

## Pathogenicity for Humans of Human Rhinovirus Type 2 Mutants Resistant to or Dependent on Chalcone Ro 09-0410

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**Mutants of human rhinovirus type 2 (HRV-2) resistant to and dependent on the antirhinoviral compound chalcone Ro 09-0410 were selected in cell culture under clean laboratory conditions. A total of 42 volunteers were challenged with either the drug-resistant mutant [SR2-410(r)] (15 volunteers), the drug-dependent mutant [SR2-410(d)] (15 volunteers), or a wild-type HRV-2 which had a similar passage level in vitro as the mutants but without the drug (12 volunteers). Of volunteers challenged with the wild-type HRV-2, 33, 67, and 82% developed cold symptoms, shed virus, and showed serological evidence of infection, respectively. In contrast, only 13, 27, and 23% of volunteers challenged with the drug-resistant mutant developed colds, shed virus, and showed serological evidence of infection, respectively. None of the volunteers challenged with the drug-dependent virus became infected or had symptoms of colds. These results demonstrate that a drug-resistant rhinovirus was capable of infecting humans and producing disease, although its infectivity was reduced when compared with that of the wild type. In contrast, a drug-dependent virus had lost its ability to infect humans.**

In recent years, highly selective, potent inhibitors of viruses, a number of which have been shown to be effective when given either prophylactically or therapeutically, have been found. These include acyclovir for herpes simplex virus (15), amantadine and rimantadine for influenza A virus (6), azidothymidine for human immunodeficiency virus (11), and ribavirin for respiratory syncytial virus (12). Furthermore, recently a new synthetic antiviral agent, R61837 or 3-methoxy-6-[4-(3-methylphenyl)-1-piperazinyl]pyridazine, has been shown to suppress colds in volunteers challenged with a rhinovirus (1). However, drug-resistant virus mutants have been isolated following exposure of susceptible virus strains to most of these drugs (8-10, 13). In view of the rapidly increasing clinical use of antiviral agents, it is clearly very important to address the potential problem of drug resistance. Indeed, the emergence of drug-resistant and drug-dependent viruses in the course of antiviral chemotherapy may complicate the management of susceptible patients who may be at particular risk of developing serious complications (9, 10).

This study addressed the question of whether drug-resistant or drug-dependent mutants are clinically significant by challenging volunteers with both rhinoviral drug-resistant and -dependent viruses which were selected in vitro. The outcome was compared with that in similar volunteers challenged with the parental wild-type virus that had undergone the same passages in vitro in the absence of drug.

### MATERIALS AND METHODS

**Virus and cell culture.** Trypsin-dispersed cultures of human diploid MRC-5 cells were cultured at 37°C in Eagle basal medium containing 10% fetal calf serum, 2.0% sodium bicarbonate, 50 µg of gentamicin per ml, 50 IU of penicillin G per ml, and 50 µg of streptomycin per ml and later maintained in Eagle basal medium containing 2.0% fetal calf serum.

Rhinovirus-sensitive Ohio HeLa cells were cultured at 37°C in Eagle basal medium containing 10% newborn calf

serum, 0.088% sodium bicarbonate, and 50 µg of gentamicin per ml. Maintenance medium consisted of Eagle basal medium, 2% fetal calf serum, 50 µg of gentamicin per ml, 5% tryptose phosphate broth, 30 mM magnesium chloride, 2.5 µg of amphotericin B (Fungizone) per ml, 1 mg of neomycin per ml, 50 IU of penicillin G per ml, and 100 µg of streptomycin per ml.

The methods used for growing and determining the infectivity of human rhinovirus type 2 (HRV-2) in tissue culture were described elsewhere (3).

**Drug.** Chalcone Ro 09-0410 (4'-ethoxy-2'-hydroxy-4,6'-dimethoxy-chalcone) is a potent inhibitor of rhinovirus uncoating (14) and was kindly supplied by Ian Lennox-Smith, Roche Research Laboratory, Welwyn Garden City, United Kingdom. It was stored as a stock solution (10 mg/ml) in dimethyl sulfoxide and diluted in maintenance medium shortly before use.

**Cytotoxicity and estimation of MIC.** The cytotoxic concentration and MIC of Ro 09-0410 were estimated as described previously (2).

**Neutralization test.** Neutralization tests were done as described previously (3). Briefly, doubling dilutions of serum were mixed with equal volumes of HRV-2 (100 50% tissue culture infective doses per ml) and incubated at room temperature for 1 h. Cells from freshly dispersed sheets of Ohio HeLa cells ( $3.0 \times 10^5$  ml) were added to the neutralization mixture. A virus control (no serum), serum control (no virus), and cell control (no virus or serum) were included in each test. Plates were incubated at 33°C and read microscopically for cytopathic effect when virus controls showed 100% cytopathic effect.

**Development of HRV-2 mutants.** Human diploid MRC-5 cell monolayers were infected with 0.2 ml of the HRV-2 parental strain ( $10^{4.86}$  50% tissue culture infective doses per ml) under clean laboratory conditions in the absence of chalcone Ro 09-0410 and in the presence of serial doubling dilutions of the drug starting with a concentration of 0.128 µg/ml (Fig. 1). When the cells showed cytopathic effect, the supernatant was collected and 0.2 ml was used to infect new MRC-5 monolayers. Virus from passage VI was purified by

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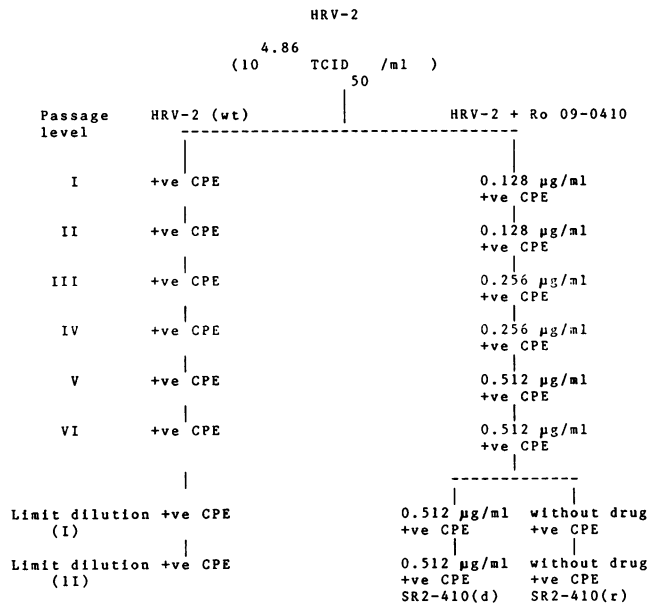


FIG. 1. Selection of HRV-2 mutants in MRC-5 cells. TCID<sub>50</sub>, 50% Tissue culture infective dose; wt, wild type; CPE, cytopathic effect.

two limiting-dilution passages in the presence and absence of 0.512 µg of Ro 09-0410 per ml and stored. Viruses were defined as drug resistant if they grew equally well in the presence and absence of 0.512 µg of Ro 09-0410 per ml and drug dependent if they grew significantly better in the presence of the drug. All virus pools were shown to be free of other pathogens by a battery of tests that included checking for growth in nutrient broth, thioglycolate broth, Sabouraud liquid medium, and blood agar. Virus was also inoculated into MDCK and C16 (clones of MRC-5 sensitive to coronavirus 229E replication) cultures (16) to detect influenza viruses and coronaviruses, respectively. Pools were also shown to be free of mycoplasmas.

**Stability of drug-resistant clinical isolates.** Nasal wash samples obtained from volunteers challenged with either the drug-resistant mutant [SR2-410(r)] or the wild-type HRV-2 and who showed evidence of infection or developed colds were passaged twice in Ohio HeLa cells in the absence of Chalcone Ro 09-0410. Virus titers were determined as previously described (3). The minimal concentration of chalcone Ro 09-0410 required to inhibit the cytopathic effect exhibited by these clinical isolates was determined as described earlier (2).

**Volunteer study.** These trials were approved by the Harrow District Ethical Committee. Healthy volunteers of either sex and aged between 18 and 50 years were recruited and housed in isolation in groups of two or three at the Medical Research Council Common Cold Unit, Salisbury, United Kingdom, according to our normal practice (5). All volunteers completed questionnaires to assess their introversion and extroversion and certain obsessional factors, since these have been shown to influence the outcome of virus challenge (7, 17). Volunteers were randomly allocated into three groups, A, B, and C, according to age, sex, psychological scores, and titer of serum antibody to HRV-2. After an observation period of 48 h, group A volunteers ( $n = 12$ ) were given the tissue culture-passaged wild-type HRV-2 strain, while group B volunteers ( $n = 15$ ) were given the drug-resistant [SR2-410(r)] mutant virus and group C volunteers ( $n = 15$ ) were given the drug-dependent [SR2-410(d)] mu-

TABLE 1. Distribution of volunteers in the three groups according to their prechallenge antibody levels

| Group               | Prechallenge antibody titer | No. of volunteers |
|---------------------|-----------------------------|-------------------|
| A (wild-type HRV-2) | <2                          | 5                 |
|                     | 2-8                         | 6                 |
|                     | >8                          | 1                 |
| B [SR2-410(r)]      | <2                          | 6                 |
|                     | 2-8                         | 7                 |
|                     | >8                          | 2                 |
| C [SR2-410(d)]      | <2                          | 8                 |
|                     | 2-8                         | 5                 |
|                     | >8                          | 2                 |

tant virus. Table 1 shows the distribution of volunteers in the three groups according to their prechallenge antibody titers. Three volunteers with high titers of antibody to HRV-2 (one had an antibody titer of 1:48 and the other two had titers of  $\geq 1:256$ ) were challenged with saline. The challenge virus consisted of 1,000 50% tissue culture infective doses per ml in Hanks balanced salt solution with 0.25% human serum albumin. Volunteers were challenged with virus on two occasions (0.5 ml per nostril) 1 h apart.

Symptoms were assessed and scored daily as described elsewhere (5). Briefly, clinical symptoms were recorded by an observer unaware of the allocation of volunteers to the challenge virus or saline. Each volunteer was assessed daily and assigned a score on the basis of the occurrence and severity of clinical signs and symptoms and nasal secretion weight. Colds were graded clinically as nil, doubtful, very mild, mild, moderate, or severe. Only mild, moderate, or severe colds were considered significant. Volunteers were excluded from the study if they developed signs of cold before virus challenge or if a rhinovirus was isolated from their prechallenge nasal wash sample.

Nasal washings for virus isolation were collected on the day before and on each day after virus challenge for 5 days and inoculated into Ohio HeLa cells. A blood sample was collected before virus challenge, and a further sample was requested from each volunteer 2 weeks after leaving the unit. The paired serum samples were tested in parallel for titers of neutralizing antibody to HRV-2. A fourfold-or-greater rise in antibody titer was considered significant as evidence of infection.

**Statistical analysis.** Differences in the frequency of colds and antibody increases between the group of volunteers challenged with the drug-resistant virus and those challenged with the wild-type parental virus were tested for significance by using the chi-square test with the correction of Yates. Clinical scores and nasal secretion weight data were tested by rank analysis of variance in which the data were blocked into three strata according to the prechallenge neutralizing titer of each volunteer (<2, 2 to 8, and >8).

## RESULTS

**Selection of SR2-410(r) and SR2-410(d) mutants.** Table 2 shows the final titers of the parental wild-type HRV-2 and the drug-resistant and drug-dependent mutants after passage and selection in MRC-5 cells. The drug-resistant mutant SR2-410(r) clearly grew equally well in the presence and absence of 0.512 µg of Ro 09-0410 per ml, whereas the drug-dependent SR2-410(d) virus grew significantly better in

TABLE 2. Selection of rhinoviral drug-resistant [SR2-401(r)] and drug-dependent [SR2-410(d)] viruses by limiting dilution<sup>a</sup>

| Ro 09-0410       | Titer (TCID <sub>50</sub> /ml <sup>b</sup> ) after limiting dilution 2 |                    |                   |
|------------------|--|--------------------|-------------------|
|                  | Wild-type HRV-2  | SR2-410(r)         | SR2-410(d)        |
| Yes <sup>c</sup> | 10 <sup>1.7</sup>  | 10 <sup>5.36</sup> | 10 <sup>4.9</sup> |
| No               | 10 <sup>5.0</sup>  | 10 <sup>5.0</sup>  | 10 <sup>2.2</sup> |

<sup>a</sup> This table describes an endpoint experiment.

<sup>b</sup> TCID<sub>50</sub>, 50% Tissue culture infective dose.

<sup>c</sup> In the presence of 0.512 µg of drug per ml.

the presence of 0.512 µg of Ro 09-0410 per ml (at least a 2-log<sub>10</sub>-higher titer in the presence of this concentration of drug than without drug).

**Pathogenicity of SR2-410(r) and SR2-410(d).** Table 3 shows the percentages of each group of volunteers who developed signs of colds, shed virus, and had serological evidence of infection. None of the volunteers given saline showed any evidence of illness or infection.

There were no significant differences in the frequency of colds between those challenged with the wild-type virus and those challenged with the drug-resistant virus (*P* = 0.437). However, the frequency of serological evidence of infection (seroconversion or significant antibody rises) was significantly higher among those challenged with the wild-type virus than among those challenged with the drug-resistant virus (*P* = 0.014). In addition, volunteers given the wild-type virus shed significantly more virus on days 4 and 7 after virus challenge than those given the resistant virus (*P* = 0.048 and *P* = 0.049, respectively).

Generally, there were no significant differences in the mean total clinical scores (*P* = 0.770) or the mean total nasal secretion weights (*P* = 0.735) between those challenged with the wild-virus and those challenged with the drug-resistant mutants (6.2 and 7.9 versus 5.4 and 6.3, respectively).

**Stability of drug-resistant clinical isolates.** Viruses cultured from volunteers challenged with the wild-type or drug-resistant virus were tested for their resistance to Ro 09-0410. Isolates obtained from the nasal washings of volunteers challenged with the SR2-410(r) mutant and who showed evidence of infection were approximately seven times less susceptible to the agent than those obtained from volunteers challenged with the wild-type HRV-2. These results suggest that SR2-410(r) isolates were relatively genetically stable in the respiratory tract and were responsible for initiating the infection in volunteers (Table 4).

**DISCUSSION**

We have shown that mutants which were resistant to or dependent on the chalcone Ro 09-0410 exhibited a reduced

TABLE 3. Pathogenicity of wild-type HRV-2, SR2-410(r), and SR2-410(d) for humans

| Characteristic | No. (%) of volunteers     |                      |                      |
|----------------|---------------------------|----------------------|----------------------|
|                | Group A (HRV-2 wild type) | Group B [SR2-410(r)] | Group C [SR2-410(d)] |
| Total          | 12                        | 15                   | 15                   |
| Colds          | 4 (33)                    | 2 (13)               | 0 (0)                |
| Virus shedding | 8 (67)                    | 4 (27)               | 0 (0)                |
| Seroconversion | 9 (82) <sup>a</sup>       | 3 (23) <sup>b</sup>  | 0 (0)                |
| Infection      | 11 (92)                   | 4 (27)               | 0 (0)                |

<sup>a</sup> Number of volunteers tested was 11.

<sup>b</sup> Number of volunteers tested was 13.

TABLE 4. MICs of Ro 09-0410 for virus isolated in Ohio Hela cells from volunteers challenged with wild-type virus and drug-resistant mutant<sup>a</sup>

| Virus                    | Volunteer         | Day after challenge when virus was isolated <sup>b</sup> | MIC of Ro 09-0410 (µg/ml) |
|--------------------------|-------------------|--|---------------------------|
| SR2-410(r) isolates      | A (no cold)       | 5  | 0.256                     |
|                          | B (mild cold)     | 1  | 0.256                     |
|                          | C (no cold)       | 2  | 0.512                     |
|                          | D (moderate cold) | 2  | 0.256                     |
| HRV-2 wild-type isolates | E (no cold)       | 1  | 0.064                     |
|                          | F (mild cold)     | 1  | 0.032                     |
| HRV-2 wild-type inoculum |                   |  | 0.032                     |
| SR2-410(r) inoculum      |                   |  | 0.512                     |

<sup>a</sup> Virus was isolated in the absence of drug.

<sup>b</sup> This represents the first nasal washing after challenge from the volunteer which yielded a positive virus isolation.

infectivity for volunteers when compared with a similarly passaged wild-type HRV-2. Early experiments in humans suggested that tissue culture-passaged human rhinoviruses are able to infect and produce disease in volunteers but that some of the viruses may become attenuated (18). Our data showed that our parental wild-type HRV-2 strain, which had undergone the same number of serial passages as our drug-resistant virus, was not attenuated for humans and was able to infect and produce disease in a high proportion of volunteers. In contrast, the drug-resistant strain, SR2-410(r), produced colds in half and infection in about one third as many volunteers as the wild-type virus. Although there were reductions in the number of colds and the rate of infection, the severity of colds produced by the drug-resistant mutants was not different from the severity of colds produced following challenge with the parental wild-type virus. Furthermore, the virus isolated from volunteers who were challenged with the drug-resistant mutant and who had colds or infection or both was still approximately sevenfold less susceptible to Ro 09-0410 than the virus isolated from volunteers who were challenged with the parental wild-type HRV-2. This suggests that these mutants were relatively genetically stable and did not revert to drug susceptibility despite replication in humans. Indeed, our *in vitro* studies showed that the drug-resistant mutant grew as efficiently as the wild-type virus in cell culture (unpublished data).

The results of this study indicate that acquiring resistance to the antiviral agent Ro 09-0410 also affects the ability of HRV-2 to infect and produce disease in humans. The mechanism by which this occurs is unknown. However, evidence from another study on rhinovirus mutants resistant to the Sterling-Winthrop compound WIN 51711, which is thought to have a mechanism of action similar to that of Ro 09-0410, suggests that a change in one amino acid in the base of the virus "canyon," where the drug interacts with the viral capsid protein VP1, is sufficient to make the virus resistant to the drug (4). Such a change may have occurred in our drug-resistant mutant and hence affected the ability of our virus to infect the nasal epithelial cells of our volunteers. This may explain the reduced rate of infectivity observed in these volunteers. In the case of the drug-dependent virus, the change may have been so great that the virus was unable to infect the nasal epithelial cells at all. Specific studies are needed to understand the molecular changes in the resistant

and dependent viruses relative to the parental wild-type virus. Indeed, we recently initiated some of these studies.

Recently, rimantadine-resistant strains of influenza A virus were frequently recovered from rimantadine-treated index patients (13). Furthermore, it was demonstrated in the same study that these resistant viruses could apparently be transmitted and cause illness in other members of the family of an individual taking the drug (13). These results showed that, as was the case with the rhinovirus drug-resistant mutants described in this study, rimantadine-resistant viruses are capable of infecting and producing illness in humans. Both the studies of Hayden et al. (13) and those recently reported by Dearden et al. (8) also show that drug-resistant mutants isolated from respiratory tract specimens may show cross-resistance to other antiviral agents that have similar mechanisms of action. Thus, Hayden et al. showed that rimantadine-resistant isolates were also resistant to amantadine (13). Similarly, Dearden et al. showed that R61837-resistant rhinoviruses were also resistant to other antiviral agents, such as the chalcone Ro 09-0410, dichloroflavan, and disoxaril, but were still susceptible to the antirhinovirus compound enviroxime, which has a different mechanism of action (8). These studies, therefore, emphasize the importance of carefully monitoring the emergence of drug-resistant viruses during the course of antiviral chemotherapy and treatment. This could be particularly important should chemoprophylaxis and treatment of viral infections be more widely applied in different clinical settings.

To the best of our knowledge, our experiments are the first to assess the pathogenic potential of drug-resistant and drug-dependent mutant viruses by actually challenging volunteers with such viruses.

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