ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 1993, p. 2239–2240
0066-4804/93/102239-02$02.00/0

Vol. 37, No. 10

Susceptibilities to Rimantadine of Influenza A/H1N1 and A/H3N2 Viruses Isolated during the Epidemics of 1988 to 1989 and 1989 to 1990

M. VALETTE,[1]* J. P. ALLARD,[1] M. AYMARD,[1] AND V. MILLET[2]

Laboratoire de Virologie, Universite Claude Bernard-Lyon 1, Unite de Formation et de Recherche Faculte de Medecine Grange-Blanche 8, Avenue Rockefeller, 69373 Lyon Cedex 08,[1] and Produits Roche Division Pharmaceutique, 52, Neuilly-sur-Seine Cedex,[2] France

Received 6 May 1993/Returned for modification 25 May 1993/Accepted 9 July 1993

Clinical isolates of influenza A viruses identified during outbreaks in two winters were tested for their rimantadine susceptibilities by an enzyme-linked immunosorbent assay modified from that described previously by Belshe et al. (R. B. Belshe, B. Burk, F. Newman, R. L. Cerruti, and I. S. Sim, J. Virol. 62:1508–1512, 1988). The infectivity titer and the 50% inhibitory concentration of rimantadine were calculated for each virus. Of 105 influenza virus A isolates tested, 28 influenza A/H1N1 isolates from the 1988 and 1989 outbreak and 77 influenza A/H3N2 isolates from the outbreak in following year, were susceptible to the antiviral action of rimantadine.

Since its commercialization in France in 1987, rimantadine has been used to prevent influenza when administered to people (health professionals or family members) in contact with ill individuals during influenza A virus epidemics (1). The emergence and possible transmission of rimantadine-resistant strains during combined therapeutic and prophylactic use of the drug in the United States during the 1987 and 1988 outbreak (2) or the 1988 and 1989 outbreak (10) led us to develop a test to evaluate the rimantadine susceptibilities of influenza A isolates. The present study focused on the strains isolated during the epidemic in the winter of 1988 (A/H1N1 subtype) and the epidemic in the winter of 1989 (A/H3N2 subtype). Rimantadine-resistant strains were obtained from World Health Organization's Influenza Center, National Institute for Medical Research, London, United Kingdom (3, 10).

An overnight enzyme-linked immunosorbent assay (ELISA) for detection of viral antigens was used, with modifications (2, 11). Vero or MDCK cells were grown on microtiter plates. Each virus stock was tested in serial dilutions ranging from 10^{-1} to 10^{-5} against six rimantadine concentrations (rimantadine was kindly provided by Roche Laboratories) ranging from 40 to 0.0026 μg/ml in fivefold dilutions (7–9). We also screened the A/H1N1 isolates at concentrations ranging from 5 to 0.002 μg/ml in fourfold dilutions. The plates were centrifuged at 225 × *g*. Each test was performed in duplicate wells, and cell controls were included on each plate to evaluate the cellular toxicity of the antiviral agent (6).

Cells were fixed with 0.1% glutaraldehyde and were then incubated with a rabbit antiserum to either A/Guizhou/54/89-like (A/H3N2) or A/Singapore/6/86-like (A/H1N1) viruses. We used a protein A-horseradish peroxidase conjugate (Bio-Rad Laboratories, Richmond, Calif.), and the substrate was a 2,2-azino-di(3-ethylbenzthiazoline) sulfonic acid (ABTS; Zymed Laboratories, San Francisco, Calif.) in ABTS buffer (Boehringer, Mannheim, Germany). After agitation for a short period of time, the optical densities at 405 nm were read by using a multichannel spectrophotometer (Titertek-Multiskan), and all the data were analyzed in a microcomputer.

In our assay, we used a chessboard titration technique that allowed simultaneous titration of the virus both in the absence and in the presence of increasing rimantadine doses. The ELISA was performed after 20 h of virus multiplication on MDCK cells and 44 h of virus multiplication on Vero cells to allow for sufficient virus multiplication.

The virus titer was the inverse value of the dilution producing 50% antigenic material. This titer was calculated by the geometrical method from the two points nearest the 50% value. The rimantadine concentration giving a 50% reduction in the production of antigenic material (EC_{50}) was evaluated at the optimal viral dilution.

In vitro testing of A/H3N2 strains. The vaccine prototype strains were tested on the two continuous cell lines. With Vero cells, the infectious titer of A/Shanghai/16/89 was $10^{4.3}$, which was 1 dilution higher than the titer observed with MDCK cells, but the susceptibility to rimantadine was comparable in MDCK cells (EC_{50}, 0.018 μg/ml) and Vero cells (EC_{50}, 0.03 μg/ml) at the optimal virus dilution. The strain A/Guizhou/54/89 reached a similar infectious titer (10^4) and also showed the same susceptibility (EC_{50}, 0.01 μg/ml) for both cell lines. For the A/Lyon/5389/88 strain, the rimantadine EC_{50} was 0.03 μg/ml in MDCK cells. For the resistant control strain A/New York/83/R6, EC_{50}s were 21 μg/ml in Vero cells and 27 μg/ml in MDCK cells. For other isolates from the two studies (3, 10) tested in MDCK cells, EC_{50}s ranged from 4 to 14 μg/ml (*P* was not significant) in the first group and from 9 to 14 μg/ml (*P* was not significant) in the second group (Table 1).

The relationship between the growth ability of influenza viruses on two continuous cell lines and the rimantadine EC_{50} was tested on 10 clinical isolates. In Vero cells the titer was an average of $10^{2.9}$, but the same isolates grew to nearly 100-fold higher titers in MDCK cells. The susceptibility to rimantadine was also very different by cell line. In Vero cells at 44 h, the mean EC_{50} for the 10 isolates was 9 μg/ml, which is typical of resistant viruses. With MDCK cells at 20 h, all isolates were susceptible; the mean EC_{50} was 0.07 μg/ml. It

* Corresponding author.

TABLE 1. Susceptibilities of influenza A viruses to rimantadine

Strain	Subtype	Cell	No. of isolates assayed	EC_{50} (μg/ml)[a]
Reference strains				
A/SHANGHAI/16/89	H3N2	Vero	1	0.03
		MDCK	8	0.018 ± 0.009
A/GUIZHOU/54/89	H3N2	Vero	1	0.012
		MDCK	3	0.018 ± 0.008
A/LYON/5389/88	H3N2	MDCK	2	0.039 ± 0.024
A/SINGAPORE/6/86	H1N1	MDCK	1	0.03
A/VICTORIA/36/88	H1N1	MDCK	1	0.02
A/NY/83/R6	H3N2	Vero	5	21.5 ± 12.7
		MDCK	7	27.5 ± 16.6
Strains from[b]				
Patient 7	H3N2	MDCK	2	7 ± 3.7
Patient 86	H3N2	MDCK	2	13.2 ± 1.4
Patient 88	H3N2	MDCK	2	4.15 ± 0.55
Patient 53	H3N2	MDCK	2	13.3 ± 1.8
Patient 90	H3N2	MDCK	2	14.5 ± 0.2
Family 296	H3N2	MDCK	2	9 ± 1.9
Family 152	H3N2	MDCK	2	14.3 ± 1
Family 241	H3N2	MDCK	2	14 ± 0.6
Family 247 index	H3N2	MDCK	2	14.8 ± 1
Family 247 contact	H3N2	MDCK	2	9.8 ± 4.5
French isolates tested				
77 isolates	H3N2	MDCK	1	0.033 ± 0.05
10 isolates[c]	H3N2	Vero	1	9.3 ± 9
		MDCK	1	0.07 ± 0.1
10 isolates	H1N1	Vero	1	0.051 ± 0.04

[a] Values are means ± standard deviations.
[b] Rimantadine-resistant strains (3, 10).
[c] Of 77 isolates, 10 A/H3N2 isolates were tested in two cell lines.

appeared that a low level of viral replication resulted in apparently resistant virus.

In MDCK cells, all of the French clinical isolates were susceptible to rimantadine; the mean EC_{50} was 0.03 μg/ml, but susceptibility varied. For one strain, the EC_{50} was 0.5 μg/ml. Among the others, 51 isolates presented the same range of susceptibilities as the reference strains; EC_{50}s were between 0.1 and 0.01 μg/ml and 25 isolates were highly susceptible; the 50% inhibitory concentration for these isolates was less than 0.01 μg/ml.

In vitro testing of A/H1N1 strains. The A/H1N1 strains were tested for rimantadine susceptibility in Vero cells, and the ELISA was performed 44 h after inoculation. Both reference strains A/Singapore/6/86 and A/Victoria/36/88 were susceptible to the antiviral action of rimantadine; the EC_{50}s were 0.03 and 0.02 μg/ml, respectively. The infectious titer calculated for the 10 A/H1N1 isolates were close to those calculated for the reference strains, varying from $10^{2.3}$ to $10^{3.2}$, and the rimantadine EC_{50} ranged from 0.1 to 0.02 μg/ml (mean EC_{50}, 0.05 μg/ml) (Table 1).

Our study of viruses isolated from two outbreaks of influenza virus A (H3N2 and H1N1) indicated that inhibitory concentrations varied widely but did not exceed 0.5 μg/ml. This is in agreement with the threshold EC_{50} of 1 μg/ml for screening susceptible from resistant strain. Genetic studies of the resistant strains showed that the loss of susceptibility to rimantadine is determined by single amino acid changes in the M2 membrane protein (residue 26, 27, 30, 31, or 34) (2, 3, 4, 5, 10). The strain A/New/York/83/R6 and the strain from patient 88 presented the same substitution of serine-31 for asparagine in M2, and EC_{50}s for the two strains were the most disparate: 27 and 4 μg/ml, respectively. For resistant isolates, there was no obvious correlation between the EC_{50} by our assay and the amino acid changes in the M2 protein.

All the influenza virus A isolates tested in the present study were susceptible to the antiviral action of rimantadine, but none of the patients was in contact or undergoing treatment with rimantadine. In France, prescription of rimantadine for the prevention of influenza virus A is restricted to those who are in contact with a patient with influenzalike illness during documented periods of virus activity in the community. Results of the present study confirm the results of previous studies, showing that naturally occurring strains of influenza virus A are uniformly susceptible to rimantadine (2, 3, 5).

REFERENCES

1. **Bektimirov, T. A., R. G. Douglas, R. Dolin, G. J. Galasso, V. F. Krylov, and J. Oxford.** 1985. Current status of amantadine and rimantadine as anti-influenza-A agents. Memorandum from a WHO meeting. Bull. W.H.O. **63**:51–56.
2. **Belshe, R. B., B. Burk, F. Newman, R. L. Cerruti, and I. S. Sim.** 1989. Resistance of influenza A viruses to amantadine and rimantadine: results of one decade of surveillance. J. Infect. Dis. **159**:430–435.
3. **Belshe, R. B., M. Hall Smith, C. B. Hall, R. Betts, and A. J. Hay.** 1988. Genetic basis of resistance to rimantadine emerging during treatment of influenza virus infection. J. Virol. **62**:1508–1512.
4. **Hay, A. J.** 1990. The molecular basis of susceptibility and resistance of influenza A viruses to adamantamines. Abstr. Seminaire Fondamental de Microbiologie Clinique "Virus et Antiviraux." Clermont-Ferrand, France, 13 to 14 December 1990.
5. **Hay, A. J., M. L. Zambon, A. J. Wolstenholme, J. J. Skehel, and M. H. Smith.** 1986. Molecular basis of resistance of influenza A virus to amantadine. J. Antimicrob. Chemother. **18**(Suppl. B): 19–29.
6. **Hayden, F. G., K. M. Cote, and G. J. R. Douglas.** 1980. Plaque inhibition assay for drug susceptibility testing of influenza virus. Antimicrob. Agents Chemother. **17**:865–870.
7. **Hayden, F. G., H. E. Hoffman, and D. A. Spyker.** 1983. Differences in side effects of amantadine hydrochloride and rimantadine hydrochloride relate to differences in pharmacokinetics. Antimicrob. Agents Chemother. **23**:458–464.
8. **Hayden, F. G., A. Minocha, D. A. Spyker, and H. E. Hoffman.** 1985. Comparative single-dose pharmacokinetics of amantadine hydrochloride and rimantadine hydrochloride in young and elderly adults. Antimicrob. Agents Chemother. **28**:216–221.
9. **Hayden, F. G., and A. S. Monto.** 1986. Oral rimantadine hydrochloride therapy of influenza A virus H3N2 subtype infection in adults. Antimicrob. Agents Chemother. **29**:339–343.
10. **Hayden, G., R. B. Belshe, R. D. Clover, A. J. Hay, R. J. Oakes, and W. Soo.** 1989. Emergence and apparent transmission of rimantadine-resistant influenza A virus in families. N. Engl. J. Med. **321**:1696–1702.
11. **Langlois, M., J. P. Allard, F. Nugier, and M. Aymard.** 1986. A rapid and automated colorimetric assay for evaluating the sensitivity of herpes simplex strains to antiviral drugs. J. Biol. Stand. **14**:201–211.