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In Vitro Selection of Human Rhinovirus Relatively Resistant to Soluble Intercellular Adhesion Molecule-1

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Variants of human rhinovirus serotype 39 (HRV-39) relatively resistant to inhibition by soluble intercellular adhesion molecule-1 (sICAM-1) were selected by serial passages in HeLa or WI-38 cells in the presence of sICAM-1. Moderate resistance (four- to fivefold increases in 50% effective inhibitory concentrations [EC_{50}s]) was observed after the second passage in HeLa cells and remained constant during six further passages in the presence of 10 μg of sICAM-1 per ml. A 7- to 17-fold increase in EC_{50}s was observed in WI-38 cells during passage with 10 μg/ml, and reversion to a nonresistant phenotype was not observed after four passages in the absence of sICAM-1. Resistance of a higher degree was obtained by passaging HRV-39 in the presence of 100 μg of sICAM-1 per ml in HeLa cells (30-fold EC_{50} increase). The sICAM-1-resistant phenotype was estimated to constitute 1 in 10^4 to 1 in 10^5 PFU of a nonexposed HRV-39 population. Low to moderate levels of resistance to sICAM-1 inhibition emerge readily during in vitro passage in the presence of sICAM-1 and appear to be phenotypically stable.

The cell receptor for human rhinoviruses (HRVs) of the major receptor group has been identified as intercellular adhesion molecule-1 (ICAM-1) (8, 18, 19), and recombinant soluble forms of ICAM-1 (sICAM-1) have been shown to inhibit HRV replication in vitro (3, 9, 14). One form of sICAM-1 (14) inhibits the replication of 88 of the 90 numbered HRV serotypes of the major receptor group, with 50% effective inhibitory concentrations (EC_{50}s) ranging from 0.1 to 41.1 μg/ml (5). Because the virus receptor binding site appears to be conserved, it has been postulated that a viral mutation which confers resistance to inhibition by a soluble cell receptor molecule might be lethal, unless attachment by an alternative receptor is possible (14). However, poliovirus mutants resistant to the antiviral effects of the poliovirus soluble cell receptor have been isolated (12). Furthermore, these resistant variants maintained the same receptor specificity as the original poliovirus, as determined by a receptor blockade with an antireceptor monoclonal antibody, and appeared to be as virulent as the wild-type virus following mouse inoculation (12).

This report describes the selection of variants of HRV serotype 39 (HRV-39), a major receptor group HRV readily inhibited by sICAM-1 in cell cultures and human adenoid explants (3), that are moderately resistant to the antiviral action of sICAM-1 in vitro. We have also estimated the frequency with which such resistant variants occur in a native population of HRV-39.

MATERIALS AND METHODS

Virus, cells, and reagents. A stock of HRV-39, initially derived from a clinical isolate in our laboratory, was grown in HeLa cells, harvested when the cells showed 100% cytopathic effects (CPE), frozen and thawed three times, and clarified by centrifugation at 250 × *g*. HeLa (strain R-19) cells were a gift from Richard Colonno (Merck Sharp & Dohme, West Point, Pa.), and human embryonic lung fibroblast (strain WI-38) cells were purchased from Whittaker M. A. Bioproducts (Walkersville, Md.). Growth and maintenance media were the same as those previously described (3). sICAM-1, prepared and purified as previously described (14, 16), was kindly furnished by Boehringer Ingelheim Pharmaceuticals (Ridgefield, Conn.).

Selection of resistance to sICAM-1. Isolation of HRV-39 resistant to sICAM-1 was achieved by end-point titration in the presence of sICAM-1. In brief, serial $\log_{10}$ dilutions of HRV-39, 10^7 50% tissue culture infective doses ($TCID_{50}$) per ml, were passaged in the absence or presence of sICAM-1 at final concentrations ranging from 1 to 100 μg/ml, depending on the experiment. The virus dilutions and the cell monolayers were preincubated separately with sICAM-1 for 1 h at 34°C. Thereafter, the virus–sICAM-1 mixture was allowed to adsorb to the cells for 1 h at 34°C. The monolayers were then washed once with Hanks balanced salt solution (HBSS) and replenished with medium containing the appropriate concentration of sICAM-1. After 5 to 7 days of incubation, the highest $\log_{10}$ dilution of virus causing CPE at each sICAM-1 concentration was harvested, and serial $\log_{10}$ dilutions of this harvest were inoculated onto new monolayers in the presence of the same sICAM-1 concentration. This procedure was repeated initially for a total of eight passages.

Determination of sICAM-1 susceptibility. The sICAM-1 EC_{50}s were determined by a CPE inhibition microtiter assay with WI-38 cells as previously described (2, 3), with minor modifications. The EC_{50} determination for passaged viruses was carried out either directly with harvested supernatants or with a pool prepared by passaging each harvest once in HeLa cells. The concentrations of sICAM-1 ranged in 0.5 $\log_{10}$ dilutions from 0.1 to 100 μg/ml. After 3 or 4 days of incubation, the extents of CPE in the monolayers were read when the virus control showed 80 to 100% CPE and the virus inoculum titration showed that 32 to 256 $TCID_{50}$s had been used per well. The EC_{50}s for stock HRV-39, which was run with each assay as a control, ranged from 0.3 to 1.5 μg/ml in WI-38 cells and from 1.0 to 3.0 μg/ml in HeLa cells (3).

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TABLE 1. Selection of HRV-39 variants with reduced susceptibility to sICAM-1 by sequential passage in HeLa cells in the presence or absence of sICAM-1

Passage	EC_{50} (μg/ml)[a]		Ratio of EC_{50}s (sICAM-1 exposed/control)
	Control	With sICAM-1 at 10 μg/ml	
1	1.0	1.4	1.4
2	0.9	3.8	4.2
3	0.8	2.9	3.6
4	0.8	2.6	3.3
5	0.7	3.1	4.4
6	0.7	3.0	4.3
7	0.5	2.7	5.4
8	0.6	2.7	4.5

[a] Determined in WI-38 cells. Values represent the average results from two assays.

Determination of the frequency of the sICAM-1-resistant phenotype. The frequency of the sICAM-1-resistant phenotype in a pool of HRV-39 was assessed by determining the frequency of survivors that could form plaques after adsorption to HeLa cells in the presence of a range of concentrations of sICAM-1. This determination was done for a wild-type, nonexposed pool of HRV-39 prepared in HeLa cells and for a pool of sICAM-1-resistant HRV-39 selected and plaque purified in HeLa cells in the presence of 100 μg of sICAM-1 per ml. Serial $\log_{10}$ dilutions of concentrated HRV-39 stocks at $\sim 10^7$ PFU/ml were mixed with different concentrations of sICAM-1 (0 to 320 μg/ml), incubated for 1 h at 34°C, and allowed to adsorb to duplicate monolayers of HeLa cells for 1 h at 34°C. After adsorption, the monolayers were washed three times with HBSS and overlaid with 0.6% agar in 1× McCoy's medium containing the appropriate concentration of sICAM-1. After 4 days of incubation at 34°C, a second overlay consisting of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide at 0.5 mg/ml in 1× McCoy's medium was added, the monolayers were incubated for an additional 3 to 4 h, and visible plaques were counted.

RESULTS

Passage in HeLa cells. An initial experiment with HeLa cells found that the EC_{50}s for HRV-39 passaged in sICAM-1 at a concentration of 3.2, 10, or 32 μg/ml increased three- to fivefold by passage 8 in comparison with those for virus comparably passaged in plain medium. The rapidity of the selection of resistant variants was assessed by determining the EC_{50}s for each pair of harvests, passaged in the presence of sICAM-1 at 10 μg/ml or in plain medium, at each passage. As shown in Table 1, the EC_{50} for the sICAM-1-exposed virus increased modestly by the second passage, and the ratio of EC_{50}s (sICAM-1-exposed virus/wild-type virus control) remained stable at three- to fivefold after the second passage. When this series of HeLa cell passages was repeated a second time in the presence of sICAM-1 at 0, 1.0, 10, or 100 μg/ml, similar results were obtained at 10 μg/ml, but an increased degree of resistance was observed at 100 μg/ml (Table 2). The ratios of EC_{50}s after eight passages were increased approximately 4-fold at 10 μg of sICAM-1 per ml and 30-fold at 100 μg of sICAM-1 per ml. No reduction in the EC_{50} was observed after passage of this relatively resistant virus four times in the absence of sICAM-1 (Table 2).

TABLE 2. Concentration-related selection of HRV-39 variants resistant to sICAM-1 in HeLa cells

sICAM-1 (μg/ml)	No. of passages[a]		EC_{50} (μg/ml)[b]	Ratio of EC_{50}s (sICAM-1 exposed/control)[c]
	With sICAM-1	Without sICAM-1		
0	0	0	1.1 ± 0.4	
0	0	9	0.9 ± 0.4	0.8
1	8	1	1.0 ± 0.2	1.1
10	8	1	4.2 ± 1.6	4.6
100	8	1	26.1 ± 11.8	29.0
100	8	4	29.4 ± 10.1	32.6

[a] Viruses were passaged sequentially in the presence of sICAM-1 at the indicated concentrations and passaged further in the absence of sICAM-1.
[b] Values represent the mean ± standard deviation for three to seven independent assays.
[c] The EC_{50} of unpassaged wild-type virus was used as a control for virus passaged in the absence of sICAM-1, and the EC_{50} value of virus passaged in the absence of sICAM-1 was used as a control for virus passaged in different concentrations of sICAM-1.

Passage in WI-38 cells. When similar experiments were performed with WI-38 cells, multiple attempts to passage stock HRV-39 in sICAM-1 at 100 μg/ml were unsuccessful because of inability to recover infectious virus. As shown in Table 3, sICAM-1 at 10 μg/ml selected virus that showed an approximate sevenfold increase in the EC_{50}. Passage in a low concentration (1 μg/ml), comparable to the EC_{50} for wild-type HRV-39, did not affect susceptibility to sICAM-1 in HeLa (Table 2) or WI-38 (Table 3) cells. The rapidity of resistance development was assessed by determining the EC_{50} in harvests from the first three passages in 10 μg of sICAM-1 per ml in WI-38 cells or in 100 μg of sICAM-1 per ml in HeLa cells. In both instances, resistance of a moderate degree was observed as early as the first passage (Table 4). This result confirmed the initial findings with HeLa cells.

To determine whether virus with a higher degree of resistance could be selected from this moderately resistant population, four further passages were carried out with WI-38 cells in the presence of 10 or 32 μg of sICAM-1 per ml. As shown in Table 5, the observed EC_{50}s only increased by approximately twofold for either further-passaged virus compared with virus that had been passaged eight times.

In addition, to determine whether the observed level of resistance was reversible, virus from passage 8 was also passaged four additional times in the absence of sICAM-1. As shown in Table 5, no reduction in the EC_{50} was observed when moderately resistant HRV-39 was passaged in the absence of sICAM-1.

TABLE 3. Selection of HRV-39 variants resistant to sICAM-1 by sequential passage in WI-38 cells

sICAM-1 (μg/ml)[a]	No. of passages		EC_{50} (μg/ml)[b]	Ratio of EC_{50}s (sICAM-1 exposed/control)[c]
	With sICAM-1	Without sICAM-1		
0	0	0	1.1 ± 0.6	
0	0	9	1.1 ± 0.6	1.0
1	8	1	0.7 ± 0.2	0.6
10	8	1	8.4 ± 2.2	7.6

[a] Attempts to passage virus in the presence of sICAM-1 at 100 μg/ml were unsuccessful because of inability to recover infectious virus.
[b] Results are expressed as the mean ± standard deviation for three independent assays.
[c] See Table 2, footnote *c*.

TABLE 4. sICAM-1 susceptibility of harvests of HRV-39 grown in WI-38 or HeLa cells in the presence or absence of sICAM-1

Passage	EC_{50} (μg/ml)[a] for WI-38 passages		Ratio of EC_{50}s (sICAM-1 exposed/control)	EC_{50} (μg/ml)[a] for HeLa passages		Ratio of EC_{50}s (sICAM-1 exposed/control)
	Control	With sICAM-1 at 10 μg/ml		Control	With sICAM-1 at 100 μg/ml	
1	1.1	19.2	17.4	1.0	38.3	38.3
2	1.0	14.9	14.9	1.3	28.0	21.5
3	1.2	16.4	13.7	1.4	37.9	27.0

[a] Determined in WI-38 cells. Values represent the average results from two assays.

Frequency of the resistant phenotype in native and sICAM-1-resistant HRV-39. As shown in Fig. 1, the fraction of wild-type, nonpassaged HRV-39 stock that was able to form plaques after incubation in the presence of sICAM-1 decreased in a concentration-dependent manner. Approximately 1 in 10^4 to 1 in 10^5 (~0.01 to 0.001%) of the input infectious virus formed plaques at 100 μg of sICAM-1 per ml. In contrast, the fraction of plaques formed by a stock of moderately resistant virus remained relatively stable at sICAM-1 concentrations of up to 100 μg/ml. No plaques were visible at 320 μg of sICAM-1 per ml in either pool.

DISCUSSION

We previously found that the HRVs belonging to the major receptor group differ substantially in their susceptibility to inhibition by sICAM-1 in cell cultures and human respiratory epithelial cell explants (3, 5). The current studies showed that an HRV serotype which was readily inhibited in vitro by sICAM-1 initially could be passaged successfully in concentrations well in excess of the EC_{50} and that relatively resistant variants could be selected in early passages with EC_{50}s roughly proportional to the concentration of sICAM-1 in which they had been selected.

Selection of a preexisting resistant subpopulation was suggested indirectly by the rapidity of resistance development, which occurred in early passages in WI-38 and HeLa cells, and directly by the plaquing experiments, in which it was estimated that between 1 in 10^4 and 1 in 10^5 PFU in a native population of HRV-39 was able to form plaques in the presence of high concentrations of sICAM-1. This frequency is in the same range as the previously observed frequencies of single-step escape mutants of HRV-14 obtained with an anti-HRV-14 antibody (17) or the capsid-binding agent WIN 52084 (10) and of poliovirus mutants resistant to neutralization with soluble receptors (12). Once the resistant phenotype was selected, it appeared to be stable, without apparent loss despite four passages in the absence of sICAM-1.

The level of resistance selected at 10 μg of sICAM-1 per ml was modest, only about 4- to 5-fold in HeLa cells and about 7- to 14-fold in WI-38 cells, compared with that of wild-type virus. Consequently, the EC_{50}s for the resistant variants selected at 10 μg/ml fall within the range reported for a number of other major receptor group HRVs, 86% of which are inhibited by concentrations of 10 μg/ml or less (5). However, passage at 100 μg of sICAM-1 per ml in HeLa cells selected for moderate degrees of resistance (approximately a 30-fold increase in EC_{50}s). One study reported the selection of poliovirus mutants resistant to the inhibitory effect of a soluble poliovirus cell receptor (12). In that study, several poliovirus mutants displayed intermediate levels of resistance (2- to 4-fold increases in inhibitory concentrations), while one variant was highly resistant (over a 10-fold increase) to neutralization. The levels of sICAM-1 resistance of HRV-39 variants that we have reported are comparable to the resistance levels previously observed for single-step HRV-14 mutants selected in vitro in the presence of low concentrations of the capsid-binding agent WIN 52084 (four-

TABLE 5. Effect of further passages in WI-38 cells in the presence or absence of sICAM-1 on the susceptibility of HRV-39 relatively resistant to sICAM-1

sICAM-1 (μg/ml)	No. of passages		EC_{50} (μg/ml)[a]	Ratio of EC_{50}s (passaged/control)[b]
	With sICAM-1	Without sICAM-1		
0	0	0	1.1 ± 0.3	
10	8	0	7.6 ± 3.6	6.9
10	12	0	15.5 ± 13.6	14.1
10[c]	8	4	9.4 ± 3.2	8.5
10 + 32[d]	8+4	0	15.5 ± 9.4	14.1

[a] Values are expressed as the mean ± standard deviation for four independent assays.

[b] For comparison, the control value was the EC_{50} of nonpassaged virus.

[c] Virus was passaged eight times in the presence of 10 μg of sICAM-1 per ml and then four times in the absence of sICAM-1.

[d] Virus was passaged eight times in sICAM-1 at 10 μg/ml and then four times in sICAM-1 at 32 μg/ml.

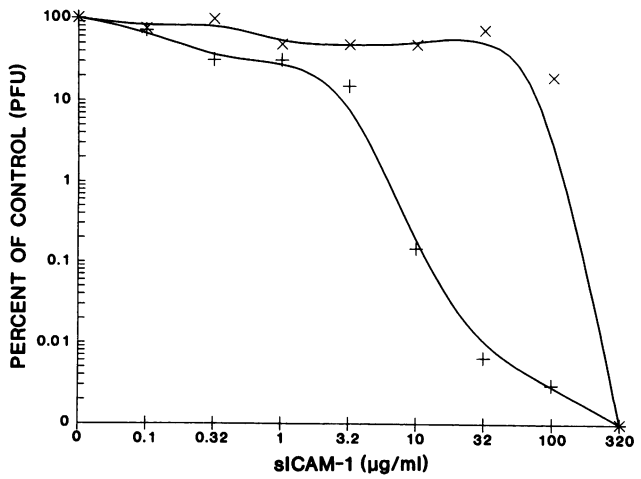

FIG. 1. Frequency of wild-type HRV-39 (+) and sICAM-1-resistant HRV-39 (×) forming plaques in the presence of increasing concentrations of sICAM-1 in HeLa cell monolayers. Plaques were counted after 4 days of incubation at 34°C, and the results are expressed as a percentage of the plaque counts obtained in the absence of sICAM-1. Titers obtained in the absence of sICAM-1 (100%) were 1.7×10^7 PFU/ml for the wild-type stock and 1.0×10^7 PFU/ml for the sICAM-1-resistant stock.

to sevenfold increases in EC_{50}s) (10). However, our observed increases in EC_{50}s are lower than those observed for HRV-14 mutants selected in the presence of high concentrations of WIN 52084 (40- to 100-fold increases in EC_{50}s). Moderate levels of resistance (16-fold increase in the EC_{50}) to a chalcone, Ro 09-0410, also have been obtained for HRV-2, a minor receptor group rhinovirus, after six passages in vitro (20). One study reported the recovery of HRV-9 resistant to a capsid-binding agent, R618367, after a human experimental infection (6), in which 32% of the treated volunteers shed resistant viruses at some point. Most of these viruses had moderate resistance (5- to 15-fold increases in EC_{50}s), but one person shed a highly resistant virus (>100-fold increase in the EC_{50}).

The mechanism of resistance to sICAM-1 is undefined at present. It has been demonstrated that although the HRV binding region comprises the first 88 residues of the N-terminal domain of ICAM-1 (15), a more extensive area comprising the second domain of ICAM-1 may be secondarily involved in virus binding (7). It has also been shown for a limited number of HRV serotypes that there is a serotype-dependent variability in the rate of receptor binding and stability of virus-receptor complexes (11). Furthermore, slight conformational changes induced by site-directed mutagenesis in residues related to receptor binding can alter the binding phenotype (4). In addition, amino acid residues which are located at or near the rim of the canyon and which interact with the second domain of ICAM-1 create an electrostatic potential which can be slightly changed by mutations, therefore creating polar alterations which may interfere to various extents with the fitting of ICAM-1 into the canyon (7, 13). Another alternative to be explored in explaining the basis for sICAM-1 resistance, initially suggested by Kaplan et al. (12) as a possibility to explain poliovirus resistance to a soluble cell receptor, is that the resistant phenotype may carry a functional mutation which influences an essential transitional conformation of the virion during viral entry or uncoating. Therefore, it is possible that complex interactions between residues on the canyon contour of certain HRV serotypes and less distal domains of ICAM-1 define the accessibility of the molecule to the binding region and may explain moderate levels of resistance to sICAM-1 in certain serotypes or in a selected subset of a heterogeneous virus pool within one serotype of major receptor group HRVs.

The significance of these observation will depend in part on the mechanism of resistance and on its possible effects on viral replication and pathogenicity. Of note, poliovirus variants selected for resistance to soluble poliovirus cell receptors, despite their reduced binding to HeLa cells, did not differ significantly from the wild-type virus with regard to the kinetics of viral replication in vitro or neurovirulence for mice (12). Although there are conflicting data on the ability of HRVs selected for resistance to a capsid-binding agent, chalcone Ro 09-0410, to grow in cell cultures (1, 21), one resistant HRV-2 variant was found to have significantly reduced infectivity for humans (20). Further characterization of the sICAM-1-resistant phenotype, including its ability to replicate in human cell lines and respiratory cell epithelium, is warranted.

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