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REVIEW ARTICLE

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Emergence of amantadine-resistant influenza A viruses: epidemiological study

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Abstract At present, three licensed antiviral influenza agents are available in Japan: amantadine, zanamivir, and oseltamivir. These antiviral agents can be used for controlling and preventing influenza, but they are not a substitute for vaccination. Amantadine is an antiviral drug with activity against influenza A viruses, but not influenza B viruses. Persons who have influenza A infection and who are treated with amantadine can shed sensitive viruses early in the course of treatment and later shed drug-resistant viruses, especially after 5–7 days of therapy. Such persons can benefit from therapy even when resistant viruses emerge. In screening for amantadine susceptibility, enzyme-linked immunoassays, plaque reduction assays, and $TCID_{50}$/0.2 ml titration are employed. The molecular changes associated with resistance have been identified as single-nucleotide changes, leading to corresponding amino acid substitutions in one of four critical sites, amino acids 26, 27, 30, and 31, in the transmembrane region of the M2 protein. The polymerase chain reaction (PCR)-restriction fragment length polymorphism analysis method is quite useful. Resistant viruses have been circulated in outbreak situations at nursing homes where amantadine was used not only for treating influenza virus infection but also for Parkinson's disease. Measures should be taken to reduce contact, as much as possible, between persons taking and those not taking antiviral drugs for treatment or chemoprophylaxis.

Key words Influenza virus · Amantadine · Resistant virus

Introduction

The antiviral agent amantadine has been shown to be effective for the treatment and prevention of human influenza A infections.[1–3] Amantadine was approved for the prophylaxis of H2N2 (Asian) influenza A infection in the United States in 1966, and for the prophylaxis and treatment of all influenza A infections in 1976. Antiviral drugs for influenza are an adjunct to influenza vaccination for controlling and preventing influenza, but are not a substitute for vaccination.

In Japan, amantadine was approved for treatment for neurological indications, including Parkinson's disease, in 1975, and for influenza A virus infections in November 1998.[4,5] Annual consumption of amantadine increased sharply after its approval for the treatment of influenza A infections in Japan in 1998, and the emergence of amantadine-resistant viruses during clinical use of the agent in infected individuals was a matter of concern. However, after the introduction of neuraminidase inhibitors, the annual consumption of amantadine decreased dramatically. Here, we review amantadine-resistant influenza A viruses on the basis of our studies.

Action of amantadine against influenza A viruses

Normally, acidification of the endosomal environment leads to a flow of protons to the inner part of the virion through ion channels formed by homotetramers of the M2 protein, a transmembrane protein. The decrease of pH causes dissociation of the M protein, the major structural protein of the virus, and the ribonucleoprotein complex is released into the cytoplasm of the infected cell to initiate virus replication. Amantadine blocks the proton flow through the M2 ion channel and thus prevents the release of viral RNA into the cytoplasm of the infected cells.[6–8]

Emergence of amantadine-resistant strains

Viral resistance to amantadine and its analogue rimantadine emerges quickly in vivo and in vitro.[1–5,9–15] Up to approximately one-third of patients may shed resistant viruses when amantadine or rimantadine is used for therapy. In

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vitro, sensitive viruses became resistant after three or five passages in the presence of 2 μg/ml amantadine. Naturally occurring influenza A viruses can be viewed as mixtures of sensitive and resistant strains with a ratio of 10000:1, the latter would be selected within 2–3 days of starting amantadine therapy.[12] The frequency with which resistant viruses are transmitted and their impact on efforts to control influenza are unknown. Persons who have influenza A infection and who are treated with amantadine can shed sensitive viruses early in the course of treatment, and later shed drug-resistant viruses, especially after 5–7 days of therapy. Such persons can benefit from therapy even when resistant viruses emerge. However, amantadine-resistant viruses are not more virulent or transmissible than -sensitive viruses.

Screening of resistant viruses

In screening for amantadine susceptibility, enzyme-linked immunosonbent assays (ELISAs) and plaque reduction assays are employed.[9,11] We used a virus titration method with comparison of 50% tissue culture infectious dose ($TCID_{50}$)/0.2 ml titration in the presence and absence of amantadine for screening, and verified change by partial nucleotide sequence analysis of the M2 gene (Table 1).[4,5] An amantadine susceptibility test was done with two series of 10-fold dilutions of virus from a cytopathic effect (CPE)-positive culture, plated in triplicate in a 96-well microplate, on Madin–Darby canine kidney (MDCK) cells, with one dilution series containing 2.0 μg/ml of amantadine in the medium. Resistant strains were identified when a less than 2.0-fold difference in log $TCID_{50}$/0.2 ml titer was observed with and without the drug 48 h after inoculation.

The molecular changes associated with resistance have been identified as single-nucleotide changes leading to corresponding amino-acid substitutions of one of four critical sites, amino acids 26, 27, 30, and 31, in the transmembrane region of the M2 protein.[15,16] With low virus isolation rates, the development of a sensitive and rapid laboratory method is required. The PCR-restriction fragment length polymorphism (PCR-RFLP) analysis method reported by Klimov et al.[17] is quite useful, but needs virus isolation and costly endonucleases. For PCR-RFLP analysis, we performed nested PCR, with three pairs of primers, corresponding to amino-acid substitutions at positions 27, 30, and 31, directly from nasopharyngeal swabs (Fig. 1).[5,18] For quick surveys, it might be of advantage to select specific primers for substitution at position 27 for H1N1 strains and at position 31 for H3N2 strains.

Amino acid substitutions in the 27 amino acids spanning the transmembrane domain in the M2 protein of resistant stains

All resistant type A influenza viruses were verified by partial nucleotide sequence analysis of the M2 gene, and it was shown that single-nucleotide changes leading to corresponding amino acid substitutions of one of four critical sites in the transmembrane region of the M2 protein confer resistance.[4,5,16,17,19] A thorough literature search and our previous reports indicated that 70% to 80% of substitutions in resistant viruses occur at position 31, around 10% each at positions 27 and 30, and 1% to 2% at position 26, in vitro and in clinical samples.[4,5,11,12,17–22] (Table 2).

We found that dominant amino acid substitution residues differed significantly with the subtype.[4,6] In two influenza seasons during which H1N1 and H3N2 co-circulated, resistance was more frequent in H3N2 strains than in H1N1 strains after amantadine treatment.[18] Predominant amino acid substitutions in M2 protein occur at position 31 (serine to asparagine) in H3N2 strains and at position 27 (valine to alanine) in H1N1 strains (Table 3). To date, no mechanism for the low level of emergence of amantadine-resistant H1N1 strains during co-circulation with the H3N2 strains has been suggested.

Table 1. Amantadine (Am) sensitivity and mutation of M2 domain after passages in the presence of Am (three to five passages under a 2 μg/ml concentration of Am)

Case (subtype)	Before			After			Amino acid substitution in M2
	$TCID_{50}$/0.2 ml		Phenotype	$TCID_{50}$/0.2 ml		Phenotype	
	Am(−)	Am(+)		Am(−)	Am(+)		
11/93 (H3N2)	5.3	0.5	S	5.3	5.3	R	Ser-31-Asn
105/94 (H3N2)	3.5	0.8	S	3.5	3.5	R	Ala-30-Val
76/95 (H3N2)	4.5	0.5	S	4.5	4.3	R	Ser-31-Asn
77/95 (H3N2)	4.5	0.5	S	4.5	4.5	R	Ser-31-Asn
32/96 (H1N1)	3.5	0.5	S	3.5	3.3	R	Ser-31-Asn
5/97 (H3N2)	5.3	0.5	S	5.5	5.5	R	Ser-31-Asn
18/98 (H3N2)	3.5	0.5	S	3.5	3.5	R	Val-27-Ala
23/98 (H3N2)	4.3	1.3	S	4.3	4.5	R	Ser-31-Asn
25/98 (H3N2)	3.8	0.5	S	3.3	2.8	R	Ser-31-Asn
78/98 (H3N2)	4.8	0.5	S	4.8	4.5	R	Ser-31-Arg
94/98 (H3N2)	3.3	0.5	S	3.8	4.3	R	Ser-31-Asn

Table from reference 4, with permission
S, Am-sensitive virus; R, Am-resistant virus; Ala, alanine; Arg, arginine; Asn, asparagine; Ser, serine; Val, valine; $TCID_{50}$, 50% tissue culture infectious dose

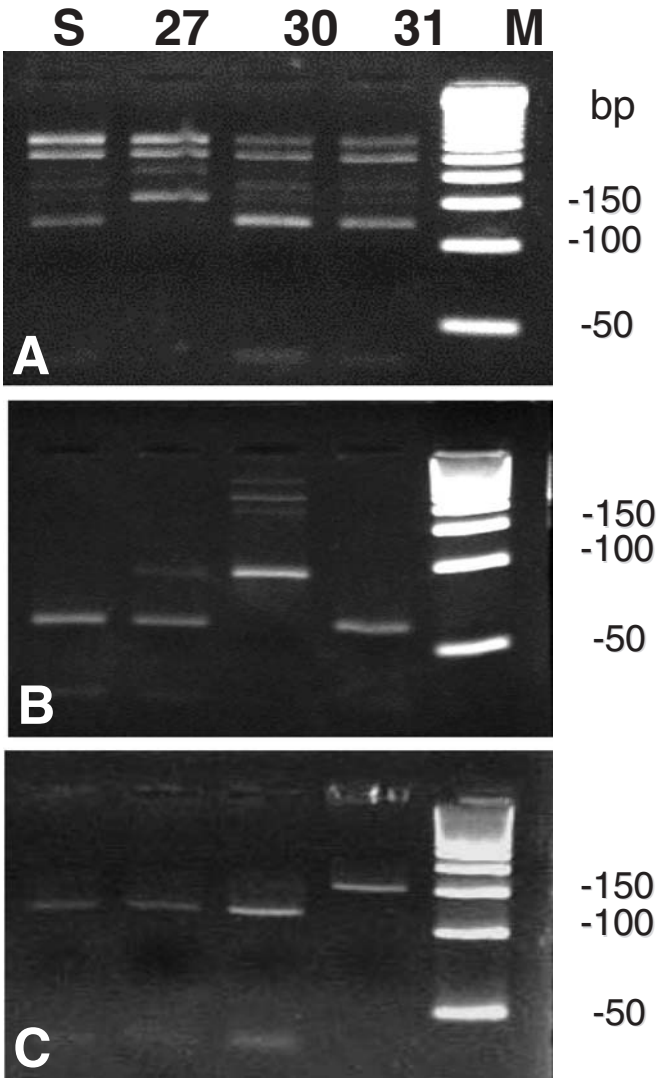

Fig. 1A–C. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of amantadine-resistant reference viruses. Each aliquot of 5 μl of reverse transcription-PCR product, amplified by specific nested primer sets, was treated with 5 U of *Bsp*Lu11I (**A**) at 48°C for 2 h and *Hha*I (**B**) or *Sca*I (**C**) at 37°C for 2 h, respectively, and then electrophoresed in 4% agarose X gels. *Lanes*, S, amantadine-sensitive virus without substitution; *27*, *30*, and *31*, strains having amantadine resistance substitution at amino acids 27, 30, and 31 of the M2 protein, respectively; *M*, 50-bp molecular size marker. From reference 18, with permission

Table 2. Frequencies of amino acid substitutions in M2 protein in amantadine-resistant strains

Position		Data from the literature (1989–1999)[17–22]	Our results (1997–2001)
26	Leu → Phe	1 (2.3%)	1 (1.4%)
27	Val → Ala	4 (9.5%)	8 (10.8%)
	Val → Thr	1 (2.3%)	0 (0.0%)
30	Ala → Thr	4 (9.5%)	3 (4.0%)
	Ala → Val	1 (2.3%)	4 (5.4%)
31	Ser → Asn	31 (73.8%)	57 (77.0%)
	Ser → Arg	0 (0.0%)	1 (1.4%)
Total		42 (100%)	74 (100%)

Ala, alanine; Arg, arginine; Asn, asparagine; Leu, leucine; Phe, phenylalanine; Thr, threonine; Ser, serine; Val, valine

In vitro, sensitive viruses became resistant after three or five passages in the presence of 2 μg/ml amantadine, and they showed an amino acid change at residue 26, 27, 30, or 31. At each passage process, we sequenced nucleic acid changes in the M2 protein for confirmation.[18] Resistant strains did not change, and the site of the M2 protein alteration in each resistant strain was also fixed.

Epidemiological data of resistant viruses

General population

About 1.54% of 1813 isolates in the United Kingdom,[23] and 0.8% of 2017 viruses collected in 43 countries and territories were found to be drug-resistant,[24] indicating that the circulation of drug-resistant viruses is actually quite rare.

Before the approval of amantadine for the treatment of influenza virus infections, we could not find resistant viruses in Niigata, Japan.[4] However, 2.2 million courses of treatment (100 mg/day for 5 days) were used for the 1999/2000 season. About 1.6% of the total Japanese population (around 126 million) was estimated to undergo amantadine treatment; when 5% of the total population of Japan is infected with influenza virus, about 33% of patients undergo amantadine treatment.[25] We detected only 3.4% of resistant viruses from children who were symptomatic and without treatment. Therefore, the circulation of drug-resistant viruses was not so high in the community, even with the excess use of amantadine.

Nursing homes

Resistant viruses can emerge quickly and be transmitted when amantadine is used in an outbreak situation at nursing homes.[26–28] However, resistant viruses were also circulated in nursing homes where amantadine was used not only for treating influenza virus infection but also for treating Parkinson's disease (Table 4). We demonstrated that 80% to 90% of elderly patients who shed resistant strains had no known prophylactic or therapeutic amantadine treatment during the study periods. Thus, the resistant strains appeared to be virulent, genetically stable, and capable of competing with wild-type, drug-sensitive strains of virus causing infection in humans. These findings also suggest the frequent transmission of resistant viruses among nursing home residents, as they stay in closed communal settings.[26–29] Measures should be taken to reduce contact as much as possible between persons taking and not taking antiviral drugs for treatment or for chemoprophylaxis. Furthermore, we also suggest that persons taking amantadine for neurological indications should be included in the reduced-contact measures when such measures are taken.

Table 3. Subtype-specific frequency of amantadine-resistant H1N1 and H3N2 strains from posttreatment samples during the 1999–2000 and 2000–2001 influenza seasons in Niigata City, Japan

Season	H1N1 strains					H3N2 strains					Total by season (%)
	No. of strains with amino acid substitution at:[a]				Total no. of resistant strains/ isolates (%)	No. of strains with amino acid substitution at:[a]				Total no. of resistant strains/	isolates (%)
	26	27	30	31		26	27	30	31		
1999–2000	0	4	0	1	5/22 (22.7)	0	1	3	15	19/59 (32.2)	24/81 (29.6)
2000–2001	1	2	0	1	4/23 (17.4)	0	0	1	2	3/7 (42.9)	7/30 (23.3)
Total by subtype (%)	1	6[b]	0	2[c]	9/45 (20.0)	0	1[b,d]	4[d]	17[c,d]	22/66 (33.3)	31/111 (27.9)

Table from reference 18, with permission

[a] Each number indicates the number of cases of amantadine-resistant influenza virus A strains with the respective amino acid substitutions in the transmembrane domain of the M2 protein

[b] The proportion of amino acid substitutions at position 27 was significantly higher in H1N1 strains than in H3N2 strains (Yates corrected χ^2 test; $P < 0.01$)

[c] The proportion of amino acid substitutions at position 31 was significantly higher in H3N3 strains than in H1N1 strains (Yates corrected χ^2 test; $P < 0.05$)

[d] The proportion of amino acid substitutions at position 31 was significantly higher than that at position 27 and 30 within H3N2 strains (Yates corrected χ^2 test; $P < 0.001$)

Table 4. Frequency of resistant strains among residents in eight nursing homes in the 1998–1999 season, Niigata Prefecture, Japan

Facility (no. of residents)	Outbreak	No. of patients receiving amantadine for:		No. of resistant strains[a]/ no. of PCR-positive strains (%)	No. of strains with substitution in M2[b] at position:		
		Flu	Parkinson's disease		27	30	31
A (95)		5	1	3/15 (20.0)	0	1	2
B (93)	+	3	1	2/11 (18.2)	0	0	2
C (94)	+	62	0	4/18 (22.2)	0	0	3
D (160)	+	34	0	18/54 (33.3)	0	1	18
Subtotal		104	2	27/98 (27.6)[c]	0	2	25
E (88)	+	0	1	3/26 (11.5)	0	1	2
F (112)		0	5	3/9 (33.3)	0	0	3
G (68)		0	3	1/4 (25.0)	0	0	1
H (50)		0	1	0/4 (0.0)	0	0	0
Subtotal		0	10	7/43 (16.3)[c]	0	1	6

Table from reference 5, with permission

[a] A sample was considered to be resistant if it showed a resistant pattern in one of three single amino acids in M2 by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis

[b] Substitution position of the amino acid in the M2 protein verified by RFLP analysis and sequencing

[c] There was no significant difference, with regard to frequency subtotals, between facilities where amantadine was used mainly for flu and facilities where amantadine was used only for Parkinson's disease

Clinical picture of resistant strains

The appropriate treatment of patients with respiratory illness depends on accurate and timely diagnosis, within 48 h of onset.[2,3] The early diagnosis of influenza can reduce the inappropriate use of antibiotics and provide the option of using antiviral therapy. After the introduction of rapid antigen testing, the use of antiviral agents increased dramatically, and appropriate treatment of patients with respiratory illness was given in many clinics in Japan. Resistance has usually been recognized late in the course of therapy, which typically is still successful in shortening the duration and severity of clinical illness. When used for prophylaxis, an antiviral agent can prevent illness while permitting subclinical infection and the development of protective antibodies against circulating influenza viruses. Therefore, amantadine does not interfere with the antibody response to a vaccine.

Limited data are available to determine the frequency and clinical significance of the recovery of drug-resistant virus from treated children.[30] In an interesting observation, we found that one-third of treated children excreted resistant viruses, and they showed reduction in fever on day 3, but a recrudescence of fever on day 5 (Fig. 2).[31] In children treated with rimantadine, however, a nonsignificant recrudescence of fever was shown on days 4 and 5.[30]

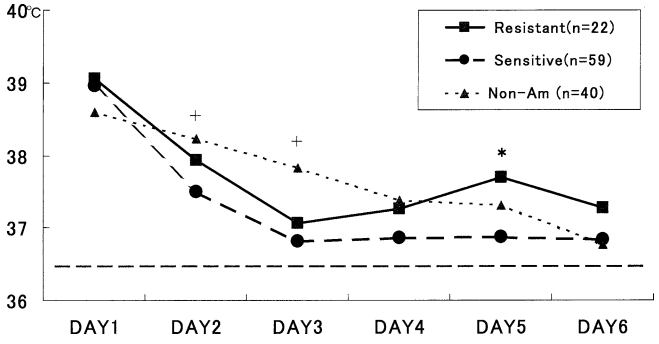

Fig. 2. Mean daily body temperatures for 22 children who shed amantadine-resistant strains on their second or third visits to clinics (*resistant*), and 59 children who remained sensitive after treatment (*sensitive*) compared with 40 controls. (*non-Am*); Statistical analysis was performed with Schefffe's test. $^{+}P < 0.01$, Between sensitive and control on day 2, and between resistant and control, and sensitive and control groups, on day 3; $*P < 0.05$, between sensitive and control groups. There were no significant differences among the three groups on days 1, 4, and 6. From reference 31, with permission

Pandemic planning and emergence of resistant strains

With regard to pandemic planning for new virulent influenza strains, a novel class of antiviral agent, neuraminidase inhibitors, are promising candidates for treatment and prophylaxis, but amantadine is still an important option in terms of cost and chemical stability.[3,32,33] When an influenza emergency arises, the potential for the rapid emergence and dissemination of resistant viruses needs to be studied, especially in relation to strategies for antiviral use and spread to unprimed populations. The risk of adverse drug effects is an additional concern. As described above, the circulation of drug-resistant viruses was not so high in the community, even with excess use of amantadine.[25] However, we need a monitoring system to survey resistant viruses, and also to investigate their frequency, clinical significance, and likelihood of transmission.

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