Polioviruses, Coxsackieviruses, and Echoviruses: Comparison of the Genomes by RNA Hybridization

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Hybridization of single-stranded RNA from virions of human enteroviruses with denatured double-stranded RNA from infected cells indicates that a minimum of about 5% of the genome is shared by these viruses. Polynucleotide sequence relationships, furthermore, are consistent with the biologic classification into polioviruses, coxsackieviruses groups A and B, and echoviruses. In general, about 30 to 50% of the nucleotide sequences are shared by different serotypes of virus within each of these major groups, whereas among different groups less than 20% homology is observed. Coxsackievirus B4 appears to be more closely related to echoviruses than to group A coxsackieviruses, whereas polioviruses are only distantly related to any of the other agents.

More than 65 serotypes of human enteroviruses are currently recognized (7). These acid-stable picornaviruses have virion diameters of 18 to 25 nm, buoyant densities in CsCl of 1.32 to 1.35 g/cm³ (5), and are protected by divalent cations against thermal inactivation (11).

Human enteroviruses have been further subclassified into three taxa—polioviruses, coxsackieviruses, and echoviruses—based on biologic and antigenic relationships. Polioviruses are pathogenic for the central nervous system of primates and primate cell cultures, whereas coxsackieviruses were initially distinguished from polioviruses by producing paralysis in suckling mice but no cytopathic effect in cultured cells (4). Moreover, group B coxsackieviruses produce different clinical syndromes and a different type of histopathologic lesion in mice than group A coxsackieviruses and frequently replicate in cell cultures. Echoviruses are characterized by the production of cytopathic effects in cultured cells but failure to cause lesions in laboratory animals. Within each of these three groups, distinct serotypes are recognized by neutralization tests. However, the existence of common antigens is indicated by minor cross-reactivity, especially by complement fixation tests, among individual serotypes of coxsackieviruses as well as between coxsackieviruses and echoviruses (9).

Problems have arisen from this biological classification because newly discovered strains antigenically like coxsackieviruses are cytopathic for cell cultures without paralyzing mice, whereas other strains are pathogenic for mice but are antigenically related to echoviruses. These difficulties have led to a taxonomic scheme in which newly recognized enteroviruses are given serial numbers beginning with enterovirus 68 rather than being classified as coxsackieviruses or echoviruses (7).

An independent means of categorizing enteroviruses, especially one capable of establishing quantitative as well as qualitative relationships, could be expected to shed light on the basic validity of the biologic classification. It was previously demonstrated by RNA-RNA hybridization that about 26 to 38% of the genome is common to the three antigenic types of polioviruses (14). These studies are now extended to representative strains of coxsackieviruses and echoviruses with a view to elucidating their interrelationships.

MATERIALS AND METHODS

Viruses and immune sera. The virus strains and their sources are listed in Table 1. The complete pedigree of most of these extensively passaged strains is unknown. Immune sera were obtained from the Research Resources Branch, National Institute of Allergy and Infectious Diseases.

TABLE 1. *Virus strains and sources*

Virus	Sero-type	Strain	Source
Poliovirus	1	Brunhilde	RRB, NIAID* Bureau of Biologics, Food and Drug Administration RRB, NIAID
	2	MEF ₁	
	3	Leon	
Coxsackie-virus	A7	AB-IV (USSR)	RRB, NIAID R. J. Huebner RRB, NIAID
	A10	1816	
	B4	JVB	
Echovirus	1	Farouk	RRB, NIAID RRB, NIAID
	12	Travis	

* Research Resources Branch, National Institute of Allergy and Infectious Diseases.

Propagation of viruses. Preliminary experiments indicated that all strains studied except coxsackievirus A10 produced infectivity titers of at least $10^{7.0}$ to $10^{7.5}$ PFU per ml in cultures of Vero cells. Accordingly, these cells were cultivated in roller bottles, approximate surface area 1,410 cm² (Bellco Glass Inc.), in medium 199 with 5% fetal calf serum. Confluent monolayers were washed with Hanks balanced salt solution and then infected at a multiplicity of 10 to 30 PFU/cell. After adsorption for 30 min at 37 C, the inoculum was replaced by 50 ml of medium 199 lacking serum and incubated until yields of infectious virus were maximal as determined by preliminary experiments, usually at 5 to 7 h after infection.

For the production of coxsackievirus A10, 60 litters of 2-day-old white mice (NIH all purpose) were inoculated intraperitoneally with $10^{4.5}$ mean lethal doses, and the carcasses were harvested when the animals were moribund by decapitating and eviscerating them.

Infectivity assays and neutralization tests. These assays were performed as previously described (14) except for the addition of 25 mmol of MgCl₂ to the agar overlay (12).

Purification of viruses. The method of purifying poliovirus after elution of virus from diethylaminoethyl (DEAE) cellulose (Whatman DE 23) columns has been described (14). This method was also used to purify coxsackievirus A7 and echovirus 1. A small amount of ³²P-labeled virus was first prepared by infecting 2×10^7 to 6×10^7 cells with virus in the presence of actinomycin D, 1 µg/ml, and 50 µCi of ³²P-orthophosphate per ml from 0 to 6 h postinfection. After freeze-thawing the cells three times and removing cellular debris by centrifugation at $2,000 \times g$ for 15 min, virus was pelleted from the supernatant fluid by centrifugation in a Beckman no. 30 rotor, $78,000 \times g$ for 2 h at 4 C. The pellet was suspended in 0.02 M K₂H-KH₂PO₄ buffer (PB) and applied to a DEAE-cellulose column (0.9 by 18 cm) after which stepwise elution was performed with increasing concentrations of NaCl in 0.02 M PB. The effluent was assayed for ³²P acid-precipitable radioactivity, and plaque assays were performed on each radioactive peak to determine the position of virus eluting from the column (Fig. 1).

A peak containing 27% of the coxsackievirus a7 applied to the column was eluted in 0.25 M NaCl, whereas only 9% of echovirus 1 was recovered in the peak eluting in 0.02 M PB. After the conditions of elution were determined, large-scale purification of each virus was achieved by applying 10^{11} to 10^{12} PFU of unlabeled virus to columns (2.5 by 40 cm). Virus eluting from the column was then repelleted in a no. 30 rotor as described above, the pellet was suspended in 0.02 M PB and adjusted to a density of 1.34 g/cm³ with CsCl, and ultracentrifugation was carried out at $88,000 \times g$ for 14 h at 4 C in an SW65 rotor. After harvesting the opalescent band of virus by puncturing the bottom of the tube and collecting fractions, the virus was dialyzed twice against 2 liters of Tris-phosphate buffer (0.01 M Tris, pH 7.4; 0.02 M K₂H-KH₂PO₄; 5×10^{-4} M EDTA, pH 8.0) overnight to remove CsCl.

For the purification of coxsackieviruses A10, B4, and echovirus 12, DEAE-cellulose chromatography was abandoned because of poor recovery (less than 10%) of infectious virus from the columns. Instead, these viruses were concentrated from cell lysates or from mouse carcasses homogenized in 8.6% sucrose in Tris-phosphate buffer by precipitation with ammonium sulfate as described by Mattern (6), after which the virus was centrifuged onto a cushion of CsCl (density = 1.40 g/cm³) in an SW25.1 rotor, $60,000 \times g$, for 2.5 h, at 4 C. The opalescent virus band was collected with a Pasteur pipette and dialyzed free of CsCl as described above. The virus was then layered on a 15 to 30% sucrose gradient in sodium dodecyl sulfate (SDS) buffer (0.5% SDS; 0.1 M NaCl; 0.01 M Tris, pH 7.2; 10^{-3} M EDTA) in an SW27 rotor and centrifuged at $81,000 \times g$ for 2.5 h at 17 C. Virus was collected by puncturing the bottom of the tube and monitoring optical density at 260 nm in each fraction. Virus-containing fractions were pooled and pelleted in a type 65 rotor, $150,000 \times g$ for 2 h at 20 C. After resuspending the pellets in 0.02 M PB, the solution containing virus was adjusted to a density of 1.34 g/cm³ and centrifuged to equilibrium followed by dialysis as described above.

Preparation of single-stranded viral RNA. The extraction of RNA from purified virions by phenol at room temperature has been previously described (14).

Preparation of radiolabeled double-stranded RNA. Approximately 1.4×10^8 Vero cells were preincubated for 1 h with phosphate-free Eagle medium containing actinomycin D, 1 µg/ml. After the fluid was decanted, virus which had previously been dialyzed against phosphate-free medium was added at a multiplicity of 10 to 50 PFU/cell and allowed to adsorb for 30 min at 37 C. The inoculum was then replaced with phosphate-free medium plus actinomycin D, 1 µg/ml. One hour after infection, ³²P-orthophosphate was added to a concentration of 100 µCi/ml and the cells were incubated at 37 C for an additional 2 to 4 h. The medium was then decanted, and the cells were harvested. The methods for extraction and purification of viral double-stranded RNA (dsRNA) from infected cells by treatment with hot phenol, differential salt preparation of single-stranded RNA (ssRNA), and chromatography on cellulose columns have been previously described

Fig. 1. Chromatography of coxsackievirus A7 and echovirus 1 on DEAE-cellulose columns. One milliliter of virus labeled with ^{32}P -orthophosphate and concentrated by ultracentrifugation was applied to a DEAE-cellulose column. Eluent buffer, consisting of 0.02 M $\text{K}_2\text{H-KH}_2\text{PO}_4$ (PB) and graded increments in NaCl concentration, was added in 40- to 50-ml amounts stepwise and 2-ml fractions were collected. The position of virus eluting from the column was determined by measuring acid-precipitable radioactivity (O) in each fraction and then measuring infectivity (●) of each radioactive peak plus selected other fractions.

(14). Purified dsRNA preparations typically had specific activities of 2×10^5 to 10^6 counts per min per μg and were 90 to 97% resistant to hydrolysis by pancreatic ribonuclease, 50 $\mu\text{g}/\text{ml}$, in $2 \times \text{SSC}$ (standard saline citrate: 0.15 M NaCl, 0.015 M sodium citrate).

Annealing reactions. Based on the results of a previous study (14), annealing reactions were carried out by "melting" viral dsRNA in 10 volumes of dimethylsulfoxide (DMSO, spectro grade, Fisher Chemical Co.) for 30 min at 37 C and then reacting less than 0.01 μg of the denatured RNA with sufficient ssRNA from the homologous virus to render approxi-

mately 45 to 50% of the input radioactivity resistant to hydrolysis by pancreatic and T_1 ribonucleases (Worthington Biochemical Corp.). In other reaction tubes, heterologous viral ssRNA, i.e., prepared from a different virus strain, or polyadenylic acid (Schwarz-Mann) was substituted for homologous viral ssRNA at the same concentration. Annealing was carried out at 67 C for 1 h in $2.25 \times \text{SSC}$ in a reaction volume of 1.25 ml. Digestion by pancreatic and T_1 RNase, 40 $\mu\text{g}/\text{ml}$ and 10 U/ml, respectively, was in $2 \times \text{SSC}$ at 37 C for 30 min. RNase-resistant material was precipitated with 5% trichloroacetic acid and collected on nitrocel-

lulose membrane filters. The dried filters were placed in toluene-Liquifluor (New England Nuclear Corp.) and counted in a liquid scintillation spectrometer.

RESULTS

Denaturation in DMSO and determination of optimal concentrations of reactants. For each preparation of radiolabeled dsRNA, the extent of denaturation in DMSO was assessed by measuring conversion to RNase sensitivity under nonannealing conditions. Generally, 6 to 10% of the RNA remained resistant to RNase after DMSO treatment. Since this undenatured dsRNA is presumably unable to participate in annealing reactions, it was treated as "background," and an equal number of counts was subtracted from the result of each annealing reaction after including this control with each set of reactions. In other experiments, each DMSO-treated dsRNA preparation was allowed to react at 67°C for 2 h; detectable self-annealing (RNase-resistant radioactivity above "background" in the absence of added ssRNA) was not observed. The amount of homologous ssRNA required to achieve complete reannealing of radiolabeled dsRNA was then determined by adding increasing quantities of ssRNA to a constant amount of dsRNA. In nearly all instances, this value was $\leq 2 \mu\text{g}$ of ssRNA. For a few preparations with low specific activity (2×10^5 counts per min per μg), 5 to 8 μg of ssRNA was required to achieve complete homologous reannealing while still having enough radioactive counts in the reaction to determine the fraction annealed for heterologous viral RNA. A representative saturation curve (Fig. 2) is shown for the reaction of radiolabeled coxsackievirus A7 dsRNA with homologous ssRNA. Saturation was achieved with $\geq 2 \mu\text{g}$ of coxsackievirus A7 ssRNA. When 2 μg of coxsackievirus A10 ssRNA was added to 0.01 μg of coxsackievirus A7 dsRNA, the decimal fraction of labeled RNA made RNase-resistant was 0.34 of the homologous value. With nonsaturating quantities of added ssRNA, the ratio of heterologous: homologous counts remained essentially constant.

Cross-hybridization experiments. Each radiolabeled denatured dsRNA was reacted with saturating amounts of homologous or heterologous ssRNA, and the results are expressed as a decimal fraction of the homologous value (Table 2). For poliovirus 1 ^{32}P -dsRNA, this fraction was 0.35 with poliovirus 2 ssRNA and 0.52 with poliovirus 3 ssRNA. For coxsackieviruses and echoviruses, lower values (0.03 to 0.07) were observed, whereas with ssRNA from MS 2 bacteriophage and uninfected Vero cells

there was no demonstrable homology (less than 0.01). When 2 μg each of coxsackieviruses A7 and A10 and echoviruses 1 and 12 ssRNA were simultaneously reacted with poliovirus 1 dsRNA, 406 counts/min (0.06) were hybridized; with 2 μg each of poliovirus 2, coxsackievirus A10, and echoviruses 1 and 12 ssRNA, 1,847 counts/min (0.32) were hybridized. Thus, the addition of multiple heterologous species of ssRNA did not result in any more hybridization with poliovirus dsRNA than did the most closely related individual ssRNA when reacted singly.

With coxsackievirus B4 dsRNA, the fraction hybridized with ssRNA from polioviruses was 0.06 to 0.08, for group A coxsackieviruses 0.10 to 0.14, and for echoviruses 0.16 to 0.33. For multiple RNA combinations (data not shown) of 2 μg each, the values were 0.17 for poliovirus 1 and echovirus 12, and 0.18 for coxsackievirus A7 and echovirus 12. The nucleotide sequences which these agents hold in common with coxsackievirus B4 therefore appear to be largely overlapping.

In Table 3, the above data are summarized along with the results of cross-hybridization experiments utilizing ^{32}P -radiolabeled dsRNA of coxsackievirus A7, echovirus 1, and echovirus 12. Relatedness values of 0.31 were obtained for coxsackieviruses A7 and A10, but only 0.11 to 0.15 between either of these agents and the RNA of a group B coxsackievirus or echoviruses, and still lower values, 0.04 to 0.05, with polioviruses. When coxsackievirus B4 dsRNA was used as the source of complementary minus strand, results of 0.10 to 0.14 were obtained with ssRNA of coxsackieviruses of group A, but 0.16 to 0.33 with echovirus RNA. Again, a more distant relationship was observed with poliovirus RNA, 0.06 to 0.08. Echoviruses 1 and 12 RNA appeared to share 0.36 to 0.50 of their sequences reciprocally, while values of 0.23 to 0.38 were obtained with coxsackievirus B4, 0.09 to 0.14 with group A coxsackieviruses, and 0.07 with polioviruses. Additive homologies were not demonstrated when multiple ssRNAs were reacted in combination with each dsRNA. In all instances, ssRNA from MS 2 bacteriophage and uninfected Vero cells resulted in no significant hybridization (<0.01).

Although, in general, less than 30% homology is present among different groups, an apparent exception is the relationship between coxsackievirus B4 and echoviruses, where 16 to 38% homologies were demonstrated reciprocally. Two possible explanations were considered: (i) the virus stocks were cross-contaminated and (ii) a closer relationship exists between these

Fig. 2. Annealing of coxsackievirus A10 and A7 ssRNA with coxsackievirus A7 dsRNA. Denatured, ^{32}P -radiolabeled coxsackievirus A7 dsRNA, $< 0.01 \mu\text{g}$ per tube, was annealed with increasing quantities of ssRNA from either the homologous (coxsackie A7, $\circ$) or the heterologous (coxsackie A10, Δ) virus. Reactions were carried out in $2.25 \times \text{SSC}$ at 67°C for 1 h. Acid-precipitable radioactivity was determined after treatment with ribonuclease. The ratios of counts hybridized for heterologous and homologous ssRNA (coxsackie A10:coxsackie A7) were then calculated at each concentration.

groups than others. To investigate the first possibility, serial dilutions of concentrated virus stocks of coxsackievirus B4 and echoviruses 1 and 12 were neutralized by the plaque-reduction method (14) in the presence of a constant amount (1:10 dilution) of each antiserum. In all instances, the log neutralization indices were > 3.75 , indicating that any hypothetical contaminant constituted less than 1/9,000 of the population of PFU. Five surviving plaques for each virus stock were cloned three times and then passaged one additional time in Vero cells. Neutralization titers of these clones, when tested with homologous antiserum, were identical to those of the uncloned virus population, indicating that even minor cross-contamination among coxsackievirus B4 and echoviruses 1 and 12 had not occurred. To examine the second possibility, that these viruses were indeed closely related, cross-neutralization tests were performed. No relationship was demonstrated (Table 4). It appears, therefore, that a closer relationship exists between the nucleotide sequences of coxsackievirus B4 and echoviruses than with group A coxsackieviruses, but that this relationship is not reflected by shared neutralizing antibodies.

To determine whether some of the nucleotide sequences apparently shared by enteroviruses might merely be due to polyadenylate present at the 3'-terminus of each virion RNA (13) rather than indicating true genetic relatedness, ^{32}P -labeled dsRNA from poliovirus 1 was an-

nealed with saturating quantities of either homologous ssRNA or polyadenylate (Table 5). The results show that polyadenylate alone accounts for a relatedness value of only 0.009.

DISCUSSION

Cross-hybridization of the RNA of human enteroviruses supports the essential qualitative validity of the biological classification into polioviruses, group A and B coxsackieviruses, and echoviruses. At least for the strains included in the present study and under the reaction conditions used, the generalization can be made that approximately 0.3 to 0.5 of the genome is shared by different serotypes within the same group, for example, coxsackieviruses of group A with each other and echoviruses with each other. With poliovirus 1, previous results (14) indicated 0.28 to 0.38 relatedness of the RNA of polioviruses 2 and 3, respectively, but in the present study highly reproducible values of 0.36 to 0.52 were obtained. The reasons for this discrepancy are unclear. It was also previously demonstrated that two different strains of poliovirus 1 shared 0.74 of their nucleotide sequences (14). Different strains of the same serotype of coxsackievirus or echovirus were not examined. When hybridization was performed among the RNAs of different groups (for example, coxsackieviruses A and B), relatedness values of less than 0.20 were observed, indicating that genetic relationships across groups are less

TABLE 2. Hybridization of ³²P-radiolabeled denatured dsRNA of poliovirus 1 or coxsackievirus B4 with other enterovirus RNAs^a

ssRNA	dsRNA			
	Polio 1		Coxsackie B4	
	Counts/min hybridized	Fraction	Counts/min hybridized	Fraction
Polio 1	6,370	1.00	286	0.06
Polio 2	2,281	0.35	295	0.06
Polio 3	3,308	0.52	361	0.08
Coxsackie A7	210	0.03	487	0.10
Coxsackie A10	312	0.05	665	0.14
Coxsackie B4	322	0.05	4,686	1.00
Echo 1	433	0.07	1,537	0.33
Echo 12	408	0.06	792	0.16
MS 2	22	0.00	-9	0.00
Vero cell	-8	0.00	21	0.00

^a ³²P-labeled poliovirus 1 dsRNA was denatured in 10 volumes of DMSO, and 0.1-ml samples (<0.01 μg) were reacted with 2 μg of homologous or heterologous ssRNA in a volume of 1.25 ml made 2.25 × SSC at 67°C for 2 h. Each sample was then treated with pancreatic ribonuclease, 40 μg/ml, in 2.25 × SSC at 37°C for 30 min. After correcting for radioactivity in dsRNA not denatured by DMSO (this value was actually 7.7% of input counts), 6,370 of 13,620 counts/min in the reaction of polio 1 ssRNA with polio 1 dsRNA were found to be RNase resistant, giving a value of 47% for homologous annealing. The reactions for heterologous ssRNA were then expressed as a decimal fraction of the homologous value. Similar calculations were made for coxsackievirus B4 dsRNA, where 4,686/9,759 counts/min or 48% homologous annealing was observed. ssRNA from MS 2 bacteriophage or from uninfected Vero cells was included as control.

than relationships within each group. Polioviruses appeared to be only distantly related (0.04 to 0.08) to all other human enteroviruses, yet these relationships appeared to be specific as judged by the absence of any hybridization with the RNA of MS 2 bacteriophage and uninfected Vero cells.

Furthermore, when polyadenylic acid was reacted with denatured poliovirus dsRNA, only 0.9% ± 0.4% hybridization occurred, presumably due to annealing with polyuridylylate present at the 5'-terminus of the complementary strand in poliovirus dsRNA. This result is in good agreement with the more precise data of Yogo and Wimmer (13) indicating the presence at the 3'-terminus of poliovirus RNA of polyadenylate with an average chain length of 89 residues, which corresponds to a molecular weight of 32,400, or 1.2% of the genome. Thus, most of the approximately 5% homologies observed among the most distantly related enteroviruses is not

TABLE 3. Summary of polynucleotide sequence relationships among RNAs of polioviruses, coxsackieviruses, and echoviruses^a

dsRNA	ssRNA									
	Polio 1	Polio 2	Polio 3	Coxsackie A7	Coxsackie A10	Coxsackie B4	Echo 1	Echo 12	MS 2	Vero
Polio 1	1.00	0.36 ± 0.03	0.52 ± 0.01	0.03 ± 0.01	0.05 ± 0.01	0.05 ± 0.02	0.07 ± 0.02	0.06 ± 0.01	0.00 ± 0.01	0.00 ± 0.01
Coxsackie A7	0.05 ± 0.01	0.04 ± 0.01	0.05 ± 0.02	1.00	0.31 ± 0.04	0.15 ± 0.01	0.13 ± 0.03	0.11 ± 0.02	0.00 ± 0.01	0.00 ± 0.01
Coxsackie B4	0.06 ± 0.03	0.06 ± 0.03	0.08 ± 0.01	0.10 ± 0.03	0.14 ± 0.02	1.00	0.33 ± 0.01	0.16 ± 0.01	0.00 ± 0.01	0.00 ± 0.01
Echo 1	0.06 ± 0.02	0.06 ± 0.02	0.05 ± 0.01	0.13 ± 0.04	0.14 ± 0.03	0.23 ± 0.01	1.00	0.36 ± 0.03	0.00 ± 0.01	0.00 ± 0.01
Echo 12	0.07 ± 0.02	0.07 ± 0.01	0.07 ± 0.04	0.09 ± 0.02	0.13 ± 0.01	0.38 ± 0.03	0.50 ± 0.03	1.00	0.00 ± 0.01	0.00 ± 0.01

^a Expressed as decimal fractions of the value for homologous annealing. Details of the reactions are given in Materials and Methods and in Table 2. Results are the mean of two to four experiments ± 2 standard errors.

TABLE 4. *Cross-neutralization tests with echoviruses 1 and 12 and coxsackievirus B4*

Virus	Reciprocal of serum dilution			
	Echo 1	Echo 12	Coxsackie B4	NHS ^a
Echo 1	20,480	20	20	20
Echo 12	40	20,480	40	20
Coxsackie B4	10	10	20,480	10

^a NHS, normal horse serum. Serial twofold dilutions of horse immune sera were reacted with 60 to 240 PFU of each virus for 1 h at room temperature and then plated on Vero cell monolayers. Reduction in plaque number of 80% was taken as neutralization end point.

TABLE 5. *Annealing of poliovirus 1 dsRNA with poliovirus 1 ssRNA or polyadenylate^a*

ssRNA added	Hybridized (counts/min)	Annealing relative to homologous (%)
Poliovirus 1 ssRNA	1,154 ± 9	100.0 ± 0.8
No added RNA	0 ± 5	0.0 ± 0.4
Polyadenylate	10 ± 5	0.9 ± 0.4

^a Denatured ³²P-labeled poliovirus 1 dsRNA, <0.01 µg and approximately 2,300 counts per min per reaction after subtracting background as described in Materials and Methods, was reacted for 1 h at 67°C in 2.25 × SSC either without added ssRNA or in the presence of 10 µg of either poliovirus 1 ssRNA or polyadenylate. Each sample was then treated with pancreatic ribonuclease, 40 µg/ml, and T₁ ribonuclease, 10 U/ml, for 30 min at 37°C. Results are expressed as mean acid-precipitable RNase-resistant radioactive counts from three experiments ± two standard deviations counting error. Approximately 50% of the input counts were rendered ribonuclease resistant by the addition of homologous ssRNA from poliovirus 1. This value is normalized to 100%, and the results for annealing in the absence of added ssRNA or in the presence of polyadenylate are expressed as a percentage of the homologous value.

accounted for by polyadenylate sequences in virion RNA.

One relationship uncovered by RNA hybridization not emphasized in the biological classification is that between a group B coxsackievirus and the echoviruses. Coxsackievirus B4 is more closely related to echoviruses (0.16 to 0.38 relatedness) than to group A coxsackieviruses (0.10 to 0.15 relatedness). Although no antigenic relationship between coxsackievirus B4 and echoviruses was found in the present study by neutralization tests with animal hyperimmune sera, nevertheless heterotypic complement-fixing antibodies between echoviruses and group B coxsackieviruses are not uncommonly observed in patients infected with these viruses (1, 3, 8),

suggesting that an antigenic relationship does exist. Furthermore, it is perhaps not surprising that coxsackievirus B4 is more closely related to echoviruses than to group A coxsackieviruses, since the former groups replicate well in primate cell cultures, whereas infant mice are the preferred host for group A coxsackieviruses. Examination of other group B coxsackieviruses by RNA-RNA hybridization will be necessary to confirm this relationship.

Not all of the nucleotide sequence homologies among the viruses of different groups were quantitatively reciprocal. For example, in hybridization reactions employing coxsackievirus B4 dsRNA, a closer relationship was observed to ssRNA from echovirus 1 (0.33) than echovirus 12 (0.16). Yet when coxsackievirus B4 ssRNA was hybridized with dsRNA from echoviruses 1 and 12, 0.23 and 0.38 homologies were observed, respectively. These results imply that the gene sequences shared by coxsackievirus B4 and echovirus 1 are not identical with the sequences shared by coxsackievirus B4 and echovirus 12, nor are these sequences identical with those shared by echoviruses 1 and 12 themselves. Nevertheless, appreciable additive homologies were not observed when multiple heterologous RNA combinations were reacted simultaneously. Although some overlap among the sequences shared by different enteroviruses is thus likely, failure to demonstrate additive homologies where nonreciprocal differences exist may indicate that intact RNA of unit length is unsuitable for this purpose. Further experiments utilizing fragmented ssRNA are contemplated which may elucidate this question.

Despite remarkable biochemical, biophysical, morphologic, and biological properties shared by human enteroviruses, their nucleotide sequences are very dissimilar. It is nevertheless possible that their proteins are more closely related than the present data would imply due to degeneracy in the genetic code. Thus, comparisons of the genes for rabbit and duck hemoglobins by DNA-RNA hybridization indicate that only 5 to 10% of the nucleotide sequences are shared by these species (10), yet protein sequencing of rabbit and avian globins shows that approximately 70% of the amino acid sequences are the same (2). Direct comparisons of the proteins of human enteroviruses are needed to resolve whether these proteins exhibit greater homologies than the nucleotide sequences coding for them.

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