Lack of a Close Relationship Between Three Strains of Human Rhinoviruses as Determined by Their RNA Sequences¹

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The possible genomic homologies between three serotypes of human rhinoviruses (HRV 1A, HRV 2, and HRV 14) were investigated. First we confirmed that these viruses were unrelated by the criterion of the absence of common antigenic determinants on the surfaces of the native virions, as detected by cross-neutralization of complement fixation. RNA-RNA hybridization was then examined with purified, highly radioactive, double-stranded, replicative-form RNA and excess single-stranded virion RNA. Single-stranded RNA showed 100% homology with the minus strand from the replicative-form RNA of the same type of virus. HRV 1A, HRV 2, and HRV 14 showed low intertypic homologies; these were not significantly greater than those found between the rhinoviruses and poliovirus, which were used as a negative control. The immunological relationship and the RNA homology between HRV 1A and HRV 1B were also examined by the above techniques. It was confirmed that HRV 1A and HRV 1B share some surface determinants and it was also found that HRV 1B RNA shares 70% homology with HRV 1A RNA.

Rhinoviruses have been categorized as a subgroup of the picornaviruses because of their acid lability and their relatively high buoyant density in CsCl (for reviews see 9, 19, 20). Most of the ever increasing numbers of recognized human rhinovirus (HRV) types (5, 6) are, by definition, unrelated to the others by neutralization with antisera. In at least one case, however, there is a common complement fixing antigen (7). There is, however, little information upon which to build any conclusion about the degree of genetic relatedness among the human rhinoviruses.

This investigation is a part of an ongoing effort to make a detailed molecular comparison of a few selected strains of human rhinoviruses (10, 12, 14, 15). Native virions of HRV 1A, HRV 2, and HRV 14 were permitted to react with high titer antisera which were produced against the native virions of these types, and the results confirm the absence of intertypic native antigenic determinants by both the criteria of cross neutralization and by complement fixation. We have then examined the degree of RNA:RNA

homology between these selected viruses. In both the immunochemical and hybridization studies, HRV 1B served as a positive control since it is already known to be related to HRV 1A. Polio 2 served as a negative control. A specific serological relatedness between denatured virions of HRV 1A and HRV 2 has been discovered and we will discuss this in the following article (13).

MATERIALS AND METHODS

Virus, virus preparations, and purification. The sources and propagation of HRV 1A (strain 2060), HRV 2 (strain HGP), HRV 14 (strain 1059), and polio type 2 (strain P712-Ch2ab) have already been described (10, 12). HRV 1B (strain B-632) was obtained from the Reference Reagent Service of the National Institute of Allergy and Infectious Diseases (NIAID) (Bethesda, Md.) and was plaque-purified and propagated by the same procedures. The identity of each strain of virus was confirmed by neutralization tests with standard reference serum obtained from the NIAID.

All highly purified virion preparations were labeled with ³H-uridine, ³H-amino acids, ¹⁴C-amino acids, or ³²P-phosphate to facilitate the process of purification (10, 12). The number of virion particles per milliliter in the dialyzed pools was calculated by the method

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already described (10).

Antiserum. Antisera against highly purified virions were prepared in young female rabbits. After CsCl-gradient centrifugation, virions were diluted with nine volumes of cold water and samples of 1 ml were either used directly or frozen at -70°C . In the first injections, 1 ml of antigen was mixed with 0.5 ml of Complete Freund's Adjuvant (Difco) and portions of the mixture were injected into all four foot pads. At 2- to 4-week intervals, additional samples of antigen were thawed and given in the ear vein. Typically, 0.2 to 0.4 absorbance units (260 nm) of virions were used per injection. The rabbits were exsanguinated 1 week after the 4th or 5th injection.

Neutralization. An appropriate amount of virus was diluted 100-fold into antiserum in medium containing 5% heat inactivated fetal calf serum. This mixture was kept for 1 h at room temperature and then diluted 10- and 100-fold. These dilutions were used for plaque assay according to the method already described (10). The neutralization titers of the antisera are the dilutions producing a 90% reduction in plaques.

Complement fixation tests. The method was essentially that of Chan et al. (4). This employed sheep erythrocytes, rabbit anti-sheep erythrocyte hemolysin, and guinea pig complement (Microbiological Associates, Bethesda, Md.). Complement was titrated daily in serial twofold dilution steps. The highest dilution which caused 100% lysis of sensitized blood (an equal mixture of 0.5% sheep-red-blood cells and hemolysin) was used in each test.

Complement fixation titrations were made in block-checkerboard patterns. Purified viruses and antisera were diluted serially with Veronal buffered saline in Kahn tubes. Samples of 0.025 ml each of virus, antiserum, and complement were deposited in microtiter plates (Microbiological Associates, Bethesda, Md.), agitated, sealed, and kept at 4°C overnight. The next morning, 0.05 ml of sensitized blood was added to each well and this was incubated at 37°C for 1 h. The plates were centrifuged at $200 \times g$ for 5 min in an I.E.C. centrifuge with the aid of special adapters (Microbiological Associates, Bethesda, Md.). The patterns obtained resembled those already described by others for the complement fixation by crude poliovirus (11, 17). The +4 (0-10% hemolysis) end point was chosen in each case (17).

Virion RNA. Nonradioactive virions were partially purified through the sucrose-gradient centrifugation step, but omitting CsCl-gradient centrifugation, as employed in the purification of radioactive virus (see above). In the cases of HRV 1A or HRV 1B, the sucrose gradients contained 1 M NaCl and 0.02 M pH 8.1 Tris-chloride buffer. The virions were visualized in the sucrose gradients by light scattering and the appropriate fractions were diluted fivefold in 0.02 M pH 7.5 Tris-chloride buffer and sedimented in the ultracentrifuge at $175,000 \times g$ for 1.5 h at 4°C . At this stage, the pelleted virions were impure but the major fraction of the RNA is probably within virions.

The pelleted virus from 20 or more roller bottles was frozen in 1.5 ml 0.2% sodium dodecyl sulfate (SDS) 0.02 M pH 7.5 Tris-chloride, 0.002 M EDTA,

pH 7.5. The pellet was thawed and dispersed with the aid of ultrasonic treatment (15 s with the Biosonic III ultrasonic generator, Bronwill Scientific, Rochester, N.Y.). RNA was extracted for 90 s with phenol at 45°C ; the phenol phase was reextracted with the buffer used above and the combined aqueous phases were reextracted with phenol. The RNA was precipitated from the final aqueous phase by addition of 0.04 volumes of 2 M NaCl and three volumes of ethanol (overnight, -20°C). The RNA was pelleted in a low-speed centrifuge and was redissolved in 1.0 ml 0.2 M NaCl containing 0.01 M pH 7.5 Tris-chloride buffer. It was then reprecipitated with ethanol at -20°C . The pellet was washed with a cold 7:3 mixture of ethanol and buffer saline and then dissolved in $2.25 \times \text{SSC}$ (SSC, 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). It was assumed that the RNA had an $E_{1\%}^{257}$ of 250. Typical preparations contained 200 μg of RNA per ml, and the ratio of adsorption at 257 nm to that at 230 nm was 2.3.

Preparation of labeled double-stranded RNA. Monolayer cultures of HeLa cells were infected at 34.5°C with rhinoviruses at a multiplicity of 50 PFU/cell in the presence of 5 μg of actinomycin D per ml. After 4 h, 50 μCi of ^3H -uridine per ml was added and the incubation was continued for 12 h. The cells were then pelleted from the medium by low-speed centrifugation and were stored frozen at -70°C . The frozen cells were thawed and suspended in buffer containing 1% SDS and 0.01 M sodium acetate, pH 5, at a density of 2×10^7 cells per ml. The suspension was extracted three times with equal volumes of phenol at 60°C for 6 min and the RNA in the aqueous phase was precipitated twice with ethanol. Single-stranded RNA was differentially precipitated with 1 M NaCl according to the method of Bishop and Koch (1). The supernatant fluid containing soluble RNA and double-stranded RNA (ds-RNA) was precipitated with alcohol and redissolved in buffer containing 0.1 M NaCl, 0.002 M sodium phosphate, 0.001 M EDTA, and 0.5% butanol, pH 7.2. RNA from approximately 2×10^8 cells in 2 ml of buffer was applied to and separated on a column of sepharose 2B (2.7 by 35 cm) at room temperature with the above buffer at a flow rate of 15 ml per h according to the method of Öberg and Philipson (16). The effluent was collected and monitored for radioactivity and optical absorbance at 260 nm.

Hybridization of RNA. Hybridization tests were performed according to a modification of the method of Young et al. (23). Ten μliters of ds-RNA containing less than 10 ng and over 1,000 counts/minute was mixed with 90 μliters of dimethyl sulfoxide (DMSO) and 5 μliters of yeast t-RNA (1 mg/ml). The mixture was denatured at 67°C for 20 min and was then quickly cooled in an ice bath. For annealing, 1.2 ml of $2.25 \times \text{SSC}$ and 10 μliters of unlabeled single-stranded virion RNA were then added. The mixture was heated again to 67°C for 1 h and then slowly cooled to room temperature. RNase A and RNase T1 (Worthington Biochemical Corp., Freehold, N.J.) were added to the final concentrations of 40 $\mu\text{g}/\text{ml}$ and 60 units/ml and the mixture was incubated at 37°C for 30 min. It was assumed that trichloroacetic acid-insoluble counts represented hybridized RNA.

RESULTS

Lack of common antigenic determinants on the native virions of three types of rhinoviruses. The anti-virion sera produced as described in the Materials and Methods section have higher potency than the usual typing sera and were able to neutralize 90% of homolytic virus at a dilution of up to 33,000 (HRV 1A), 43,000 (HRV 2), or 14,500 (HRV 14). These sera showed no heterotypic neutralization within this group at concentrations 1,000-fold greater than those at the homologous end point. HRV 1B, however, was 90% neutralized by a 480-fold dilution of anti-HRV 1A serum.

Further attempts were made to detect heterotypic antigenic determinants on the surface of native virions by complement fixation. The +4 end point (17) was chosen for each virus-serum pair, and for purposes of representation, the dilution of the antiserum at this point was multiplied by the dilution of the virus. This product was then multiplied by 10^{10} and then normalized by dividing it by the number of particles per milliliter in the undiluted virus sample. The titers for all virus-antisera pairs have been represented in this manner and are given in Table 1 which gives the results of both heterotypic and homotypic tests. Except for the anti-HRV 1A and HRV 1B pair, the native virions fix complement only with homotypic sera.

Characterization of labeled double-stranded RNA. A typical profile of the separation of ^3H -labeled cell-associated rhinovirus RNA on a sepharose 2B column is shown in Fig. 1. The peak at 48 ml, which is the void volume, contained ds-RNA. It usually contained less than $1.0\text{ }\mu\text{g}$ of RNA per ml with 10^5 to 5×10^5 counts per min per μg . This material eluted in the void volume again when rechromatographed on sepharose 2B and it also migrated during gel-electrophoresis as a single peak which coincided with the replicative form of rhinovirus RNA, as previously reported by Yin and Knight (22).

The double-stranded RNA was 95 to 100%

TABLE 1. Complement fixation titers^a of purified rhinovirions (average values)

Serum	Virus				
	1A	1B	2	14	Polio
1A	320	67	<4	<4	<2
2	<2	<2	800	<4	<2
14	<2	<2	<2	2,300	<2

^a Dilution of antiserum $\times$ dilution of virus at end point $\times 10^{10}$ divided by virus particles per ml.

FIG. 1. Isolation of double-stranded RNA. HRV 2 RNA was labeled with ^3H -uridine, extracted from infected cells, and fractionated with 1 M NaCl. RNA from 2×10^8 cells was applied to a sepharose 2B column. The radioactivity of the effluent was determined as counts per minute per 0.1 ml, and optical absorbance was determined at 260 nm.

resistant to digestion by pancreatic RNase A and RNase T1 under the conditions described in Materials and Methods, but was about 10% resistant after first being denatured in DMSO and then being annealed in the presence of added yeast t-RNA, as also described. In the absence of added yeast RNA, a larger and variable fraction became RNase resistant. The fact that the radioactive RNA became RNase sensitive after denaturation-annealing indicates that its concentration during annealing is too low for most of the separated strands to find each other. The reason for a 10% residual resistance is not entirely clear but this residual portion was shown to be RNA since it was completely digested by RNase if diluted in water instead of $2.25 \times \text{SSC}$. DNase also did not digest the resistant fraction and it was completely digested by 0.5 M NaOH. Without annealing (i.e., with rapid cooling), the RNase-resistant counts were found to be 7 to 8%. The replicative form of poliovirus RNA has also been found to contain such an RNase-resistant fraction after denaturation and annealing under similar conditions (personal communication, N. A. Young).

Hybridization of homologous RNA. Labeled HRV 2 ds-RNA was hybridized with increasing amounts of single-stranded virion

RNA. The percentage of hybridization was calculated by (i) subtracting the RNase-resistant counts of the denatured ds-RNA alone as a background from all other samples, and (ii) assuming that the RNase-resistant counts of the undenatured ds-RNA were equal to 100% annealing. The hybridization increased with increasing quantities of added virion RNA (Fig. 2). When more than 2.5 μ g of single-stranded RNA was added, the percentage of hybridization approached the theoretical value of 50% (i.e., all labeled minus strands were hybridized). In the case of HRV 1A and HRV 14, the dependency of hybridization upon added homologous virion RNA was precisely the same as shown for HRV 2 in Fig. 2. The mean deviation for replicate samples was also found to be $\pm 2\%$.

Lack of hybridization of heterologous RNA. Attempts were made to anneal heterologous virion RNA with ds-RNA. In these experiments, 4 μ g of virion RNA was usually used per sample, but two to three times this quantity was also employed in a few cases. Homologous single-stranded RNA was also used as a positive control and single-stranded poliovirus RNA as a negative control. The results are shown in Table 2. Rhinovirus 1B, which is known to be immunologically related to HRV 1A, was also included in order to test the sensitivity of the technique.

The results of Table 2 show that single-stranded RNAs are able to hybridize with approximately 50% of the homologous ds-RNA. Except in the case of HRV 1A and HRV 1B, heterologous HRV single-stranded RNAs show no more than about 2% hybridization of HRV

TABLE 2. Hybridization of HRV RNAs

Double-stranded labeled RNA	Single-stranded unlabeled RNA	Hybridization (%)
1A	1A (3) ^a	52.3
	1B (1)	36.3
	2 (2)	1.3
	14 (2)	0.9
	polio (3)	0.8
1B	1B (1)	51.0
	1A (4)	34.8
	polio (3)	1.0
2	2 (2)	46.0
	14 (2)	0.0
	1A (2)	1.5
	polio (3)	0.1
14	14 (2)	49.0
	1A (1)	0.0
	2 (2)	2.0
	polio (3)	0.0

^a Parentheses indicate number of experiments performed to obtain average value of percentage of hybridization.

1A, HRV 2, or HRV 14 ds-RNA; this is probably not significantly greater than the hybridization produced by poliovirus RNA. In the case of HRV 1A and HRV 1B, either single-stranded RNA can hybridize about 35% of the complementary ds-RNA.

DISCUSSION

The three serotypes of human rhinoviruses chosen for this study represent two members of one cell-receptor family (HRV 2 and HRV 1A) and one member of another family (HRV 14) (12). They also differ, each from the other, in buoyant density in CsCl and in the size of their constituent polypeptides but have similar sedimentation coefficients (10) (Korant and Longberg-Holm, unpublished data). Although these viruses do differ from each other by other than serological criteria, it is not known whether they may be considered as representative examples for the large HRV family.

It has already been established that the strains of HRV designated as types 1A, 2, and 14 differ in their neutralizing determinants. Typing sera are generally of relatively low potency and it was of interest to reexamine the question of cross-neutralization with the relatively highly potent serum which was produced against highly purified virions. This serum was able to neutralize 90% of homotypic virus infectivity at a dilution of greater than 10^4 . Our results confirmed that the rhinovirus types investi-

FIG. 2. Dependence of the extent of hybridization upon the concentration of unlabeled single-stranded RNA. Double-stranded RNA (1,120 counts per min per sample) was hybridized with increasing amounts of virion RNA. The percentage of hybridization was calculated as described in Results.

gated did not share neutralizing determinants. However, anti-HRV 1A serum showed about 1 to 2% of its homotypic reactivity against HRV 1B and this confirmed that HRV 1A and HRV 1B may be considered as subtypes. The possible existence of non-neutralizing determinants was also examined by complement fixation tests and the data of Table 1 show that there was no significant reaction between heterotypic pairs of virions and sera. There was, however, a significant reaction between HRV 1A serum and HRV 1B virions, as expected on the basis of the neutralization data. This reaction is also relatively more prominent (67/320) than the corresponding cross neutralization (480/33,000).

It was hoped that RNA:RNA hybridization would detect a significant homology between at least two of the three serotypes examined. Other groups of viruses have shown such relationships. For example, the RNAs of the three serotypes of poliovirus share about 25 to 33% sequence homology although the virions do not share appreciable neutralizing determinants (23). Three strains of foot-and-mouth disease virus also share RNA homology (8) as well as certain neutralizing antigens (2; G. E. Cottral, 1972. *Bull. Off. Int. Epiz.*, in press). Six strains of influenza A share 70% RNA homology and influenza A and B share 20% homology (18).

In the case of hybridization between homologous HRV RNAs, for example HRV 2 ds-RNA and HRV 2 virion RNA as shown in Fig. 2, half the label of the ds-RNA could be annealed to nonradioactive virus RNA and this was presumably the minus strands. This simply confirms that the virions contained only plus strands, and hence 50% hybridization indicates 100% homology. The sensitivity of our test for RNA homology was verified by the hybridization found with RNAs of HRV 1A and HRV 1B. There is about 35% homology using either pair of single-stranded and ds-RNAs and this indicates that about 70% of the genomes are composed of homologous sequences.

The results of hybridization tests made with various pairs of ds- and single-stranded RNAs are shown in Table 2. It can be seen that heterologous pairs do not produce more than 2% hybridization, a figure which is probably not significantly greater than the 0 to 1% observed with poliovirus plus strand RNA. Two percent hybridization would indicate that 4% of the genome is homologous, under the conditions used in the test. This must then be considered an upper limit for the specific intertypic hybridization of HRV 2 and 1A. Since the rhinovirus genome has a molecular weight of about 2.5×10^6 (3, 14), it can code for about 2.5×10^5

molecular weight units of protein. Four percent of this represents 10^4 molecular weight units or roughly one polypeptide at the most, if all homology resides in a single portion of the genome.

It is also possible that hybridization is not sensitive enough to detect genetic homology between certain related polypeptides because of misfits produced by degeneracy in the genetic code (21) and because of genetic drift. In this regard, it has recently been found that the messenger RNA of human hemoglobin cannot hybridize effectively with DNA complementary to rabbit hemoglobin message, despite the fact that human and rabbit hemoglobin have very similar structures (S. Packman, H. Aviv, J. Ross, D. Swan, and P. Leder; private communication).

The possibility still exists that there could be common polypeptide sequences which cannot be detected at the surface of HRV virions by cross-neutralization or by complement fixation tests. Some common antigenic determinants have indeed been discovered between denatured HRV 1A and HRV 2 virions, and this is the subject of the following paper (13).

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