis-

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tant sH1N1 viruses, resistant pH1N1 viruses have not yet spread in the human population.

Based on these observations we focused our study on the behavior of reassortant viruses harboring a H275Y NA from sH1N1 in a pH1N1 genetic background. We previously showed (Ottmann et al., 2010) that pH1N1 could acquire oseltamivir-resistance by reassortment with the H275Y NA from sH1N1. Co-circulation of seasonal and pandemic H1N1 viruses was observed during the first stage of the pandemic, giving initial cause for concern over the possibility of co-infection, although this now seems less and less likely (Peacey et al., 2010). In March 2011, only six former sH1N1 strains closely related to A/Brisbane/59/2007 were detected (WHO, 2011a).

Here we investigated in a murine model the properties of oseltamivir-resistant viruses either isolated from patients or obtained by reassortment. We assessed the infectivity of each virus by measuring the quantity of virus necessary to infect fifty percent of mice (mouse infectious dose, i.e. MID_{50}).

2. Materials and methods

2.1. Facilities and ethics statement

Experiments performed with reassortant viruses obtained by co-infection were conducted in the Biosecurity level 4 laboratory "Jean Mérieux", Lyon, France, according to the current recommendations of the French health authorities during the onset of the pH1N1 virus. Animal studies were approved by the ethics committee of animal experimentation of the Université Lyon 1.

2.2. Viruses

We used A/Lyon/1364/2007 and A/Lyon/1337/2007 clinical isolates antigenically related to A/Brisbane/59/2007 H1N1 and A/Lyon/969/2009 and A/Lyon/48.425/2009 clinical isolates related to the A/California/07/2009 H1N1 pandemic reference strain.

The reassortment between A/Lyon/969/2009 and the oseltamivir-resistant virus A/Lyon/1337/2007 was performed by *in vitro* co-infections within the BSL4 facilities (Ottmann et al., 2010). Viral genomes were extracted (QIamp virus RNA mini kit, Qiagen) and sequenced by the Plateforme de Génotypage des Pathogèneset Santé Publique, PF8-Génopole (Institut Pasteur, Paris).

In order to obtain additional data on neuraminidase enzymatic properties, we generated by reverse genetics recombinant viruses RG-969 (eight segments from A/Lyon/969/2009) and RG-R16 (NA segment from A/Lyon/1337/2007 and the seven other segments from A/Lyon/969/2009). Complementary DNAs from each segment of A/Lyon/969/2009 and from the HA and NA segments of A/Lyon/1337/2007 were cloned in the pHW2000 plasmid (Hoffmann et al., 2000) as already described (Moules et al., 2010). All recombinant plasmids were sequenced (GATC Biotech). HEK 293T cells (ATCC CRL-11268) were transfected with eight cDNAs (1 μg per plasmid) using Superfect (Qiagen) in Opti-MEM medium (Gibco) and then incubated at 37 °C. At 24 h post-transfection, 1 μg/ml of trypsin (Roche) was added. Supernatants were harvested 72 h post -transfection, clarified by centrifugation at 1800*g* for 5 min, and virus yield was titrated in MDCK cells (ATCC CCL34).

All virus titers were determined in MDCK cells by end point titration and expressed as $CCID_{50}$ (cell culture infective dose-50) per ml values determined using the Reed and Muench statistical method (Reed and Muench, 1938).

2.3. Kinetic analyses of neuraminidase activity

Fluorometric NA activity assay and the determination of half-maximal inhibitory concentration (IC_{50}) for oseltamivir carboxylate (kindly provided by GSK) were performed twice for each isolate as described previously (Ferraris et al., 2005). The Michaelis–Menten constant (Km) was evaluated from viral supernatants using the MUNANA substrate (Sigma–Aldrich) as already described (Casalegno et al., 2010; Rameix-Welti et al., 2006). The 4-methylumbelliferone fluorescence was measured with the FLUOstar OPTIMA fluorometer (BMG LABTECH) or TECAN fluorometer. The initial velocities (Vi) were calculated for each substrate concentration and integrated in a non-linear Michaelis–Menten equation by the MARS program (BMG) for Km calculation. The results were expressed as the mean of three experiments. The Ki (affinity of NA for an inhibitor) was evaluated at a concentration range between 0.01 and 10 nM for oseltamivir-sensitive viruses and between 50 and 3200 nM for oseltamivir-resistant viruses. The substrate was used at 20 μM. The Vi was calculated for each inhibitor concentration and integrated in a non-linear competitive inhibition equation by GraphPad software (Prism) for Ki calculation.

2.4. Mouse experiments

Six-week-old female Balb/cByJ mice (Charles River Laboratories) were anesthetized with 5% isoflurane (1-chloro-2,2,2-trifluoroethyl difluoromethyl ether) before infection. Infectious viruses were serially diluted in PBS to obtain infectious doses corresponding to 10^6, 10^5, 10^4, 10^3 and 10^2 $CCID_{50}$/20 μl. Mice were inoculated intranasally with 20 μl of one of the infectious virus doses. The 50% mouse infectious dose (MID_{50}) and 50% mouse lethal dose (MLD_{50}) titers were calculated by inoculating groups of eight mice (or eleven mice in the 10^5 $CCID_{50}$/20 μl infectious dose group). For MID_{50} determination, three mice from each group were euthanized 3 days post infection (p.i.) and their lungs, spleen, heart, liver, brain and blood collected and stored at −70 °C. The frozen tissues were thawed, weighed and homogenized in 0.5 ml of PBS, and clarified by centrifugation (2000*g*) at 4 °C. Supernatants were titrated by end point dilution using the MDCK cell line and confirmed by hemagglutination assays using chicken erythrocytes (Charles River). The viral load of blood samples was determined on viral RNA extracted from 140 μl serum using the QIAmp virus RNA mini kit (Qiagen). The amount of M viral genomic segment was then determined by real-time RT-qPCR as described previously (Duchamp et al., 2010). Three mice from the group inoculated with the 10^5 $CCID_{50}$/20 μl infectious doses were euthanized 6 days p.i, and their lungs removed, treated and titrated as described above. The five remaining mice of each group were monitored daily for clinical signs for up to 14 days p.i. Antibodies in blood samples from mice inoculated with the highest infectious dose and still alive 14 days p.i. were measured by hemagglutination inhibition assay.

2.5. Affinity of hemagglutinin (HA) for the sialic acid receptors

The HA affinity assays were performed by coating viruses on 96-well plates overnight at room temperature (Richard et al., submitted for publication). Biotinylated sialylglycopolymers Neu5Acα2–3Galß1–4Glc-PAA-Biotin (3′SL) and Neu5Acα2–6Galß1–4GlcNAc-PAA-Biotin (6′SLN) (Lectinity) were serially diluted in a 10 nM zanamivir solution and incubated 2 h at 4 °C. After washing, peroxidase labeled anti-biotin antibodies (Euromedex) were added. TMB (KPL) was used as substrate and absorbance was recorded at 450 nm (Microplate reader UMV340 Asys, Bioserv). The dissociation constant (Kd) for specific binding was calculated with a non-linear one site binding equation using GraphPad software.

3. Results

3.1. Characterization of reassortant H1N1 viruses

Most of the reassortant viruses obtained by the co-infection of A/Lyon/1337/2007 sH1N1 virus and A/Lyon/969/2009 pH1N1 virus as previously described (Ottmann et al., 2010) were completely characterized by whole genome sequencing (Table 1).

The parental pH1N1 strain was found in five clones (R15, R19, R22, R30 and R39), none of which contained sH1N1 segments. The polymerase complex PB2/PB1/PA sequenced in 27 reassortant clones was mainly composed of segments of pH1N1 origin (20/27, 74%). Five clones (R28, R4, R17, R43 and R8) harbored a pH1N1 background with two segments from sH1N1 (NP and NA, HA and NA or HA and M). Six clones (R7, R18, R44, R32, R6, R45) had three segments from sH1N1 and clones R35 and R24 had four and seven segments (all except M) from sH1N1, respectively (Table 1).

Altogether, the partial and complete genotypes of recovered clones indicated a higher frequency of reassortment of the segments HA (82.6%) and NA (52.2%) than other segments. Reassortant viruses containing other segments occurred at lower frequencies (25% or less); the reassorted segments were NP, PB1, PB2, PA, NS and M in decreasing order of frequency. In addition, the most frequent constellation comprised a pH1N1 genetic background with the sH1N1 HA.

The threat of an emergent oseltamivir-resistant pandemic virus led our experiments to further characterize the R16, R28 and R43 clones, all of which carried the H275Y NA of sH1N1 (Table 1). The *in vitro* replication of reassortant and oseltamivir-resistant pH1N1 viruses was not impaired compared to the parental pH1N1 and sH1N1 strains (Ottmann et al., 2010).

3.2. Sialidase activities of parental and reassortant H1N1 viruses

Neuraminidase activities of native, reassortant and recombinant viruses were measured using cell culture supernatant (Table 3). The NA enzymatic characteristics (IC_{50}, Km, Ki) of reassortant viruses were not influenced by the genetic background of HA origin, but were by NA origin (Table 3).

The oseltamivir-resistant strains A/Lyon/1337/2007 sH1N1 and A/Lyon/48.425/2009 pH1N1 and the RG-R16 virus showed similar values for IC_{50} of oseltamivir between 230 and 260 nM (Table 3).

The Ki determination confirmed neuraminidase inhibitor assays (Table 3), since a high oseltamivir IC_{50} value correlated with a high Ki for A/Lyon/1337/2007, reassortant viruses and A/Lyon/48.425/2009 viruses (Table 3). These high Ki values reflected the low inhibitor affinity of H275Y NA regardless of origin; seasonal or pandemic strain. No significant difference ($p > 0.1$) was observed between the seasonal and the pandemic oseltamivir resistant neuraminidase. So, despite the large antigenic distance between seasonal and pandemic NA, oseltamivir and zanamivir susceptibilities and affinities were comparable for all of the N1 NA (Stoner et al., 2010).

Regarding the substrate affinity (Km), three NA groups were highlighted. A/Lyon/1364/2007 NA showed the highest substrate affinity with a Km value of 14.2 μM. The H275Y mutation within the related NA induced a 2.7-fold decrease in substrate affinity with a Km value of 38.4 μM for A/Lyon/1337/2007. Remarkably, although the Km of pandemic NA was significantly different to Km of seasonal NA, the p value between pH1N1 NA and A/Lyon/1337/07 sH1N1 was equal to 0.04 whereas the p value between pH1N1 NA and A/Lyon/1364/07 sH1N1 was lower than 0.0001. The substrate affinity of the pandemic H275Y NA was reduced by 1.8-fold compared to the wild type pH1N1 and appeared very low compared to H275Y sH1N1. Finally, neuraminidase characteristics were unaffected by the genetic background since for the three recombinant viruses (R16 or RG-R16, R28 and R43) the affinity (Km) of NA for its substrate was of the same order of magnitude compared to that of the parental virus A/Lyon/1337/2007.

3.3. Infectivity, virulence and persistenceof A H1N1 reassortant viruses in mice

The MLD_{50} of the three reassortant viruses R16, R28 and R43 was determined in BALB/c mice and compared with values

Table 1
Genotypes of H1N1 viruses recovered from co-infection assay.

number of viral clones	name of viral clones[a]	PB2[b]	PB1	PA	HA	NP	NA	M	NS
5	R15, R19, R22, R30, R39								
1	**R16**								
10	R1, R3, R11, R20, R27, R31, R34, R36, R38, R46								
1	**R28**								
3	R4, R17, **R43**								
1	R8								
3	R7, R18, R44								
1	R32								
2	R6, R45								
1	R35								
1	R24								
2	R9, R33				nd				
1	R26				nd				
10	R5, R12, R13, R14, R23, R41, R42, R47, R49, R50	nd	nd	nd		nd		nd	nd
4	R2, R10, R37, R48	nd	nd	nd		nd		nd	nd
3	R21, R25, R29	nd	nd	nd		nd	nd	nd	nd
	Percent % of reassortment	11.1	14.8	7.4	82.6	25.9	52.2	3.7	7.4
	(ratio : reassortants/sequenced segments)	(3/27)	(4/27)	(2/27)	(38/46)	(7/27)	(24/46)	(1/27)	(2/27)

[a] The name of reassortant viruses characterized further are in bold.
[b] Gray boxes: segment from the A/Lyon/969/2009 pH1N1 virus; black boxes: segment from A/Lyon/1337/2007 oseltamivir-resistant sH1N1 virus; nd: not determined.

Table 2
Wild type and reassortant viruses.

A/H1N1	HA	NA	NA Position and amino acid			HA sequence	NA sequence
		Origin	106	248	275		
A/Lyon/969/2009	p	p	V	N	H	JF429402	JF429403
RG-969	p	P	V	N	H		
A/Lyon/1364/2007	s	s	I	N	H	HQ658484	HQ658486
A/Lyon/1337/2007	s	s	I	N	Y	HQ658483	HQ658485
R16	p	s	I	N	Y		
RG-R16	p	s	I	N	Y		
R28	p	s	I	N	Y		
R43	s	s	I	N	Y		
A/Lyon/48.425/2009	p	p	I	D	Y	JF429393	JF429398

p: segment from the A/Lyon/969/2009 pH1N1; s: segment from A/Lyon/1364/2007 or A/Lyon/1337/2007 sH1N1 related to A/Brisbane/59/2007. All segments were sequenced by the Genotyping of Pathogens and Public Health platform, Institut Pasteur, Paris.

Table 3
Enzymatic parameters for wild-type and reassortant H1N1 viruses recovered from co-infection assays.

	NA					HA	
	NI assay[a]		Enzymatic properties			HA affinities	
	IC50 (nM)		Ki (nM)		Km (μM) [b]	Kd (nM)	Kd (nM)
	Oseltamivir	Zanamivir	Oseltamivir	Zanamivir		6′SLN	3′SL
A/Lyon/969/09	0.62	0.49	0.25 ± 0.04	0.27 ± 0.04	34,7 ± 13,24	50	1500
RG-969	0.40	0.49	0.21 ± 0.004	0.21 ± 0.01	27.03 ± 1.48		
A/Lyon/1364/07	0,4	0,6	0.23 ± 0.04	0.21 ± 0.02	14.2 ± 1.25		
A/Lyon/1337/07	260	0,2	84.13 ± 1.30	0.28 ± 0.01	38.4 ± 1.91	20	950
R16[c]	nd	nd	nd	nd	21.1		
RG-R16	231.7	0.58	79.38 ± 1.15	0.26 ± 0.01	27.53 ± 0.97		
R28[c]	nd	nd	nd	nd	22.7		
R43[c]	nd	nd	nd	nd	23.2		
A/Lyon/48.425/09	232	0.29	99.95 ± 4.66	0.33 ± 0.02	62.6 ± 4.68		

nd: not determined.
[a] The neuraminidase inhibitors assays were performed on cell supernatant with MU-NANA as substrate. Results are given as means IC_{50} value ± standard deviation (SD) calculated using values derived from three separate assays performed in duplicate.
[b] Results are given as means ± SD for three independent Km determinations for duplicate samples of cell supernatant on BMG Labtek fluorometer.
[c] Km determinations were performed in the BSL4 laboratory on a TECAN fluorometer.

Table 4
Pathogenicity and infectivity of the parental and reassortant viruses.

	Pathogenicity MLD_{50}[a]	Infectivity D3 MID_{50}[b]	Seroconversion D14 %[c]
A/Lyon/969/2009 pH1N1	$>10^6$	10^4	100
A/Lyon/1337/2007 H275Y NA sH1N1	$>10^6$	$>10^6$	100
R16 (H275Y NA sH1N1)	$>10^6$	$10^{2,5}$	100
R28 (H275Y NA and NP sH1N1)	$>10^6$	$10^{2,2}$	100
R43(H275Y NA and HA sH1N1)	$>10^6$	10^6	100
A/Lyon/48.425/2009 pH1N1 (H275Y NA)	$>10^6$	$10^{2.7}$	100

Eight mice were infected for each virus and at each infectious dose (10^6, 10^5, 10^4, 10^3, 10^2 TCID50/20 μl) by intranasal inoculation.
nd: not determined.
[a] Groups of 5 mice per inoculation dose (see Materials and methods) were used to determine MLD_{50}. Mice were monitored 14 days p.i. MLD_{50} are expressed as the $TCID_{50}$/20 μl required for one MLD_{50}.
[b] Groups of 3 mice per inoculation dose were euthanized 3 days p.i. to determine MID_{50}. Virus titers in lungs were determined, and MID_{50} values were expressed as the $TCID_{50}$/20 μl required for one MID_{50}.
[c] Seroconversions were assayed against the virus used for the inoculation on sera from mice infected with 10^6 $TCID_{50}$ and alive 14 days p.i.

obtained for pH1N1 and the oseltamivir-resistant sH1N1 viruses. As shown in Table 4, parental and reassortant H1N1 viruses did not demonstrate the ability to cause severe disease or death in this mouse species despite high infectious doses. However, seroconversion was confirmed in mice infected with a dose of 10^6 $CCID_{50}$, demonstrating an effective infection.

The pH1N1 virus was able to replicate in mouse lungs without prior host adaptation with a mouse infectious dose (MID_{50}) of 10^4, while the A/Lyon/1337/2007 sH1N1 failed to be detected efficiently (Fig. 1). The reassortant viruses R16 and R28, bearing the H275Y sH1N1 NA in the pandemic background, were also able to replicate efficiently in mouse lungs. The oseltamivir-resistant pH1N1 A/Lyon/48.425/2009 replicated in mouse lungs as efficiently as did reassortant viruses. The MID_{50} of the two reassortant viruses R16 and R28 and the oseltamivir-resistant non-reassortant pH1N1 virus were 150 fold lower than the sensitive pH1N1 virus. The reassortant virus R43 with HA and H275Y NA from sH1N1 had a high MID_{50} (10^6 $CCID_{50}$), as did the parental sH1N1 strain (Table 4).

Virus yield and persistence were measured in lungs and blood by end point titration and RT-qPCR, respectively, at 3 and 6 days p.i., in

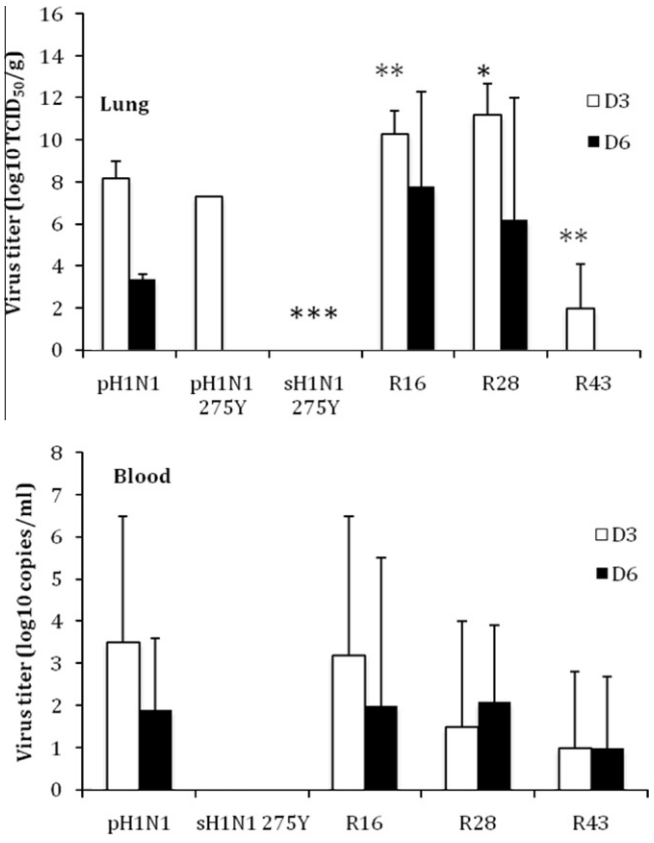

Fig. 1. Virus detection in lungs and blood of infected mice. Lungs and blood viral titers of mice infected with pH1N1 reassortant R16 (pH1N1 background with NA from sH1N1): R28 (NA and NP from sH1N1): and R43 (NA and HA from sH1N1) (see Table 2) or with clinical isolate viruses pH1N1 A/Lyon/969/2009, pH1N1 A/Lyon/48.425/2009 (pH1N1 275Y) and H275Y sH1N1 A/Lyon/1337/2007. Mice were inoculated with an infectious dose of 10^5 $TCID_{50}$. Virus in lungs and blood was titrated at 3 and 6 days after inoculation by end point titration on MDCK cells and by RT-qPCR on M segment, respectively. Lung: (log TCID50/g) mean of lung infectious titers at 3 or 6 days post inoculation. Lung infectious titers was only determined at day 3 for pH1N1 A/Lyon/48.425/2009. Negative result: infectious titer of <1. Blood: (log10 copies/ml) mean of blood infectious titers at 3 or 6 days post inoculation. The mean values for two or three mice with standard deviations are presented. $^{*}p < 0.05$; ** $p < 0.01$; $^{***}p < 0.001$: p values of the t test when compared to the pH1N1 A/Lyon/969/2009.

mice inoculated with 10^5 $CCID_{50}$ (Fig. 1). No virus was detected in the liver, spleen, heart or brain of mice. Oseltamivir-resistant sH1N1 failed to be detected either in the lungs or the blood. The reassortant R43 virus was detected in one mouse only, at low titers, in the lungs (<10^2 $CCID_{50}$/ml) and blood (<10^2 log10 copies/ml) at 3 and 6 days p.i. At day 3 p.i., pH1N1 virus was detected at 10^8 $CCID_{50}$/g of lung, and in the blood at $10^{3.5}$ log10 copies/ml. The MID_{50} of pH1N1 H275Y was of the same order as MID_{50} of R16 and R28 reassortants ($p > 0.1$) (Table 4). This high infectivity efficiency was counterbalanced by a replication in lung not statistically significant to pH1N1 but lower than reassortants R16 and R28. Reassortant viruses R16 and R28 were detected at levels that were 200-fold higher than those of parental pH1N1 virus, at $10^{10.3}$ and $10^{11.2}$ $CCID_{50}$/g of lung, respectively. Statistically significant differences were also observed between R28 and pH1N1 viruses ($p < 0.05$). Six days after inoculation, pH1N1 virus was detected at relatively low titers (less than 10^4 $CCID_{50}$/g of lung), whereas the R16 and R28 viruses were detected at high levels in 2 out of 3 mice ($10^{7.5}$ $CCID_{50}$/g of lung).

3.4. Receptor binding affinity of HA

One hypothesis that can be drawn from the above results could be that a distinct HA binding affinity for sialic acid exists for sH1N1 and pH1N1 viruses. Assessment of the receptor binding affinity was therefore performed on both pH1N1 and sH1N1 HA (Table 3). The sH1N1 HA protein showed a higher affinity than the pH1N1 HA for both sialic acid with alpha 2–3 (950 and 1500 nM, respectively) and alpha 2–6 (20 and 50 nM, respectively), linked to galactose.

4. Discussion

Since the emergence of the pandemic H1N1 virus in 2009 and despite the use of neuraminidase inhibitors, the isolation of oseltamivir-resistant pH1N1 has remained sporadic. We have recently obtained reassortant pH1N1-sH1N1 viruses by co-infection of a cell line (Garten et al., 2009; Ottmann et al., 2010). Here, we have studied the biochemical characterization and the viral fitness of these reassortant viruses in a murine model. The genetic contents analysis of our reassortant viruses revealed a very relative frequency of reassortment for HA and NA segments.

Oseltamivir-sensitive NA from sH1N1 or pH1N1 can be distinguished primarily by its affinity towards the neuraminidase substrate. A low substrate affinity for NA and HA seemed to allow a better infectivity in mice. Indeed, we reported the mouse infectivity advantage of two reassortant viruses R16 and R28, both harboring the H275Y sH1N1 NA and the pH1N1 HA. The sole replacement of the pH1N1 HA by the sH1N1 HA (R43 virus) mitigated infectivity in mice suggesting a predominant role of HA in this case. Moreover, the sH1N1 NP in the R28 virus had no detectable influence on viral fitness when compared with the R16 reassortant virus. Another striking result we observed was the boost of infectivity (MID_{50}) in the presence of a H275Y NA from either sH1N1 or pH1N1, in a pandemic genetic background, when compared to strains with the oseltamivir-sensitive NA.

Although the data available so far suggest that the rare cases of oseltamivir-resistant pH1N1 are mostly related to the acquisition of the H275Y mutation in treated patients, oseltamivir resistance could also be the result of a reassortment event. Despite the lack of demonstration of such a reassortment in humans to date (2010), it was nevertheless feasible due to the co-circulation of these two strains (sH1N1 and pH1N1) during the early part of the pandemic (Peacey et al., 2010), and one year after the onset of the pandemic with 53 areas of virus co-circulation identified (Janies et al., 2010). Moreover, triple-reassortant influenza H1N1 of swine origin has been shown to easily reassort with sH1N1 *in vivo* (Bastien et al., 2010). We have shown that reassortment by co-infection of MDCK cells with pH1N1 and sH1N1 (Ottmann et al., 2010) or H5N1 (O. Ferraris and M. Ottmann, data not shown) is easily achieved. In contrast we previously failed to isolate reassortant viruses by co-infection of H3N2 and H5N1 strainsusing the same method.

Although the molecular mechanism of reassortment is not clearly understood, the most transferable segments appeared to be identical in human and swine hosts: HA, NA and PB1 (Khiabanian et al., 2009). While *in vitro* replicative kinetics demonstrated little variability among all viruses tested, mouse infectivity analyses revealed subtle differences between strains (Schrauwen et al., 2011; Xu et al., 2011). The replicative capacity of sH1N1 in mice was very weak since this virus was not detectable in the lungs. The slight increase in mouse infectivity of R43 reassortant virus, with HA and NA from sH1N1, reinforced the importance of the genetic background of viruses for mice infectivity, as already observed in other studies (Ilyushina et al., 2010; Itoh et al., 2009; Munster et al., 2009; Schrauwen et al., 2011).

Although R16 and R28 reassortant pH1N1 viruses, with H275Y NA from sH1N1, displayed the same infectivity as oseltamivir-resistant pH1N1 mutant, they exhibited a higher mouse infectivity

than the parental viruses. The susceptibility of the NA to oseltamivir or zanamivir was similar with any antigenically related virus whether seasonal or pandemic (Puzelli et al., 2011). Moreover, the H275Y NA mutation conferred a comparable increase in IC_{50} and Ki, regardless of neuraminidase antigenicity. The mouse infectivity of viruses with a pandemic background and an H275Y NA (of seasonal or pandemic strain origin) was higher than that of the pH1N1 virus. This result highlights the advantage that the H275Y mutation in seasonal or pandemic NA confers on mouse infectivity. A second 248D mutation in pandemic NA has been found but has never been associated with severe cases (Graham et al., 2011). Neither the infection duration nor the pathogenicity of such viruses was modified in mice compared with the wild-type counterpart, as described by Boivin's and Kawaoka's groups (Hamelin et al., 2010; Kiso et al., 2010).

Controversial results were reported on the viral fitness and transmissibility of oseltamivir-resistant pandemic viruses, since they were shown to be as effective as oseltamivir-sensitive pandemic viruses (Hamelin et al., 2010; Kiso et al., 2010; Seibert et al., 2010) and associated with a loss of viral fitness (Brookes et al., 2011; Duan et al., 2010). However, contrary to the H275Y sH1N1 mutant, the H275Y pH1N1 mutant has not spread in the human population to date. In human infection, the predominance of the oseltamivir-sensitive strain after the end of treatment also suggests a better fitness of the oseltamivir-sensitive versus oseltamivir-resistant pH1N1 viruses, in the absence of drug selection pressure (Campanini et al., 2010; Chen et al., 2009).

Oseltamivir-resistant NAs from sH1N1 and pH1N1 are closely related regarding inhibitor susceptibility and affinity but differ in their substrate affinity. The Km of the oseltamivir-resistant sH1N1 NA was similar to the Km of the wild type NA pH1N1. The increased Km due to the H275Y mutation in pH1N1 NA could be one of the factors explaining the low spread of oseltamivir-resistant pH1N1 virus in the human population. The ability of oseltamivir-resistant sH1N1 NA to support the H275Y defective mutation correlates with the presence of permissive mutations R222Q and V234M (Bloom et al., 2010). Such mutations were not found in H275Y pH1N1 NA. Although viruses with H275Y mutated NA from pH1N1 are associated with a lower enzymatic affinity and a lack of any fitness advantage (Baz et al., 2009; Brookes et al., 2011; Pizzorno et al., 2011), these viruses seem to be able to accommodate the single H275Y mutation as infectivity was maintained.

The balance between HA and NA activities seems to be an important condition for efficient virus replication. Replicative competence could be acquired by a decrease in viral HA binding affinity to sialylglycoconjugates (Mitnaul et al., 2000). HA binding to sialylglycoconjugates is thought to be one of the factors enabling viral replication in humans. Moreover, pH1N1 HA binding to alpha 2.6 sialylglycoconjugate is approximately three times lower than sH1N1 HA binding. This relatively low alpha 2.6 affinity compared to human viruses could be one of the factors that helped H275Y pH1N1 mutant to maintain efficient viral replication (Wagner et al., 2002). Moreover, the high alpha 2.6 affinity of both NA and HA from sH1N1 may explain the lack of replicative efficacy of these viruses in mice but their efficient spread in humans (Rameix-Welti et al., 2011). Our findings reinforce the importance of an appropriate balance of HA and NA activity depending on host species for efficient virus virulence and transmissibility.

In conclusion, we have shown that reassortment between pH1N1 and H275Y NA sH1N1 viruses results in a virus as fit as the H275Y pH1N1 virus in mice. Oseltamivir-resistant pH1N1 exhibited higher infectivity *in vivo* than oseltamivir-sensitive pH1N1 or sH1N1 viruses without evidence of altered pathogenicity or transmissibility, as described by others (Hamelin et al., 2010; Octaviani et al., 2011). Emergence of permissive mutations in the oseltamivir-resistant pH1N1 might (Hurt et al., 2011) or might not (Pizzorno et al., 2011) induce changes in viral fitness, infectivity and pathogenicity. Moreover, changes in the HA could also be a major determinant for transmission efficiency, as described recently (Jayaraman et al., 2011). All these modifications need to be carefully surveyed to anticipate a possible increase in the viral fitness of oseltamivir-resistant pH1N1 viruses. In parallel, our study also underlines the urgent need to develop new antiviral drugs.

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