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Influenza Virus Strains with a Fusion Threshold of pH 5.5 or Lower Are Inhibited by Amantadine

Brief Report

By

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With 2 Figures

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Summary

Nineteen influenza virus strains were examined for susceptibility to amantadine-HCl (AMT) and for pH-thresholds of haemagglutinin-induced haemolysis. Whereas pH-thresholds below 5.5 were not seen in AMT-resistant strains, AMT-sensitive strains showed pH-thresholds either below or above 5.5.

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The haemagglutinin (HA) molecule of influenza A and B viruses undergoes a conformational change at low pH *in vitro*. This property, well established in biological, biochemical, antigenetical, and morphological studies (for review see 13), is genetically determined by the HA-gene (7, 18). The critical pH-threshold value is strain-dependent and differs in a wide range between pH 5.0 and 6.0. It has been found that endosomal and lysosomal pH can reach low values, up to 5.0 (16) or 4.5 (9). Thus, it has been argued that the conformational change of HA also occurs *in vivo* and forms a necessary step during cell infection after endocytosis of the virus particles, triggering the fusion between viral and cellular membranes (17).

Amantadine-hydrochloride (AMT), a potent inhibitor of many influenza strains *in vivo* and *in vitro*, increases the endo- and lysosomal pH. The maximum effect of AMT on intralysosomal pH is reported as an increase up to 5.5, in experiments with mouse peritoneal macrophages (9). It has been suggested that the inhibitory effect of AMT is caused by preventing the confor-

mational change of HA and, consequently, the membrane fusion of those influenza virus strains which require a low pH. Thus, sensitivity and resistance to amantadine could be expected to be determined by the HA-gene. In contrast, several authors have described influenza A strains and recombinants whose susceptibility to AMT seemed to be determined by the matrix-protein gene or by a combination of genes other than the HA-gene (8, 3, 14).

Bearing in mind the studies of Scholtissek & Faulkner (14), Hay & Zambon (4), Oxford (10) and Daniels *et al.* (1), we established the following working hypothesis:

AMT has at least two different and genetically independent modes of inhibiting virus production, one of these involving HA as a target. Any virus strain can be sensitive to AMT either on both locations or steps in the replication circle, or on one or on none. Thus, a strain not requiring an endo- or lysosomal pH lower than 5.5 for its HA to undergo the conformational change, may be sensitive or resistant depending on the susceptibility of the alternative target. However, a strain which does require a pH lower than 5.5 for membrane fusion is always sensitive to AMT, regardless of other mechanisms.

This study describes the correlation between AMT-susceptibility in a monkey kidney cell-line model and the pH-haemolysis-threshold for 16 influenza A strains of 5 different subtypes and 3 influenza B strains, among them strains from our laboratory collection and some recombinants and mutants with well-documentated AMT-susceptibility. For names, abbreviations and source see Table 1.

Viruses were propagated in the allantoic cavity of 11-day-old embryonated chicken eggs and then adapted to a continuous monkey kidney cell-line (LLC-MK 2), Flow Laboratories Ltd., Irvine, Scotland). For haemolysis tests, virus-containing fluids were clarified by slow centrifugation and purified by adsorption to and elution from chicken erythrocytes in PBS. 25 µl of virus suspensions (>1000 haemagglutination units/ml) were added to 2.0 ml of 2 per cent fresh chicken erythrocytes in saline buffered with 0.1 M citric acid – sodium citrate at pHs varying from 5.0 to 6.0 in steps of 0.1 units. After two incubation steps at 4 °C for 1 hour and 37 °C for 1 hour, respectively, the erythrocytes were sedimentated by centrifugation and supernatants were measured photometrically for haemoglobin at 540 nm. This was a slightly modified version of the haemolysis assay of Kida *et al.* (5). Erythrocytes not coated with virus served as controls for each pH. Haemolysis of controls was not dependent on pH between 6.0 and 5.0, so that their extinctions over the whole pH-range could be used to form a threshold (mean ± 2 SD) between spontaneous and virus-induced haemolysis. The pH-threshold of the virus was defined as the pH-value, at which virus-induced haemolysis occurred first with decreasing pH. Figure 1 shows a typical ex- periment involving A/Bk/79. Experiments were done in dupli-

Table 1. *Virus strains and abbreviations used in this study*

Haemagglutinin-subtype	Strain	Abbreviation
A-H 1	A/Wilson Smith/33 (H 1 N 1)	A/WS/33
	A/Puerto Rico/8/34 (H 1 N 1)	A/PR/8
	A/Brazil/11/78 (H 1 N 1)	A/Bra/78
A-H 2	A/Japan/305/57 (H 2 N 2)	A/Jap/57
A-H 3	A/equine/Miami/1/63 (H 3 N 8)	A/eq/63
	A/Aichi/2/68 – A/PR/8 – recombinant (H 3 N 2)*	A/X 31
	2 AMT-resistant mutants of A/X 31*	A/X 31-1 a
		A/X 31-ab 4
	A/Victoria/3/75 (H 3 N 2)	A/Vic/75
	A/Texas/1/77 (H 3 N 2)	A/Tex/77
	A/Bangkok/1/79 (H 3 N 2)	A/Bk/79
A-H 7	A/FPV/Rostock/34 (H 7 N 1)	A/FPV
	2 A/FPV – A/eq/63 – recombinants with HA from A/FPV**	A/FPV-19
		A/FPV-263
	A/FPV – A/turkey/England/63 (H 7 N 3)-recombinant with HA from the latter**	A/FPV-11
A-H 10	A/FPV – A/chick/Germany/N/49 (H 10 N 7)-recombinant with HA from the latter**	A/FPV-5
B	B/Lee/40	B/Lee/40
	B/Hong Kong/8/73	B/Hk/73
	B/Singapore/222/79	B/Sing/79

* Kindly provided by Dr. J. J. SKEHEL, London, England. Production and characterization of mutants has been described by DANIELS *et al.* (1), where A/X 31/1 a is designated as "X-31 mutant 1 a". A/X 31-ab 4 is a mutant with a single amino acid change His – Arg in position 17 of HA 1 (J. J. SKEHEL, personal communication).

** Kindly provided by Dr. C. SCHOLTISSEK, Giessen, Federal Republic of Germany. Production of recombinants from A/FPV has been described by SCHOLTISSEK & FAULKNER (14). In A/FPV-19 genome segments 2 and 5 and in A/FPV-263 segments 3 and 8 are replaced by A/eq/63, in A/FPV-11 segments 1, 2, 4, 6, 7 and 8 by A/turkey/England/63 and in A/FPV-5 segments 3, 4, 5 and 8 by A/chick/Germany/N/49 (C. SCHOLTISSEK, personal communication). Genome 4 codes for HA.
The other virus strains were from our collection. For passage history see RUIGROK *et al.* (13).

cate and repeated at least twice. The assay was highly reproducible, with only a few discrepancies of 0.1 between experiments. The results for all viruses are presented in Table 2. We have already published the pH-threshold-values of some of the strains presented here, determined by monitoring morphological changes of HA by electronmicroscopy and by trypsin dige-

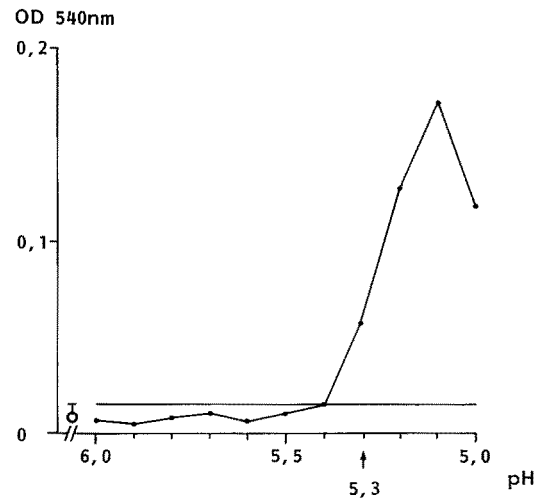

Fig. 1. Single haemolysis experiment for A/Bk/79. With decreasing pH, haemolysis of virus-coated erythrocytes (● = mean of two determinations) exceeds spontaneous haemolysis, as determined by 22 controls (± = mean of controls ō 2 SD), first at a pH of 5.3. This value was defined as the pH-threshold of A/Bk/79 in this experiment

stion (13). The results of this paper are in agreement with those previous findings.

In the literature, a great discrepancy exists with respect to the AMT-susceptibility of several influenza A reference strains. For instance, A/PR/8 is reported to be resistant (8), relatively resistant (14) or sensitive (2). Several reasons for these conflicting results may be considered: Firstly, old reference strains such as A/PR/8 (isolated in 1934), have a propagation history differing for each laboratory as regards host cell systems and passage number. This can influence the biological properties of, among others, their HA (11, 15) by genetic mutation and make comparison of results between laboratories difficult or impossible. Secondly, several assays broadly measure any decrease in the net virus production, while others may focus on certain stages during penetration and replication. SCHOLTISSEK & FAULKNER (14) demonstrated strains which changed their susceptibility to AMT when measured either by a single or by a multiple growth cycle assay, and suggested the existence of different and independent targets of AMT-susceptibility. Thirdly, there are differences in the choice of host cells and in the actual concentration of AMT.

For our purposes, an AMT-susceptibility assay should meet the following conditions:

The virus pools used for both the haemolysis assay and the susceptibility assay should be obtained from the same host cell system and the same passage.

The susceptibility assay should measure any difference of the net virus production between absence and presence of a maximum concentration of AMT and should lead to clear-cut results.

We chose a test similar to the monkey kidney cell model of GRUNERT & HOFFMANN (2). In short, confluent monolayers of LLC-MK 2 cells, produced in tissue culture cluster plates and maintained in 5 ml serum-free Dulbecco's modification of Earle's medium 199, were pretreated or not with 25 µg/ml AMT (no A-1260, Sigma Chemical Company, St. Louis, MO, U.S.A.) for 2 hours. Then, all plates were infected with 0.2 ml of 10-fold virus dilutions through the end point of infectivity. The AMT-pretreated cells also received 25 µg/ml AMT in the maintenance medium. Cell-controls contained either maintenance medium only or medium with 25 µg/ml AMT. AMT-concentrations higher than 25 µg/ml rapidly led to rounding up and death of the cells. After two days the cells were harvested by three circles of freeze-thawing and the fluids were tested for haemagglutination activity by standard titration with fresh chicken erythrocytes. Cell controls without virus and with or without AMT showed no HA-titres. Virus titres were expressed as the logarithmated reciprocal of the dilution of the seed virus which was associated with a 50 per cent decrease of the maximum haemagglutination activity (12). Two typical examples of virus growth with or without 25 µg/ml AMT are presented in Figure 2. In an experiment with A/PR/8, untreated cells produced a virus titre of 5.1, but cells treated with 25 µg/ml AMT produced a

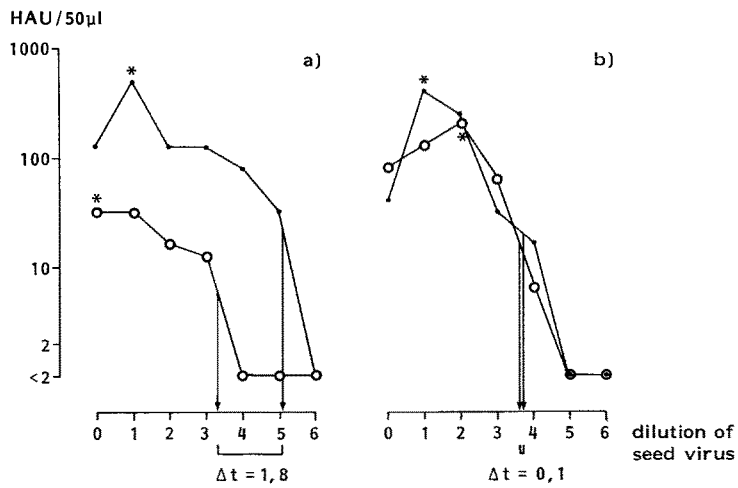

Fig. 2. Virus production in AMT-treated or untreated LLC-MK 2-cells. On abscissa, the logarithmated reciprocals of the dilutions of the virus used to infect LLC-MK 2-cells on day 0. On ordinate, the HA concentration of harvested virus on day 2 from untreated (●) or AMT-treated (○) cells (<2 = no haemagglutination observed). Maximum HA-activity (*) and calculated dilution of 50 per cent maximum HA-activity (↓) (= virus titre) are marked. *a* Single experiment with A/PR/8. Difference between virus titres from untreated and AMT-treated cells (Δt) is 1.8. *b* Single experiment with A/FPV showing a Δt of 0.1.

titre of 3.3, resulting in a difference between titres (Δt) of 1.8 (Fig. 2 a). However, when A/FPV was used, Δt was only 0.1 (Fig. 2 b). Repeated experiments allowed the calculation of 95 per cent confidence intervals (95 per cent CI) for Δt. A virus strain was regarded as AMT-resistant when Δt included zero in the 95 per cent CI of repeated experiments, and vice versa. A clear-cut decision between resistance and sensitivity to AMT was possible for all strains (Table 2).

Table 2. *pH-threshold-values and amantadine suceptibility of 19 virus strains*

Strain	pH-threshold	AMT-susceptibility no*	Δt**	95% CI	susceptible***
A/WS/33	5.7	3	1.6	1.1–2.1	+
A/PR/8	5.3	4	1.6	1.2–2.0	+
A/Bra/78	5.2	3	1.7	1.1–2.3	+
A/Jap/57	5.7	3	2.8	2.1–3.5	+
A/eq/63	5.3	3	0.7	0.5–0.9	+
A/X 31	5.4	3	1.6	1.0–2.3	+
A/X 31-1 a	5.6	3	0.2	0.1–0.4	−
A/X 31-ab 4	5.9	3	−0.1	−0.8–0.6	−
A/Vic/75	5.2	4	2.7	1.3–5.3	+
A/Tex/77	5.1	3	2.7	2.0–3.3	+
A/Bk/79	5.3	3	2.7	2.3–3.1	+
A/FPV	6.0	3	−0.2	−0.8–0.4	−
A/FPV-19	6.0	3	0.1	−0.6–0.8	−
A/FPV-263	6.0	3	−0.3	−1.2–0.6	−
A/FPV-11	5.9	3	2.0	1.5–2.5	+
A/FPV-5	5.3	5	1.6	0.6–2.5	+
B/Lee/40	5.5	4	−0.1	−0.6–0.5	−
B/Hk/73	5.8	3	0.1	−0.2–0.4	−
B/Sing/79	5.9	3	−0.1	−0.4–0.2	−

* Number of experiments

** Mean difference between virus titres produced by untreated and AMT-treated LLC-MK 2-cells

*** +, sensitive to AMT (zero not included in 95% CI)
−, resistant to AMT (zero included in 95% CI)

All naturally occurring human influenza A strains, including A/PR/8, were found to be sensitive to AMT, in contrast to the three influenza B strains and the avian strain A/FPV. All strains provided by Dr. Scholtissek (see Table 1, remark **), showed a susceptibility in accordance with the results of his multiple cycle test (14). Those A/FPV-recombinants which had obtained the HA-gene from A/FPV, were also resistant (A/FPV-19 and A/FPV-263), whereas A/FPV-11 and A/FPV-5, recombinants with the HA-gene from an AMT-resistant parent (14), turned out to be sensitive. The AMT-resistance of the two mutants of A/X 31 was confirmed.

It was not possible to perform direct measurements on endo- or lysosomal pH. Moreover, it is not yet clear whether influenza virus uncoating takes place in secondary lysosomes or already in primary endosomes (19, 20); drug-effects may be different for the two organelles (F. R. MAXFIELD, New York, personal communication). Nevertheless, we assumed that the monkey kidney cells used in this study would not show significant differences to those data reported (9).

Our findings were compatible with the working hypothesis saying that influenza strains fusing at pH-values lower than 5.5 are inhibited by AMT, regardless of other possible mechanisms of this drug. Indeed, none of the 19 strains exhibited a combination of low pH-threshold and resistance to AMT, while the other three combinations could be found (Table 3). In particular, three influenza A strains were sensitive to AMT, but possessed a high pH-threshold demonstrating the existence of drug-effects during viral entry and replication other than that on the conformational change of HA. Moreover, while the AMT-sensitive mother strain fused at low pH (5.4), the two mutants A/X 31-ab 4 and A/X 31-1 a which had obviously obtained resistance to AMT only but by a single point mutation on the HA-gene (1), showed a rise in the pH-threshold beyond 5.5, suggesting that they had acquired their resistance by escaping the need for a low pH for membrane fusion.

Table 3. *Influenza A and B strains subdivided according to their AMT-susceptibility and their pH haemolysis threshold*

		pH-haemolysis-threshold	
		pH < 5.5	pH ≧ 5.5
AMT-susceptibility	sensitive	A/PR/8 A/Bra/78 A/eq/63 *A/X 31 A/Vic/75 A/Tex/77 A/Bk/79 A/FPV-5	A/WS/33 A/Jap/57 A/FPV-11
	resistant	none	A/FPV A/FPV-19 A/FPV-263 *A/X 31-1 a *A/X 31-ab 4 B/Lee/40 B/Hk/73 B/Sing/79

* Note position of sensitive mother strain A/X 31 (low pH-threshold) and AMT-resistant mutants A/X 31-1 a and A/X 31-4 ab (high pH-threshold).

It would be interesting to study the AMT-susceptibility of low pH-mutants from mother strains resistant to AMT and with a high pH-threshold which must be mutagenized and selected under low pH conditions (methods described by Kilian *et al.* [6] for Semliki Forest virus). These mutants would be expected to become sensitive to AMT. It could even be attempted to produce AMT-sensitive influenza B strains in this way.

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