JOURNAL OF VIROLOGY, Mar. 1990, p. 1117–1123
0022-538X/90/031117-07$02.00/0

Vol. 64, No. 3

Two Groups of Rhinoviruses Revealed by a Panel of Antiviral Compounds Present Sequence Divergence and Differential Pathogenicity

KOEN ANDRIES,* BART DEWINDT, JERRY SNOEKS, LUC WOUTERS, HENRI MOEREELS, PAUL J. LEWI, AND PAUL A. J. JANSSEN

Janssen Research Foundation, Turnhoutseweg 30, B-2340 Beerse, Belgium

Received 27 July 1989/Accepted 7 November 1989

A variety of chemically different compounds inhibit the replication of several serotypes of rhinoviruses (common-cold viruses). We noticed that one of these antiviral compounds, WIN 51711, had an antiviral spectrum clearly distinctive from a consensus spectrum or other capsid-binding compounds, although all of them were shown to share the same binding site. A systematic evaluation of all known rhinovirus capsid-binding compounds against all serotyped rhinoviruses was therefore initiated. Multivariate analysis of the results revealed the existence of two groups of rhinoviruses, which we will call antiviral groups A and B. The differential sensitivity of members of these groups to antiviral compounds suggests the existence of a dimorphic binding site. The antiviral groups turned out to be a reflection of a divergence of rhinovirus serotypes on a much broader level. Similarities in antiviral spectra were highly correlated with sequence similarities, not only of amino acids lining the antiviral compound-binding-site, but also of amino acids of the whole VP1 protein. Furthermore, analysis of epidemiological data indicated that group B rhinoviruses produced more than twice as many clinical infections per serotype than group A rhinoviruses did. Rhinoviruses belonging to the minor receptor group were without exception all computed to lie in the same region of antiviral group B.

Human rhinoviruses (HRV) represent a large genus within the class of the picornaviruses, containing 100 antigenically different serotypes (10). Because they are the predominant cause of the common cold in humans, the study of their biological, serological, and genetic properties is of particular medical importance.

The three-dimensional structures of HRV14 (23) and poliovirus 2 (11) have recently been studied in atomic detail and were shown to share an unanticipated similarity. The three larger structural proteins (VP1, VP2, and VP3) of both viruses from the exterior of the viral capsid, whereas VP4 is at the interface between the capsid and the RNA. VP1, VP2, and VP3 are each folded into an eight-stranded antiparallel β-barrel. Neutralizing-antibody-binding sites were found at insertions in the β-barrel structure, on the extreme surface of the virus, surrounding a 2.5-nm-deep "canyon" on the viral surface. The canyon structure has been proposed to be the site of receptor binding (6). The floor of the canyon is formed by relatively conserved sequences of the known picornaviruses, whereas the neutralizing-antibody sites are at the hypervariable regions of the viral capsid proteins (23).

Several series of structurally unrelated antiviral compounds have been found to inhibit picornavirus replication. After the discovery in 1983 of the antiviral properties of R 61837 (2), a pyridazinamine, we initiated a study to compare the potency and spectrum of this molecule with that of other antirhinovirus compounds known at that time, such as sodium dodecyl sulfate (18), dichloroflavan (5), and chalcone (13). Although individual MICs for the same serotype were not identical, a consensus spectrum for these compounds could be identified. A distinction could be made between rhinovirus serotypes which were susceptible to most or all of these antiviral compounds (e.g., HRV1A, HRV9, HRV15, HRV29, HRV30, HRV61, HRV76, and HRV88) and serotypes which were not susceptible to any of them (e.g., HRV4, HRV5, HRV70, HRV72, HRV84, and HRV86) (K. Andries, unpublished data).

The reported spectrum of WIN 51711, an antipicornavirus agent first described in 1985 (20), was for some serotypes clearly aberrant from the above-mentioned consensus spectrum of the other compounds (e.g., WIN 51711 is active against HRV5 and HRV86). By using X-ray diffraction, WIN 51711 was subsequently shown to bind into a hydrophobic pocket beneath the canyon floor of HRV14 (24). We raised mutants of HRV9 and HRV51 resistant to R 61837 and showed them to be cross-resistant to both WIN 51711 and several structurally different reference compounds. From the observed pattern of cross-resistance, it was shown that these capsid-binding compounds probably all bind to essentially the same site in rhinoviruses, i.e., the place corresponding to the hydrophobic pocket in HRV14 (3).

In an attempt to try to understand why compounds binding to a similar site can display essentially different spectra of antiviral activity, we systematically tested the antiviral sensitivity of all 100 typed rhinoviruses, 4 untyped strains, 3 poliovirus serotypes, and coxsackievirus A21 against a panel of 15 antiviral compounds (see Fig. 1) belonging to structurally different chemical classes but all sharing the binding site of WIN 51711.

MATERIALS AND METHODS

Virus and cells. Laboratory-passaged HRV1B, HRV2, HRV3, HRV5, HRV7, HRV8, HRV9, HRV13, HRV14, HRV17, HRV18, HRV19, HRV20, HRV22, HRV23, HRV 25, HRV27, HRV31, HRV32, HRV40, HRV42, HRV43, HRV45, HRV46, HRV47, HRV48, HRV50, HRV52, HRV 54, HRV77 and the untyped strains EL, JM, and SM were obtained from D. A. J. Tyrrell, Common Cold Unit, Salis-

* Corresponding author.

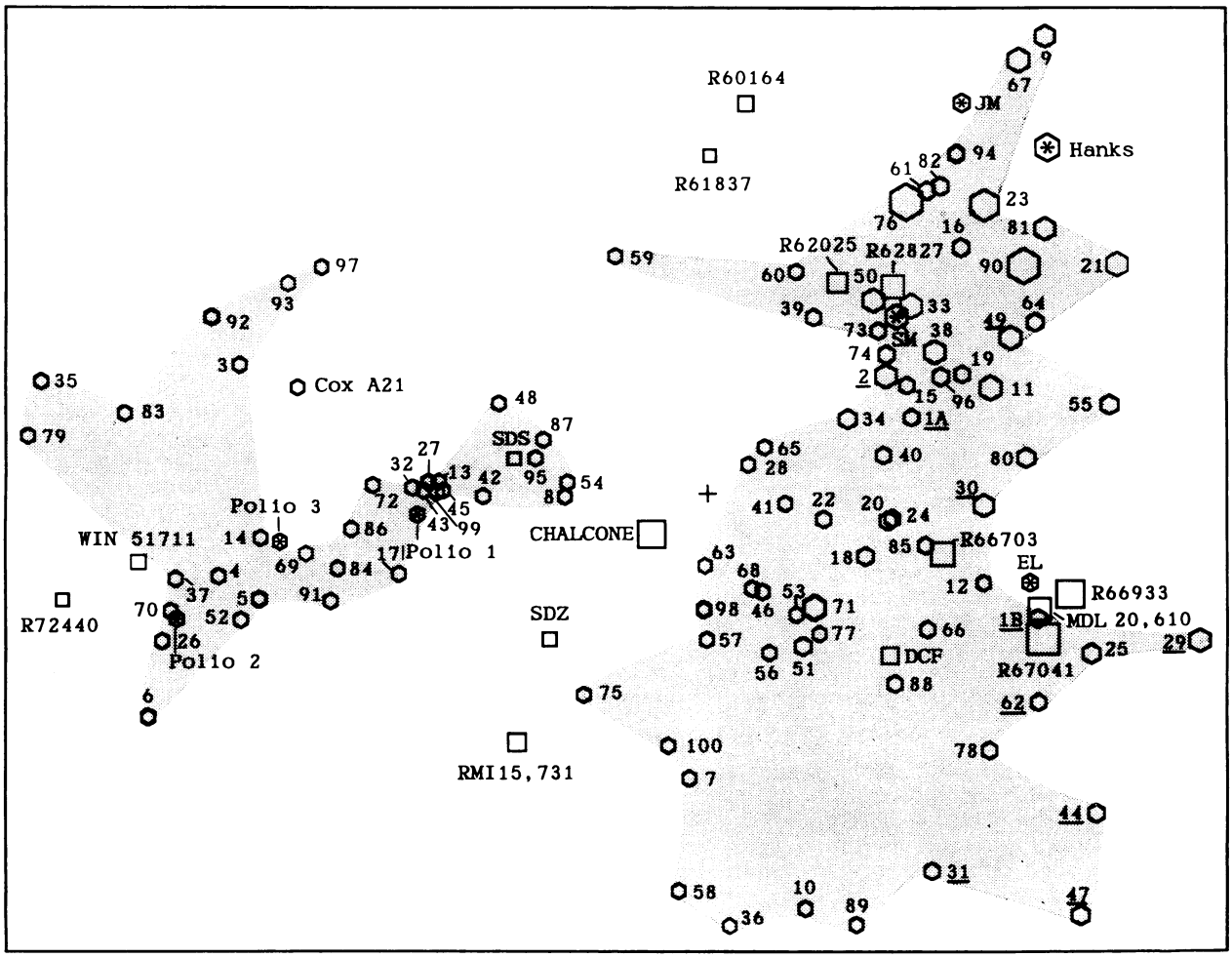

FIG. 1. Spectral map obtained by multivariate analysis of antiviral tests. A panel of 15 antiviral compounds were tested against all rhinovirus and poliovirus serotypes and coxsackievirus A21. Three rules govern the interpretation of the spectral map. (i) Hexagons represent serotypes; squares denote antiviral compounds. (ii) Areas of hexagons are proportional to the mean sensitivities of the serotypes; areas of squares are proportional to the mean potencies of the compounds. (iii) Positions of hexagons and squares on the map are the result of interactions between serotypes and compounds. Two compounds have the same positions on the map when their activities against the 100 serotypes are proportional (although the compounds may differ in average potency). Likewise, two serotypes occupy the same position when their sensitivities for the 15 compounds are proportional (although the two serotypes may possess different average sensitivities). If a serotype is more than averagely sensitive for a compound, they attract each other. On the other hand, if a serotype is less than averagely sensitive for a compound, they repel each other. These mutual attractions and repulsions determine a visible pattern of interactions between serotypes and compounds. The two-group pattern of serotypes that appears from the figure has been established by cluster analysis, as explained in the text. Serotypes belonging to the minor receptor group are underlined.

bury, United Kingdom. The untyped strain Hank's was obtained from F. G. Hayden, University of Virginia, Charlottesville. All other rhinovirus serotypes used in this study and coxsackievirus A21 were obtained from the American Type Culture Collection, Rockville, Md. Polioviruses (type 1 Mahoney, type 2 Mefie, and type 3 Saukett) were obtained from A. Billiau, Rega Institute, Leuven, Belgium. All viruses were grown in Ohio HeLa cells maintained in Eagle basal medium supplemented with 5% fetal calf serum.

Antiviral compounds. Compounds tested in the antiviral assay were R 61837 (2), R 60164 (analog of R 61837 with the methoxy group replaced by a bromo group), R 62025 (3-chloro-6-[3,6-dihydro-4-(3-methylphenyl)-1(2*H*)-pyridinyl] pyridazine), R 62827 (analog of R 62025 in which the chloro group is replaced by a bromo group), R 66703 (3-chloro-6-[4-[(3-methylphenyl)methyl]-1-piperazinyl]pyridazine), R 66933 (analog of R 66703 in which the methyl group is replaced by a chloro group), R 67041 (analog of R 66703 in which the methyl group is replaced by a bromo group), and R 72440 (3-chloro-6-[4-[3-[4-(4,5-dihydro-4,4-dimethyl-2-oxazolyl)phenoxy]propyl]-1-piperidinyl]pyridazine). Reference compounds included chalone (13), dichloroflavan (DCF) (5), MDL 20,610 (14), RMI 15,731 (4), WIN 51711 (20), a Sandoz antirhinovirus compound (SDZ) (ethyl 2-[4-[(2,5-dimethylphenyl)methyl]-1-piperazinyl]-4-thiazolecarboxylate monohydro-chloride; EP 187618), and sodium dodecyl sulfate (SDS) (18). Chalcone, dichloroflavan, MDL 20,610 and RMI 15,731 were kindly supplied by Nippon Roche, Wellcome Research Laboratories, and Merrell Dow Pharmaceuticals, Inc., respectively. Other reference compounds such as WIN 51711 and the Sandoz compound were synthesized in our own laboratories. SDS was from Bio-Rad

Laboratories. All compounds except RMI 15,731 were dissolved in dimethyl sulfoxide (10 mg/ml) and then diluted in growth medium to achieve the final concentration needed. RMI 15,731 was initially dissolved in ethanol as described by Ash et al. (4).

Aantiviral assay. MIC assays were used to assess the antiviral potency of the compounds. Ohio HeLa cell maintenance medium (Eagle basal medium with 5% fetal calf serum) was added to all wells of a microdilution 96-well tissue culture plate at 60 μl per well. An appropriate starting dilution of compound (initially dissolved in dimethyl sulfoxide) was added to duplicate wells at 60 μl per well, and twofold dilutions were made in the maintenance medium to cover a wide range of compound concentrations. Eagle basal medium (120 μl) with 2% *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, 2% fetal calf serum, and 30 mM $MgCl_2$ containing approximately 100 50% tissue culture infective doses of virus was added to all wells except cell and compound controls. Then, 150 μl of each of these virus-compound mixtures was transferred to microdilution plates with subconfluent Ohio HeLa cells, grown in 100 μl of maintenance medium. Appropriate virus controls, cell controls, and compound controls were included in each test. Plates were incubated for 4 to 5 days at 33°C in a 5% CO_2 atmosphere. They were checked by light microscopy without staining and read when the virus controls showed 100% cytopathic effect and the virus back titration showed that between 32 and 256 50% tissue culture infective doses had been used in the test. The MIC_{50} was taken as the lowest concentration of compound that protected 50% of cells from a cytopathic effect. Maximal tested concentrations of the antiviral compounds were 16 μg/ml for chalcone, R 66703, and the Sandoz compound and 32 μg/ml for the others.

Multivariate analysis. Results of antiviral tests were analyzed by using spectral map analysis (15–17). This multivariate analysis includes the following steps. First, logarithms were taken of the reciprocal MICs. Reciprocal values are required because biological activities are inversely related to MICs. Logarithmic MICs are better approximated by a normal distribution than the original MICs are. Next, we computed from the transformed data the mean sensitivity of each serotype (for the 15 compounds) as well as the mean potency of each antiviral compound (in the 100 serotypes). Then we subtracted from each value in the transformed data table the corresponding mean sensitivity and mean potency. Technically, this operation is called double-centering. If double-centering were not applied, the intrinsic interactions between serotypes and compounds would be confounded by differences in the sensitivity of the serotypes and by differences in the potency of the compounds. Next, we applied principal components analysis to the logarithmically transformed and double-centered data. Basically, principal components analysis is a mathematical technique which reduces the number of dimensions in the data. In our case, the apparent number of dimensions is determined by the 15 different antiviral compounds. One can imagine each of the 100 serotypes to form as many points in a 15-dimensional space. However, because of varying degrees of similarity between antiviral activity spectra of the 15 compounds, the relevant number of dimensions is far smaller than 15. (Similarity produces reduncancy in the data and, hence, an excessive number of dimensions). In our application, we found that three dimensions accounted for 91% of the information in the logarithmically transformed data table. These computed dimensions are usually called principal components or factors. A residual of 9% was attributed to experimental noise and was not considered further. Thus, we reduced the original 15 dimensions to three factors.

The result of PCA is in the form of a table of coordinates of the 15 compounds and 100 serotypes in three-dimensional factor space. The so-called spectral map (Fig. 1) represents all compounds and serotypes as points in a two-dimensional plane defined by the two most important factors. The rules of interpretation of the spectral map are detailed in the legend to Fig. 1.

TABLE 1. MICs of two antiviral compounds against rhinovirus serotypes

HRV serotype	MIC (μM)[a] of:		MIC ratio (R 61837/ WIN 51711)	Antiviral group
	R 61837	WIN 51711		
9	0.02	10.22	0.002	B
21	0.06	8.18	0.007	B
64	0.37	6.28	0.059	B
74	0.71	6.64	0.107	B
44	4.57	6.94	0.659	B
42	112.53	10.51	11	A
69	98.47	0.62	158	A
4	77.37	0.32	241	A
79	28.13	0.05	602	A
70	42.20	0.05	903	A

[a] On average, lowest and highest values of replicate measurement ranged between 0.59 and 1.48 times the median MIC.

RESULTS AND DISCUSSION

Rhinovirus groups based on antiviral sensitivity. Some antiviral compounds were found to be particularly active against one subset of rhinoviruses while being inactive or less active than the average against another subset of serotypes. Table 1 illustrates this with the MICs of WIN 51711 and R 61837 against 10 rhinovirus serotypes. The potency of these compounds is about the same. Indeed, the MIC to inhibit the replication of 50% of all 100 serotypes is 4,400 μM for WIN 51711 and 9,800 μM for R 61837. Nevertheless, a group of serotypes displayed a higher than average sensitivity to WIN 51711, whereas another group of serotypes had a higher than average sensitivity to R 61837.

Results of all antiviral tests were analyzed by multivariate analysis, thus allowing a detailed graphic assessment of the observed differences in sensitivities of antiviral compounds. The resulting spectral map (Fig. 1) is a two-dimensional plot (three-dimensional coordinates available on request) which shows the mutual interactions of compounds and serotypes. The map represents 91% of the total variance of the MIC data. Of this, 14% is attributable to the differences in average potency of the compounds and 49% is attributable to the differences in average sensitivity of the serotypes. Interactions between compounds and serotypes contribute 28% to the total variance. The residual 9% of the total variance, caused mostly by HRV52, HRV72, HRV79, and HRV84, is not accounted for by the spectral map.

Using hierarchical and nearest-neighbor cluster analysis (17) of the computed results, we have identified two groups of rhinoviruses, which we have designated antiviral group A and antiviral group B. Antiviral group B contains twice as many serotypes as antiviral group A does (67 and 33 serotypes, respectively). The division of the rhinoviruses in two groups is not arbitrary, but is based on cluster analysis of the three-dimensional result of the multivariate analysis described above.

Numbering of residues in HRV14	1104	1106	1107	1116	1128	1152	1174	1176	1186	1188	1191	1197	1199	1219	1221	1224	3024
HRV14	I	L	S	L	Y	Y	P	V	F	V	V	Y	C	N	M	M	A
HRV1A	I	L	Q	F	I	Y	M	I	F	I	L	Y	M	N	M	I	A
HRV1B	I	L	Q	F	V	Y	M	I	F	L	L	Y	M	N	M	I	A
HRV2	I	L	Q	F	I	Y	A	V	F	L	L	Y	M	N	M	L	A
HRV39	I	L	Q	F	I	Y	A	V	F	L	L	Y	M	N	M	L	X
HRV49	I	L	Q	F	I	Y	A	I	F	L	L	Y	M	N	M	L	X
HRV89	V	L	Q	Y	I	Y	A	V	F	I	M	Y	M	N	M	I	A
Poliovirus 1M	I	Y	K	L	L	Y	P	I	I	V	V	Y	H	N	F	L	A
Poliovirus 1S	I	Y	K	L	F	Y	P	I	I	V	V	Y	H	N	F	L	A
Poliovirus 2La	I	Y	K	L	F	Y	P	V	I	V	V	Y	H	N	F	L	A
Poliovirus 2S	I	Y	K	L	F	Y	P	V	I	V	V	Y	H	N	F	L	A
Poliovirus 3Le	I	Y	K	L	F	Y	P	I	I	V	V	Y	H	D	F	L	A
Poliovirus 3F	I	Y	K	L	F	Y	P	I	I	V	V	Y	H	D	F	L	A
Poliovirus 3S	I	Y	K	L	F	Y	P	I	I	V	V	Y	H	D	F	L	A
Coxsackievirus A21	I	Y	T	L	M	Y	P	I	M	I	V	Y	H	N	F	L	A

FIG. 2. Alignment (from reference 21) for amino acids corresponding to those lining the hydrophobic pocket in HRV14 (from reference 24).

Antiviral compounds that belong to different chemical classes can all bind in the same hydrophobic pocket, and the binding characteristics of the compounds discriminate between two groups of viruses. Therefore, we assume that the hydrophobic pocket, which is the putative binding site for all these compounds, is dimorphic in shape and/or composition. Binding of WIN 51711 in HRV14 shows that this pocket is long and narrow (24). A similar shape in serotypes belonging to antiviral group A could explain why these are more than averagely sensitive for longer molecules. The shape of the pocket in antiviral group B viruses, on the other hand, is different; this pocket is probably shorter, to accept the generally shorter molecules active against these serotypes.

Relationship of antiviral groups to the amino acid sequence. In a further approach, we studied the correlation between our multivariate analysis and protein sequence information by considering the VP1 amino acid alignment of Palmenberg (21). At first, we selected the 17 amino acids that correspond to the lining of the hydrophobic pocket in HRV14 (24) (Fig. 2). The degree of similarity of two serotypes in the spectral map (Fig. 1) can be derived from their distance in the computed three-dimensional space. The smaller the distance, the larger the degree of similarity. Degrees of similarities between sequenced rhinovirus serotypes obtained from the multivariate analysis (Table 2) were compared with the percentages of pocket amino acid identity of the same serotypes (Table 3). The two data sets revealed a high and significant correlation ($r_1 = 0.89$, $P < 0.001$; Spearman test). This correlation indicates that our panel of antiviral agents was a suitable probe for scanning the amino acids of the binding site. The antiviral compounds were really used as a panel of small molecular probes to characterize the composition of the antiviral binding site, which is a process very similar to the characterization of epitopes by using a panel of monoclonal antibodies.

However, when we went further and compared similarities obtained from the multivariate analysis with percent amino acid identity of the whole VP1 proteins of the same serotypes (Table 4), we still found a very high correlation ($r_2 = 0.87$ for degrees of similarities compared with VP1 amino acid identities; $P < 0.001$). This relationship exists because there is a strong correlation between the amino acid sequence of the pocket and the amino acid sequence of VP1. Indeed, we found a correlation of $r_3 = 0.84$ for pocket amino acid identities compared with VP1 amino acid identities ($P < 0.001$). When polioviruses and coxsackievirus A21 were included, the correlations were somewhat lower but still very significant ($r_1 = 0.73$, $r_2 = 0.76$, $r_3 = 0.91$; $P < 0.001$ in each case). Degrees of similarity also correlated with VP1 sequence similarities of rhinoviruses within the same antivi-

TABLE 2. Degree of multivariate similarities between viruses[a]

Serotype	% Similarity with:										
	HRV2	HRV39	HRV49	HRV1A	HRV1B	HRV89	HRV14	Poliovirus 1	Poliovirus 2	Poliovirus 3	Coxsackievirus A21
HRV2	100	92	91	95	77	57	53	65	42	52	54
HRV39		100	85	90	72	54	57	68	46	56	57
HRV49			100	90	77	53	43	55	33	43	46
HRV1A				100	82	61	52	64	42	52	51
HRV1B					100	73	44	54	37	45	38
HRV89						100	48	54	46	50	35
HRV14							100	87	84	92	72
Poliovirus 1								100	72	82	77
Poliovirus 2									100	89	57
Poliovirus 3										100	65

[a] Calculated from the data in Fig. 1.

TABLE 3. Percent identity of amino acids corresponding to the 17 amino acids lining the hydrophobic pocket in HRV14[a]

Serotype	% Identity of amino acids with:										
	HRV2	HRV39	HRV49	HRV1A	HRV1B	HRV89	HRV14	Poliovirus 1	Poliovirus 2	Poliovirus 3	Coxsackievirus A21
HRV2	100	94	88	77	77	71	53	35	41	29	35
HRV39		100	94	71	71	65	48	29	35	24	29
HRV49			100	77	77	59	42	35	29	29	35
HRV1A				100	88	71	47	35	29	29	41
HRV1B					100	59	47	35	29	29	35
HRV89						100	47	24	29	18	29
HRV14							100	53	59	47	47
Poliovirus 1								100	88	88	77
Poliovirus 2									100	88	71
Poliovirus 3										100	71

[a] Calculated from Fig. 2.

ral group ($r_1 = 0.72$, $r_2 = 0.71$, $r_3 = 0.60$ as assessed for six serotypes of the antiviral group B; $P = 0.002$, 0.003, and 0.02, respectively).

An amino acid alignment (21) based on the complete capsid (P1 region) of seven HRV serotypes also correlates very well ($r = 0.92$, $P = 0.006$) with our data. Even an amino acid alignment of the 3CD regions (coding for nonstructural proteins) of HRV2, HRV89, HRV14, the three polioviruses, and coxsackievirus A21 displayed a surprisingly high ($r = 0.70$, $P = 0.002$) correlation with our data set (A. Palmenberg, personal communication).

Even when complete genome sequences of rhinoviruses are compared with our data, the similarity is remarkable. Deuchler et al., for instance, described extensive sequence similarity between HRV89 and HRV2 (both located in antiviral group B), not only in stretches coding for structural proteins, but also in those coding for nonstructural proteins (8). Furthermore, the similarity between HRV2 and HRV89 was much greater than that between HRV89 and HRV14 (antiviral group A). Hughes et al. compared the complete nucleotide sequence of HRV1B with those of other serotypes and found that HRV1B and HRV2 were the most closely related serotypes overall, followed by HRV89 (12). In contrast to the relationship to these serotypes, the similarity to HRV14 was much lower. HRV14 has been shown to share a surprising degree of amino acid sequence similarity with the polioviruses (25) which appear in the same region of the spectral map as HRV14.

Relationship of antiviral groups to receptor groups and antigenic groups. We tried to correlate our antiviral groups with other biological data on rhinoviruses. Rhinoviruses are already subdivided into a major and a minor group on the basis of their use of one of two possible receptors upon entry into the cell (1). Rhinoviruses belonging to the minor receptor group (hexagons with underlined labels in Fig. 1) were, without exception, all computed to lie in the same region of antiviral group B. Our model suggests that during the evolution of picornaviruses, there was first a divergence between polioviruses and group A rhinoviruses, both sharing a similar antiviral compound-binding site. Next, a further divergence from rhinovirus group A occurred, resulting in group B rhinoviruses with the same receptor specificity but a different antiviral compound-binding site, and finally, a group of minor receptor-binding viruses branched off. Indeed, if the divergence between the receptor groups took place before the divergence of the antiviral groups, minor receptor group viruses would have distributed randomly over both antiviral groups.

Antigenic cross-relationships among rhinoviruses have been evaluated conscientiously by cross-immunization of rabbits with 90 serotypes (7). Antigenic clustering was found for the following serotypes: 2+49, 3+6+14, 5+17+45+70, 15+74, 11+15+40+73+76, 22+61, 29+44, 38+60, 39+54, 66+77, 13+41, 22+61, 1A+1B, and 9+67+32. Although the antigenically related viruses belonging to the four last antigenic clusters partitioned in more distant regions in the multivariate analysis of our MIC data, viruses belonging to the first nine antigenic clusters partitioned very close to each other (Fig. 1). This suggests an unexpected correlation between the external antigenic composition of rhinoviruses and the composition of their binding site for antiviral compounds.

TABLE 4. Percent identity of all amino acids of VP1 (283 to 292 amino acids)[a]

Serotype	% VP1 amino acid identity with:										
	HRV2	HRV39	HRV49	HRV1A	HRV1B	HRV89	HRV14	Poliovirus 1	Poliovirus 2	Poliovirus 3	Coxsackievirus A21
HRV2	100	72	92	73	71	65	43	40	37	36	40
HRV39		100	72	77	77	70	43	41	41	38	38
HRV49			100	73	71	64	43	38	37	37	40
HRV1A				100	91	69	41	37	38	35	37
HRV1B					100	68	42	38	38	35	36
HRV89						100	41	39	38	35	39
HRV14							100	44	44	44	42
Poliovirus 1								100	77	76	56
Poliovirus 2									100	79	58
Poliovirus 3										100	59

[a] Calculated from the alignment in reference 21.

TABLE 5. Frequency of rhinovirus colds related to antiviral group allocation

Study period[a]	No. of isolates of major antiviral group[b]	No. of isolates of minor antiviral group[c]	Ratio of no. of isolates per serotype observed/ no. expected[d]
1976–1981 (19)	159 (x = 2.56)	35 (y = 1.30)	1.97 (P = 0.03)
1966–1971 (19)	223 (x = 3.60)	27 (y = 1)	3.60 (P = 0.0001)
1975–1979 (9)[e]	636 (x = 10.26)	125 (y = 4.63)	2.22 (P = 0.002)
All studies	1,018 (x = 16.42)	187 (y = 6.93)	2.37 (P = 0.0002)

[a] Isolates were serotyped by exposure to antisera against 89 HRVs of which 62 fall into antiviral group B.
[b] x is the mean frequency per serotype.
[c] y is the mean frequency per serotype.
[d] P values assessed by the randomization test.
[e] Details of Table 1 in reference 9 were kindly provided by Marion K. Cooney.

Analysis of polioviruses and unnumbered rhinovirus serotypes. Because we wanted to limit our data set to capsid-binding antiviral agents interacting with serotyped rhinoviruses, we positioned the four untyped rhinovirus isolates (EL, JM, SM, and Hank's) and three polioviruses by fitting them on the results of Fig. 1. Multivariate analysis allows us to predict the most likely identity of unknown serotypes by looking at their nearest neighbors (this is preferentially done by using a computer program that rotates the three-dimensional plot). The symbol size and location of the SM isolate, for instance, indicate a very close similarity, if not identity, with HRV33. The same holds for the EL isolate and HRV12. Polioviruses all fit in the antiviral group A region of rhinoviruses, indicating that they have a antiviral compound-binding site comparable to that of HRV14.

Relationship of antiviral groups to occurrence of clinical colds. We used our data to analyze available epidemiological data on rhinoviruses. In total, 1,205 rhinovirus isolates have been collected form people with colds and serotyped by two independent groups during three periods of about 5 years each (9, 19) (Table 5). Although the number of isolates does not allow for reliable evaluation of the pathogenicity of individual serotypes, the isolates can be used to calculate the mean frequency of isolations of rhinoviruses belonging to antiviral group A as well as viruses belonging to antiviral group B. Given the fact that antiviral group B contains twice as many serotypes as antiviral group A, one would expect that serotypes of that group would account for exactly twice as many colds. However, this was not the case. Serotypes of antiviral group B accounted for five times as many colds as serotypes of antiviral group A (Table 5). The chance that this is just coincidence was calculated to be less than 1 in 10,000. The difference is unlikely to be due to selection pressure during virus isolation. Indeed, the clinical samples were inoculated each time into a panel of different rhinovirus-susceptible cell cultures, and the difference was observed in each study (Table 5). Furthermore, viruses from antiviral group A are known to use the same cellular receptor as the majority of the viruses from antiviral group B (Fig. 1). The difference in the pathogenicity of viruses belonging to antiviral groups A and B could be confirmed (P = 0.0001) when only those viruses attaching to the ICAM-1 receptor were used for analysis. Within antiviral group B we could not identify differences in pathogenicity between serotypes belonging to the different receptor groups (P = 0.15). We therefore assume that something in the structure of antiviral group B viruses endows them with a higher pathogenicity.

In summary, we have identified two groups of rhinoviruses by analyzing the binding characteristics of small molecular probes for the various serotypes. Additional evidence for the existence of two groups of rhinoviruses was obtained from comparing our results with the distribution of receptor groups, with antigenic relationships, and with sequence data of amino acids of VP1, P1, and 3CD regions and, finally, from observing a difference in epidemiological profile.

Our observations indicate the existence of a dimorphic binding site shared by a group of different antiviral compounds. The dimorphism of this binding site is apparently a reflection of a divergence of rhinovirus serotypes on a much broader level. Activities of antiviral compounds could be correlated with amino acid sequence divergence. This is probably a consequence of the fact that all 17 amino acids lining the binding site of the antiviral compounds are part of a very conserved protein structure. Indeed, many animal and plant picornaviruses use the same structural pattern, i.e., the eight-stranded antiparallel β-barrel structure, as building units of the viral capsid (23). Constraints in the construction of this building unit may explain why the binding site appears to be dimorphic rather than polymorphic. It is difficult to understand, however, why small variations in the binding site turn out to be correlated with diversities in structural and nonstructural proteins. Mutations in the pocket appear to be a reflection of random mutations in other parts of the genome. Differences in pocket composition and distances in the spectral map may therefore be representative of amino acid differences of these viruses in general.

The hypothesis of the presence of two groups may therefore have implications not only for the development of antiviral compounds, but also for the understanding of evolutionary relationships between rhinoviruses at the molecular, antigenic, and epidemiological levels.

ACKNOWLEDGMENTS

We acknowledge Raymond Stokbroekx for chemical support, Marcel Janssen and Mark De Brabander for helpful discussions, Rudy Willebrords for assistance in the collection of some of the MIC data, Jules Van Hoof and Koen Van Reusel for their help with the multivariate analysis, and Gert Verheyen for her help in the design of Fig. 1.

LITERATURE CITED

1. **Abraham, G., and R. J. J. Colonno.** 1984. Many rhinovirus serotypes share the same cellular receptor. Virology **51**:340–345.
2. **Andries, K., B. Dewindt, M. De Brabander, R. Stokbroekx, and P. A. J. Janssen.** 1988. In vitro activity of R 61837, a new antirhinovirus compound. Arch. Virol. **101**:155–167.
3. **Andries, K., B. Dewindt, J. Snoeks, and R. Willebrords.** 1989. Lack of quantitative correlation between inhibition of replication of rhinoviruses by an antiviral drug and their stabilization. Arch. Virol. **106**:51–61.
4. **Ash, R. J., R. A. Parker, A. C. Hagan, and G. D. Mayer.** 1979. RMI 15,731(1-[5-tetradecyloxy-2-furanyl]-ethanone), a new antirhinovirus compound. Antimicrob. Agents Chemother. **16**: 301–305.
5. **Bauer, D. J., J. W. T. Selway, J. F. Batchelor, M. Tisdale, I. C. Caldwell, and D. A. B. Young.** 1981. 4',6-Dichloroflavan (BW683C), a new anti-rhinovirus compound. Nature (London) **292**:369–370.
6. **Colonno, R. J., J. H. Condra, S. Mitzutani, P. L. Callahan, M. Davies, and M. Murcko.** 1988. Evidence for the direct involvement of the rhinoviruses canyon in receptor binding. Proc. Natl. Acad. Sci. USA **85**:5449–5453.
7. **Cooney, M. K., J. P. Fox, and G. E. Kenny.** 1982. Antigenic

groupings of 90 rhinovirus serotypes. Infect. Immun. **37**:642–647.

8. **Deuchler, M., T. Skern, W. Sommergruber, C. Neubauer, P. Greundler, I. Fogy, D. Blaas, and E. Keuchler.** 1987. Evolutionary relationships within the human rhinovirus genus: comparison of serotypes 89, 2, and 14. Proc. Natl. Acad. Sci. USA **84**:2605–2609.
9. **Fox, J. P., M. K. Cooney, C. E. Hall, and H. M. Foy.** 1985. Rhinoviruses in Seattle families, 1975–1979. Am. J. Epidemiol. **122**:830–846.
10. **Hamparian, V. V., R. J. Colonno, M. K. Cooney, E. C. Dick, J. M. Gwaltney, J. H. Hughes, W. S. Jordan, A. Z. Kapikian, M. J. Mogabgab, A. Monto, C. A. Phillips, R. R. Rueckert, J. H. Schieble, E. J. Stott, and D. A. J. Tyrrell.** 1987. A collaborative report: rhinoviruses—extension of the numbering system from 89 to 100. Virology **159**:191–192.
11. **Hogle, J. M., M. Chow, and D. J. Filman.** 1985. Three-dimensional structure of poliovirus at 2.9 A resolution. Science **229**:1358–1365.
12. **Hughes, P. J., C. North, C. H. Jellis, P. D. Minor, and G. J. Stanway.** 1988. The nucleotide sequence of human rhinovirus 1B: molecular relationships within the rhinovirus genus. J. Gen. Virol. **69**:49–58.
13. **Ishitsuka, H., Y. T. Ninomiya, C. Ohsawa, M. Fujiu, and Y. Suhara.** 1982. Direct and specific inactivation of rhinovirus by chalcone. Antimicrob. Agents Chemother. **22**:617–621.
14. **Kenny, M. T., J. K. Dulworth, T. M. Bargar, H. L. Torney, M. C. Graham, and A. M. Manelli.** 1986. In vitro antiviral activity of the 6-substituted 2-(3′,4′-dichlorophenoxy)-2H-pyranol[2,3-B]pyridines MDL 20,610, MDL 20,646, and MDL 20,957. Antimicrob. Agents Chemother. **30**:516–518.
15. **Lewi, P. J.** 1989. Spectral map analysis. Analysis of contrasts especially from log ratios. Chemometrics Intelligent Lab. Syst. **5**:105–116.
16. **Lewi, P. J.** 1976. Spectral mapping, a technique for classifying biological activity profiles of chemical compounds. Arzneim. Forsch. **26**:1295–1300.
17. **Lewi, P. J.** 1980. Multivariate data analysis program. End user's guide. IBM International Field Program LB11-5392-0, file no. 5370-79, Program number 5787 FAE(F). IBM Corp., Uithoorn, The Netherlands.
18. **Lonberg-Holm, K., and J. J. Noble-Harvey.** 1973. Comparison of in vitro and cell-mediated alteration of a human rhinovirus and its inhibition by sodium dodecyl sulfate. Virology **12**:819–826.
19. **Monto, A. S., E. R. Bryan, and S. Ohmit.** 1987. Rhinovirus infections in Tecumseh, Michigan: frequency of illness and number of serotypes. J. Infect. Dis. **156**:43–49.
20. **Otto, M. J., M. P. Fox, M. J. Fancher, M. F. Kuhrt, G. D. Diana, and M. A. McKinlay.** 1985. In vitro activity of WIN 51711, a new broad-spectrum antipicornavirus drug. Antimicrob. Agents Chemother. **27**:883–886.
21. **Palmenberg, A. C.** 1989. Sequence alignments of picornaviral capsid proteins. p. 211–241. *In* B. Semler and E. Ehrenfeld (ed.), Molecular aspects of picornavirus infection and detection. American Society for Microbiology, Washington, D.C.
22. **Rossmann, M. G.** 1987. The evolution of RNA viruses. BioEssays **7**:99–103.
23. **Rossmann, M. G., E. Arnold, J. W. Erickson, E. A. Frankenberg, J. P. Griffith, H. Hecht, J. E. Johnson, G. Kamer, M. Luo, A. G. Mosser, R. R. Rueckert, B. Sherry, and G. Vriend.** 1985. Structure of a human common cold virus and functional relationship to other picornaviruses. Nature (London) **317**:145–153.
24. **Smith, T. J., M. J. Kremer, M. Lou, G. Vriend, E. Arnold, G. Kamer, M. G. Rossmann, M. A. McKinlay, G. D. Diana, and M. J. Otto.** 1986. The site of attachment in human rhinovirus 14 for antiviral agents that inhibit uncoating. Science **233**:1286–1293.
25. **Stanway, G., P. J. Hughes, R. C. Mountford, P. D. Minor, and J. W. Almond.** 1984. The complete nucleotide sequence of a common cold virus: human rhinovirus 14. Nucleic Acids Res. **12**:7859–7875.