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Molecular relationships between 21 human rhinovirus serotypes

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We have analysed, by PCR using consensus primers followed by sequencing, 12 human rhinoviruses (HRVs) in a genomic region including that corresponding to the immunogenic site NIm-II. Together with published information, 21 sequences are available for comparison. In the region analysed, which encodes 112 amino acids, the majority (18) of the serotypes exhibited at least 70% amino acid identity to one another and some serotypes are very closely related. These include HRV-36, -58 and -89, known to exhibit antigenic cross-reactivity, which were shown to differ at only three amino acid positions. Three serotypes, HRV-3, -14 and -72, share at least 84% identity with one another but are less than 66% identical to the majority group. Interestingly, membership of these two molecular clusters correlates with the groupings determined by sensitivity to antivirus drugs, suggesting that they reflect a fundamental division of HRVs. In contrast, there is no correlation with receptor grouping, since the majority group contains members belonging to both HRV receptor groups.

Introduction

Human rhinoviruses (HRVs) are the major cause of the common cold and are therefore among the most frequently occurring human pathogens (Stanway, 1994). The large number of serotypes (around 100) is an important factor in this high incidence, since little, if any, immunological protection is afforded by prior exposure to a heterologous serotype. The diversity of HRVs has also hindered the development of therapeutic agents and vaccines.

It has been shown that HRVs can be sub-grouped in various ways and this may eventually aid their control if shared structural and biological properties can be exploited. One of the most fundamental groupings is based on receptor specificity (Abraham & Colonno, 1984; Uncapher *et al.*, 1991). Around 90% of serotypes (the major receptor group) are known to utilize the cellular protein, intercellular adhesion molecule 1 (ICAM-1), as a receptor to gain entry to susceptible cells (Uncapher *et al.*, 1991; Greve *et al.*, 1989; Staunton *et al.*, 1989; Tomassini *et al.*, 1989). According to the canyon hypothesis, ICAM-1 interacts with a deep depression (the canyon) on the HRV surface, enabling virus residues critical for receptor binding to be conserved, free from immune pressure (Rossmann *et al.*, 1985; Rossmann & Palmenberg, 1988). A single serotype (HRV-87) recognizes an as yet unknown receptor, while 10 serotypes (the minor receptor group) utilize members of the low density lipoprotein receptor family for cell entry (Uncapher *et al.*, 1991; Hofer *et al.*, 1994). Whether the canyon is involved in receptor binding in these cases is not known.

HRV serotypes have also been assigned into subgroups according to their sensitivity towards a spectrum of antivirus agents which interact with the virus capsid and interfere with uncoating and/or receptor binding (Andries *et al.*, 1990). Two such subgroups, A containing 33% and B containing 67% of serotypes, have been described. A third criterion is low-level immunological cross-reactivity observed between some HRV serotypes. Sixteen antigenic groups, including a total of 50 HRV serotypes, have been defined in this way (Fox, 1976; Cooney *et al.*, 1982). Finally, a number of nucleotide and predicted amino acid sequences have been derived (Stanway *et al.*, 1984; Skern *et al.*, 1985; Duechler *et al.*, 1987; Hughes *et al.*, 1988; Leckie, 1988; Palmenberg, 1989; Kim *et al.*, 1989; Horsnell, 1990; Oliveira *et al.*, 1993; J. Margolis & B. E. Clarke, unpublished). Of the HRVs for which data are available, the majority appear to be closely related to one another while, in contrast, one of the most extensively studied viruses, HRV-14, appears to be much more distinct (Stanway, 1990). There

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is some agreement between these sub-grouping criteria: for instance, all the minor receptor group viruses are contained within drug group B (Andries *et al.*, 1990). However, HRV-14 is classified within the major receptor group but is relatively distinct in molecular terms from other sequenced members of this group, which are in turn closely related to minor group members. The molecular basis of these properties thus remains obscure.

Here we describe how a PCR-based approach has enabled nucleotide sequencing of a specific genomic region, encoding the region flanking part of the immunogenic site NIm-II, from several HRV serotypes (Sherry *et al.*, 1986). The results allow a clearer understanding of the relationship between HRV serotypes and offer insights into the factors underlying shared properties.

Methods

Viruses. ATCC reference strains of a number of HRVs were provided in the form of tissue culture supernatants by Professor W. Al-Nakib, Common Cold Unit, UK, Dr D. Rowlands, Wellcome Research Laboratories, UK and Dr K. Andries, Janssen Research, Belgium.

Nucleic acid. RNA isolation from virus-containing tissue culture supernatant (100 µl), reverse transcription and PCR amplification of HRV-specific DNA were performed essentially as described by Gama *et al.* (1988, 1989). The primers used for PCR were OL145 (5′ CGCTCTAGAACTAGTGGATCCCCCGGGTGGTGGTGGAAA[T,C]T[A,G] CC 3′) and OL146 (5′ CTTGATATCGAATTCCTGCAGCC CCATTGAGTCCATTGG [T,A]AC[T,A]GC 3′). PCR products were purified by agarose (1%) gel electrophoresis, electroelution, phenol extraction and ethanol precipitation. Nucleotide sequence analysis was performed on HRVs 3, 7, 11, 21, 29, 36, 49, 50, 58 and 65 following cloning of the products into the plasmid vector pUBS or bacteriophage M13. In the case of HRVs 62 and 72 the PCR product was sequenced directly by cycle sequencing with a primer end-labelled with [^{33}P]ATP. A commercial system was used (Gibco BRL) and the procedure was performed exactly as described by the manufacturers. Sequences were determined on both strands.

Sequence analysis. Sequence data were assembled and analysed using the Staden-Plus package (Amersham), in particular the programs DBAUTO, ANALYSEQ and ANALYSEP (Staden, 1984*a*, *b*). Amino acid sequence identities were expressed in the form of a dendrogram using the program KITSCH, part of the PHYLIP suite (Felsenstein, 1989).

Results

The NIm-II region was chosen for study as it is a major immunogenic site in the HRVs studied to date (Sherry *et al.*, 1986). Furthermore, in the case of HRV-2, synthetic peptides representing this region have been shown to induce neutralizing antibodies (Francis *et al.*, 1987). To select sites suitable for use as targets for broadly reactive PCR primers, alignments of published sequences were used to identify regions of conserved amino acid sequence flanking the NIm-II site (data not shown). Two amino acid sequences, virtually invariant among HRVs, GWWWKLP and AVPMDSM, specified by nucleotide positions 1058–1078 and 1451–1471 respectively in HRV-1B, were chosen as target sites in view of the low potential degree of degeneracy in cognate RNA sequences (Hughes *et al.*, 1988). The primers OL145 and OL146 were synthesized to recognize these target sequences. The primers were designed to include the restriction sites *Bam*HI (OL145) and *Eco*RI (OL146) to facilitate cloning of the PCR products.

PCR amplification using OL145 and OL146, following reverse transcription using OL146 as primer, yielded a product of the predicted size for all 12 HRVs tested (data not shown). These products were sequenced directly or following cloning into M13 or a plasmid vector (data not shown). Amino acid sequences, predicted from the nucleotide sequences derived from these 12 HRVs (serotypes 3, 7, 11, 21, 29, 36, 49, 50, 58, 62, 65 and 72), are shown aligned, together with published sequences in Fig. 1. Within the region analysed, there is a high degree of identity and 50 out of the 112 residues are invariant in all of the 21 HRV serotypes. These are shown in bold in the consensus sequence in Fig. 1. Comparison with those viruses which have been studied at atomic resolution by X-ray crystallography (HRVs 1A, 14 and 16), shows that many invariant residues are located in the β-pleated sheets which make up the core β-barrel structure of VP2, or in α-helices (Rossmann *et al.*, 1985; Kim *et al.*, 1989; Oliveira *et al.*, 1993). βD and βF are particularly well conserved. These residues are largely buried inside the capsid and not exposed to the surface. However, outside these areas, there is striking conservation of the sequences IPEHQ and THPGEXG located respectively N-terminal to and within the VP2 'puff', the large loop which links β-pleated sheets E and F. These sequences are known to lack definable secondary structure elements and the latter is at least partially exposed on the surface of the HRVs whose structures are known (Oliveira *et al.*, 1993).

The NIm-II region has previously been defined by neutralizing monoclonal antibodies to include VP2 residues 41–44 and 62–71 (Sherry *et al.*, 1986). These residues, in particular the latter, are the most variable between the HRVs studied and it is likely that they contribute to antigenicity in the majority of serotypes (Fig. 1). One striking feature of the results is, however, the close similarity among some of the serotypes, in particular HRV-36, -58 and -89. In the region under study, these serotypes differ only within NIm-II itself and even here, by only either two or three amino acids (see Fig. 3). HRV-7 is a rather more distinct member of this cluster. There is also strong similarity between HRVs -1A and -1B; between -2 and -49; to a lesser extent between -11 and -21; and also between HRV-14 and -72.

Molecular relationships between the HRVs, based on the amino acid identity of the region of VP2 under study

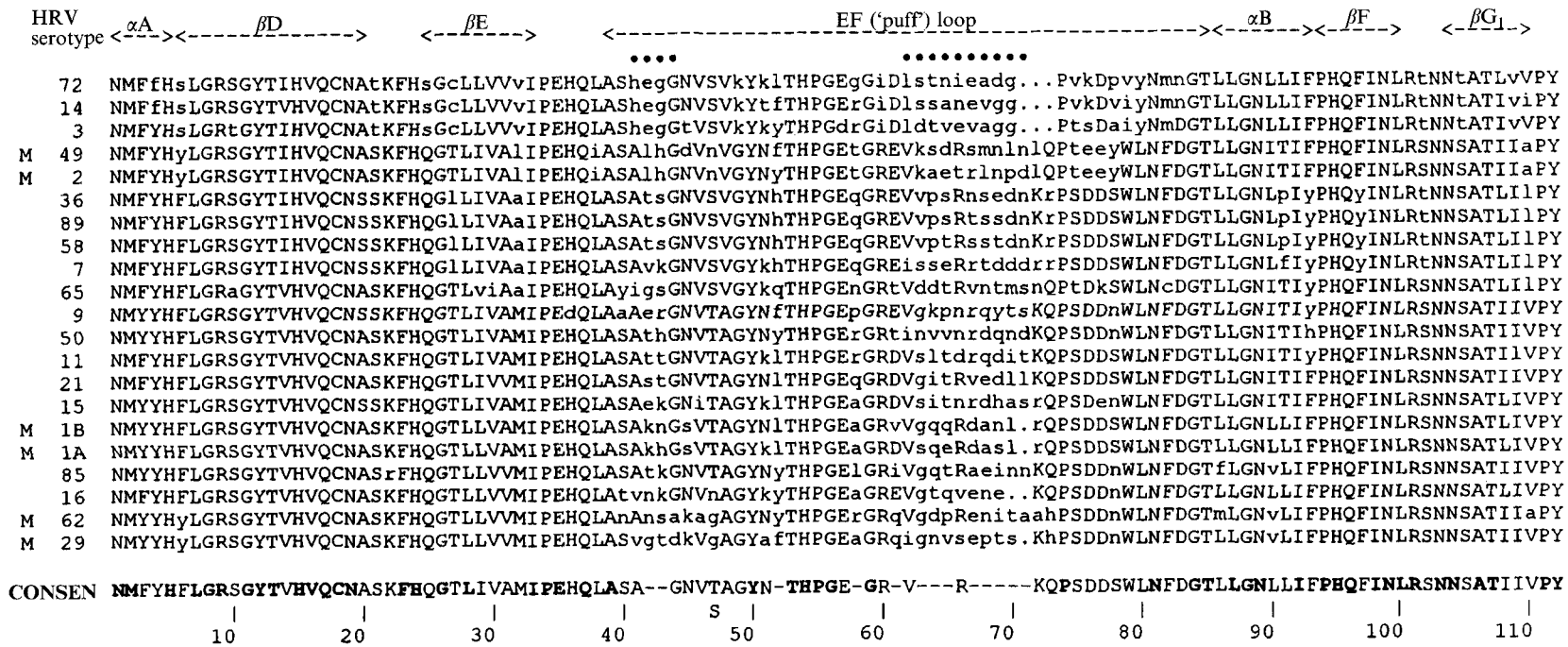

Fig. 1. Alignment of the predicted amino acid sequence of part of VP2 from several HRV serotypes. Gaps introduced to optimize the alignment are marked with dots. Residues within the NIm-II region are indicated (●) and regions of secondary structure, based on the structures of HRV-1A, -14 and -16 derived by X-ray crystallography are also marked. Largely conserved residues are in upper-case and variable in lower-case. Members of the minor receptor group are denoted by M. Invariant amino acids are denoted in the consensus sequence (CONSEN) by bold type while a hyphen represents a position where there is no clear consensus. The region has been numbered arbitrarily for convenience but represents amino acids 95–205 of VP2 in HRV-1B (Hughes *et al.*, 1988).

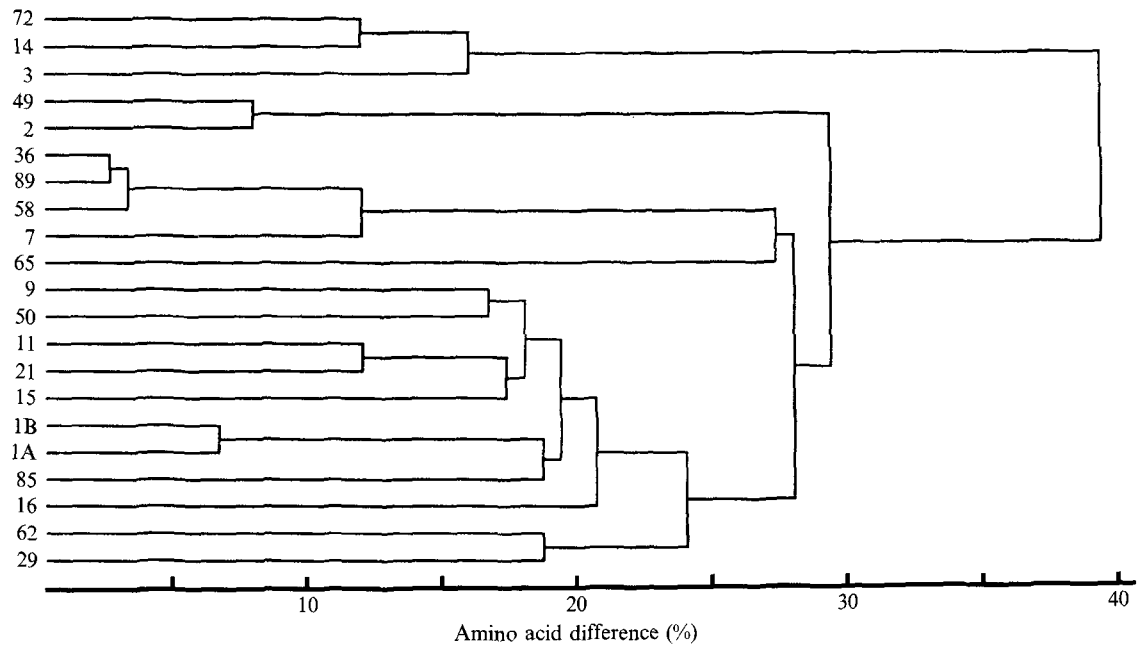

Fig. 2. Dendrogram, based on percentage amino acid sequence difference, expressing the relationship between the 21 HRV serotypes in the region under study. The dendrogram was derived using the program KITCH, part of the PHYLIP suite (Felsenstein, 1989).

Table 1. *Summary of the genetic and biological properties of the 21 HRVs analysed*

HRV serotype	Receptor group	Drug group	Genetic group	Known antigenic relatives*	Close genetic relatives†
1A	Minor	B	1	1B	1B
1B	Minor	B	1	1A	1A
2	Minor	B	1	49	49
3	Major	A	2	14	–
7	Major	B	1	–	36, 58, 89
9	Major	B	1	–	–
11	Major	B	1	15	21
14	Major	A	2	3	72
15	Major	B	1	11	–
16	Major	B	1	–	–
21	Major	B	1	29	11
29	Minor	B	1	21	–
36	Major	B	1	50, 58, 89	7, 58, 89
49	Minor	B	1	2	2
50	Major	B	1	36, 58, 89	–
58	Major	B	1	36, 50, 89	7, 36, 89
62	Minor	B	1	–	–
65	Major	B	1	–	–
72	Major	A	2	–	14
85	Major	B	1	–	–
89	Major	B	1	36, 50, 58	7, 36, 58

* Fox (1976), Cooney *et al.* (1982). Only known antigenic relationships between the 21 serotypes analysed in molecular terms are given.

† Greater than 85% amino acid identity in the region under study.

are expressed in the form of a dendrogram in Fig. 2. On this basis the 21 HRVs analysed appear to fall into two groups. Most have an amino acid identity of greater than 70% in the region analysed. For convenience, these will be termed here genetic group 1 (GG1). A smaller group of serotypes (termed genetic group 2; GG2), consisting of HRV-3, -14 and -72, share at least 84% identity with one another but are linked to GG1 by an amino acid identity of less than 60%. These latter viruses also share a number of consistent molecular characteristics, including a three amino acid deletion relative to most GG1 members in the NIm-II antigenic site itself and the replacement of the sequence WLNFD (position 79–83), well conserved in GG1 HRVs, with the distinct and more variable sequence (I/V)YNM(N/D). Within GG1, there is little length heterogeneity in the region under study, although HRV-16 has a two amino acid deletion relative to other GG1 members, while HRV-1A, -1B and -29 are shorter by a single amino acid. All minor and most major receptor group HRVs are contained within GG1 and there is no obvious clustering of receptor groups in overall molecular terms.

A summary of the genetic and biological relationships between the HRV strains studied is given in Table 1.

Discussion

The large number of HRV serotypes means that a complete molecular analysis of reference strains is unlikely to be achieved. We have shown here, however, that a PCR approach can be exploited to generate sequence data from a specific region of biological significance. The primers used gave a PCR product, corresponding to a region encoding part of the capsid

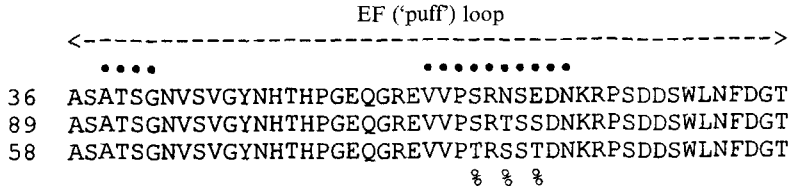

Fig. 3. Relationship between HRV-36, -89 and -58 in the NIm-II region (denoted by ●), contained within the loop linking β-pleated sheets E and F. Amino acid differences between any pair of serotypes are indicated by percentage signs.

protein VP2, for all of the HRVs tested and it is likely that they could be used to study all other HRV serotypes.

A striking feature of the results is the close molecular relationship observed between some of the HRV serotypes (Table 1). The most obvious examples are HRV-36, -58 and -89, which are virtually identical in the region under study (Fig. 3). The differences between these serotypes are in amino acids in NIm-II, which by analogy with the structure of HRV-16, are in particularly exposed locations on the virus surface. Differences between another pair of closely related viruses, HRV-1A and -1B, are again seen in particularly prominent residues in NIm-II, as can be seen from the known structure of HRV-1A (Kim *et al.*, 1989). In contrast to the low numbers of amino acid differences seen between these HRVs in NIm-II (up to three between any pair of HRV-36, -58 and -89; four between HRV-1A and -1B), another pair of closely related viruses, HRV-2 and HRV-49, differ by eight consecutive residues in NIm-II. Overall, the results indicate a wide diversity between HRVs in the NIm-II region. Thus, although it might be possible in some cases to design peptides or chimeric HRVs with consensus sequences in order to produce an immune response against more than one HRV, it seems unlikely that these would reduce significantly the number of vaccine components required to induce immunity against all HRVs. Even in the case of HRV-36, -58 and -89, the fact that the limited amino acid differences seen are located in prominent regions of NIm-II suggests that these would have a radical effect on antigenicity, making such consensus sequences difficult to design. An alternative approach might be to target regions of the HRV capsid which are highly conserved (McCray & Werner, 1987). The present work has defined several sequences which are conserved, but most are located within the capsid and are unlikely to be important antigenically. However, one conserved sequence, THPGEXG (position 53–59), is at least partially exposed on the virus surface in the HRVs whose structures are known (Rossmann *et al.*, 1985: Kim *et al.*, 1989; Oliveira *et al.*, 1993). This is devoid of recognizable secondary structures but is well conserved structurally among these HRVs, possibly suggesting an important role in the morphology or function of the capsid (Oliveira *et al.*, 1993). Intriguingly, the polioviruses and poliovirus-like enteroviruses have an analogous sequence, but other enteroviruses, such as coxsackie B viruses, lack the sequence (Palmenberg, 1989). Appropriate presentation of this sequence to the immune system could be a route towards a broadly effective vaccination strategy.

A further interesting aspect of the data obtained is that they go some way towards an explanation of the antigenic cross-reactivity which is observed between certain HRVs (Table 1). In view of the close molecular relationship between HRV-36, -58 and -89, their inclusion in the same antigenicity group is not surprising (Cooney *et al.*, 1982). Less consistent is the inclusion of HRV-50 in this antigenic group, since this serotype is well separated from the HRV-36, -58 and -89 molecular cluster. This may imply that it is difficult to predict antigenic relationships, which are probably based on very small regions of the capsid, from overall molecular identity. Alternatively, it may be possible that HRV-50 is more similar to HRV-36, -58 and -89 in other areas of the capsid.

A similar equivocal pattern emerges from a consideration of the other HRVs studied. The antigenic relationship between HRV-1A and -1B is matched by the degree of molecular identity, as is the case for HRV-2 and -49. Although the molecular relationship between HRV-3 and -14 is not as close as that seen in the examples above, the observed antigenic cross-reactivity is unsurprising given the degree of identity which does exist (Cooney *et al.*, 1982). In contrast, the basis for the antigenic relationship between HRV-21 and HRV-29 is not clear since these are relatively diverse in molecular terms in the region under study (Fig 2).

As concluded from previous sequence analysis, overall amino acid identity does not appear to influence receptor grouping (Duechler *et al.*, 1987; Hughes *et al.*, 1988). However, the data obtained allow a detailed analysis of other possible correlations in the region studied. For instance, one part of the region, residues 45–47, contributes to the ICAM-1 footprint, the region of the capsid shown recently by cryo-electron microscopy to be spatially juxtaposed with the ICAM-1 molecule in complexes between HRV-16 and soluble ICAM-1 (Olson *et al.*, 1993). Interestingly, there is strong conservation of the sequence NV(T/S) in the equivalent footprint residues of 12/15 of the major group HRVs. The exceptions are HRV-3 where the sequence TVS is found, HRV-15 (NIT) and HRV-16 itself (NVN). The latter sequence is found in the minor receptor group member HRV-2. In all six of the the minor receptor members

analysed, the NV(T/S) motif is at least partially disrupted, particularly so in HRV-29, -49 and -62. Although not an absolute correlation, it is possible that the absence of this motif at least contributes to the inability of minor group HRVs to bind to ICAM-1. It would be interesting to analyse other ICAM-1 footprint residues, which are mainly located in VP1, in the panel of viruses studied here. Despite this correlation with the lack of ICAM-1 binding, the data do not shed light on the ability of minor group HRVs to bind to their receptor. Mutagenesis of canyon floor residues in one major group member, HRV-14, has a profound effect on receptor binding, while analogous mutations in the minor group member, HRV-2, have little effect (Colonno *et al.*, 1988; Duechler *et al.*, 1993). It is therefore possible that minor group HRVs do not use a receptor/canyon interaction and that the significant residues are located elsewhere in the capsid.

Finally, the results suggest that the HRVs analysed, at least in the region under study, belong to one of two genetic groups, termed GG1 or GG2 (Table 1). These share around 60% amino acid identity within the region under study, while members of the larger group, GG1, share at least 70% identity and the minority group members share at least 84% identity. All members of the groups defined here also exhibit a number of specific characteristics, such as a three amino acid deletion in GG2 relative to GG1 and a number of amino acid motifs conserved within one group but absent in the other. In cases where the complete sequences of the capsid region are known (HRV-1A, -1B, -2, -9, -14, -15, -16, -85 and -89), comparisons throughout the whole structural protein region give results consistent with those derived using the smaller region studied in detail here (data not shown). In particular, the distinctive nature of HRV-14, the only completely sequenced GG2 member, is evident throughout the genome (Stanway *et al.*, 1984; Skern *et al.*, 1985). Thus, although only a relatively limited portion of the capsid has been analysed, it is likely that the genetic groupings derived are valid for the 21 HRVs studied. This is emphasized by sequence analysis of part of VP1 from HRV-3, which again shows a close relationship with another GG2 serotype, HRV-14 (data not shown). It is, however, possible that these apparent groups would not be sustained by the analysis of additional serotypes, since this may reveal a continuum of molecular identities. Recombination, which is known to occur in picornaviruses, could also play a role in HRV evolution and further complicate attempts to define genetic groups.

The results of the sequence comparisons are included in Table 1, which also gives a summary of the previously reported properties of the HRVs studied. These include receptor grouping (major or minor receptor group), classification in terms of sensitivity towards antivirus agents (groups A or B) and previously observed antigenic relationships (Uncapher *et al.*, 1991; Andries *et al.*, 1990; Cooney *et al.*, 1982). It can be seen that there is a good correlation between the putative genetic grouping proposed here and grouping based on drug sensitivity, since all three members of drug group A fall into GG2, while all group B serotypes are included in GG1. These antivirus agents bind within a hydrophobic pocket located below the canyon floor and interfere with uncoating and/or receptor binding (Andries *et al.*, 1990; Smith *et al.*, 1986; Pevear *et al.*, 1989; Kim *et al.*, 1993). In the native structure of most studied picornaviruses, but not HRV-14, the pocket contains a fatty acid-like molecule (the 'pocket factor'), possibly important in uncoating or assembly (Filman *et al.*, 1989; Kim *et al.*, 1993; Oliveira *et al.*, 1993). Binding of the antivirus drugs to the normally empty pocket in HRV-14 causes a major conformational shift which alters the shape of the canyon floor, hence explaining the effect on receptor binding seen in this serotype (Pevear *et al.*, 1989). In HRV-1A, drug-binding seems to merely replace the pocket factor and causes a less pronounced conformational change, which prevents uncoating but not interaction with the receptor (Kim *et al.*, 1989, 1993; Oliveira *et al.*, 1993). Since none of the residues making up the pocket was analysed in the present work (most are in VP1), it is not possible to shed further light on the precise molecular basis of the definition of the drug groups. However, it is possible that the drug-binding studies, together with the genetic analysis described here, reveal a fundamental difference in the biological properties of HRVs and divide these into two groups.

In conclusion, the results presented here demonstrate the usefulness of PCR in generating data from serotypically distinct viruses. The information obtained has given insights into the molecular basis of some of the biological properties of HRVs and has suggested that those analysed here belong to one of two genetic groups, which may reflect differences in morphology and biology. The techniques now available make feasible the eventual analysis of all the HRVs in molecular terms, at least in specific regions of the genome.

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