# Altered Receptor Specificity of Coxsackievirus B3 After Growth in Rhabdomyosarcoma Cells

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Serial "blind" passages in human rhabdomyosarcoma (RD) cells of prototype viruses from each of the six immunotypes of the group B coxsackieviruses (CB) resulted in the isolation of intratypic variants of CB1, CB3, CB5, and CB6. Each variant virus strain acquired the capacity to agglutinate human erythrocytes and produce small plaques on HeLa cells, although their serological specificity remained unchanged. An alteration in VP1 mobility in sodium dodecyl sulfate-polyacrylamide gel electrophoresis was noted for CB3-RD. The CB3-RD variant was plaque purified on RD cells and studied for receptor interactions on both HeLa and RD cells. An attachment restriction appeared to exist for prototype CB3 on RD cells, whereas CB3-RD attached well to both cells. In attachment interference assays, HeLa cells saturated with CB3-RD blocked the attachment of CB3. In contrast, saturation of cells with CB1 (which shares a common receptor with parental CB3) failed to block the attachment of CB3-RD. This unidirectional receptor blockade suggested that a second site for the attachment of virions to receptors was acquired by the CB3-RD variant. Thus, more than one virus receptor specificity may be operative in the selection of host range virus mutants. The implications of this phenomenon as they may relate to pathogenesis are discussed.

Variation in the biological and biochemical characteristics of picornaviruses is common (15). Among the more significant examples of this variation is the propensity for antigenic drift (12, 26, 29) and host range alterations which cause disease syndromes not ordinarily associated with the prototype virus (37). Selection of attenuated viruses (20) or virulent virus strains (28, 38) is enhanced by serial passage of viruses in assorted tissues. The mechanism involved in the selection of host range variants is poorly understood but is believed to involve alterations in the virion surface (17) which perhaps affect interactions with cell surface receptors (9).

The study of the selection of variant viruses by cells is best accomplished with cells in culture, since they are more homogeneous than tissues. For example, the human rhabdomyosarcoma (RD) cell line (24) serves as a sensitive host for the replication of most group A coxsackieviruses (32, 35; R. L. Crowell and B. Goldberg, Abstr. Annu. Meet. Am. Soc. Microbiol. 1974, V44, p. 208), which have a restricted host range in cultured cells (14, 33). However, RD cells are refractory to infection by the group B coxsackieviruses (CB) (32), which have a relatively wide host range. To help evaluate the nature of the restricted event for CB in RD cells, we made serial "blind" passages of each of the prototype strains of the six CB. Intratypic virus variants with an extended host range were recovered and compared with parental viruses. Of special interest were the results of an examination of variant virus receptor specificity, since prototype CB are known to share a common receptor on HeLa cells (7). The data suggest a phenomenon whereby variant viruses may acquire more than one type of virion attachment site, allowing an extension of the host range.

## MATERIALS AND METHODS

**Cell cultures and viruses.** Human RD cells obtained from R. M. McAllister (University of California at Los Angeles,

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Los Angeles, Calif.) were maintained as monolayer cultures by methods described previously (33). The propagation of suspension and monolayer cultures of HeLa cells also has been described previously (10).

The origin of the parental strains of the six HeLa cell-grown CB and poliovirus T2 (PT2) is described elsewhere (16). Variant CB, hereafter designated RD strains, were selected by serial passage in RD cells as described herein. Procedures for the preparation of seed virus and purified virus have been described earlier (10). Radiolabeled virus was prepared by the addition to cells 2 h postinfection of [$^{35}$S]methionine (4 µCi/ml; Amersham Corp., Arlington Heights, Ill.) in methionine-free minimal essential medium containing 2% calf serum. The labeled virus was propagated and purified as described above.

Virus infectivity was titrated on either HeLa or RD cell monolayers with a modification of the procedures described by Crowell and Syverton (11). These modifications included an addition of 100 mg of sodium dextran sulfate 500 (Pharmacia, Uppsala, Sweden) per ml to the 0.5% Bacto-Agar (Difco Laboratories, Detroit, Mich.) overlay. The maximum plaque size of the variants occurred after 3 days of incubation in HeLa cells or 2 days of incubation in RD cells, respectively.

The microtechnique of Melnick and Wenner (25) was used to assay the ability of virus strains to agglutinate type O, Rh$^+$ human erythrocytes in 0.01 M phosphate-buffered 0.85% saline (pH 6.5). Microtiter plates were incubated for 3 h at room temperature and then read for agglutination patterns.

**Virus attachment.** As is routine, the amount of virus infectivity (PFU) remaining unattached after virus-cell interaction was determined by methods described previously (6). Samples containing viable, washed cells (RD and HeLa) were distributed among sterile test tubes, pelleted, and suspended in 1.0 ml of minimal essential medium in Hanks salts–0.02 M HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), pH 6.5 (binding medium), containing infectious virus with an input multiplicity of 0.4 PFU per cell. The mixture was incubated at 20°C for 1 h with intermittent shaking. Samples (100 µl) of virus-cell suspen-

sions were removed at timed intervals and diluted in 9.9 ml of Hanks balanced salt solution with 3% added calf serum to stop further attachment. The cells in diluted suspensions were pelleted by centrifugation at 1,100 × $g$ for 10 min, and the supernatant was assayed for unattached virus. To control for losses of infectivity due to factors other than receptor binding, control tubes containing the virus inoculum in binding medium without cells were included in each experiment. All values were expressed as the percentages of these virus controls. The binding of purified [$^{35}$S]methionine-labeled virions to suspended cells was used as an alternative method for analyzing virus attachment and consisted of techniques described elsewhere (22).

**Virus attachment interference.** Competition among virus groups for the same receptor was assessed by methods described by Crowell (6, 7). Briefly, 7 × 10$^7$ washed, viable cells were suspended in a concentration of coxsackievirus B1 (CB1) sufficient to obtain an input multiplicity of 1,000 PFU per cell (in a volume of 1 to 2 ml). After a 1-h attachment at 20°C, cells were washed three times with Hanks balanced salt solution with 3% added calf serum to remove unattached virus, and 10$^7$ cells were distributed among sterile tubes. The attachment of the challenge virus, a CB3 strain, took place under conditions described above. The titration of unattached CB3 required pretreatment of supernatants with type-specific anti-CB1 serum (6).
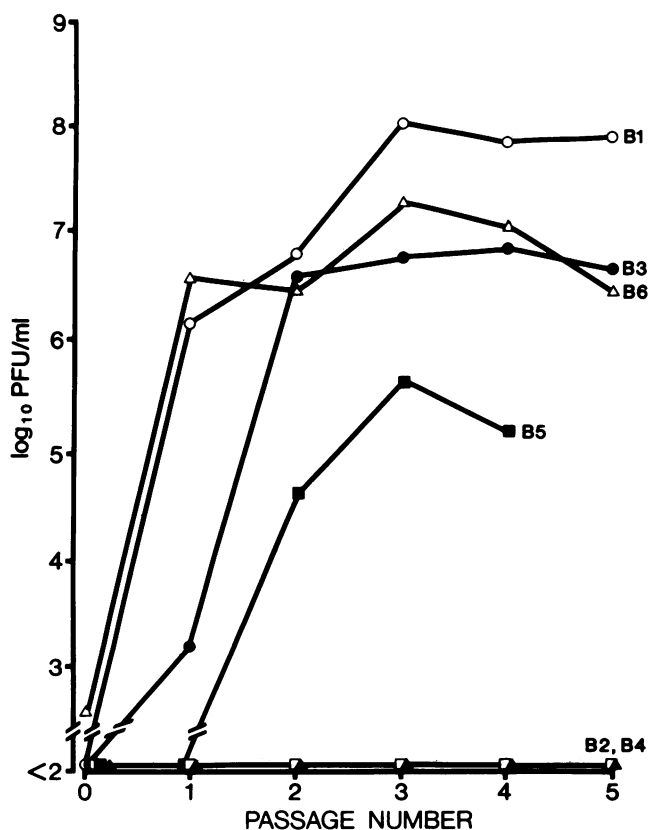


FIG. 1. Serial passage of CB in RD cell monolayers. Monolayer cultures of RD cells were infected with parental CB, incubated for 24 to 72 h, and frozen-thawed, and clarified extracts were used to infect fresh cultures. Samples of culture fluids were assayed for plaque production on RD cells. Input inocula (PFU) of parental CB, as determined on HeLa cells, were as follows: CB1, 6 × 10$^8$; CB2, 4 × 10$^8$; CB3, 3 × 10$^9$; CB4, 3 × 10$^8$; CB5, 1 × 10$^8$; and CB6, 8 × 10$^7$.

Alternatively, washed cells were exposed to unlabeled, purified virus at concentrations of 3 × 10$^5$ to 5 × 10$^5$ particles per cell in a volume not exceeding 1 ml. After a 1-h incubation at 19 to 20°C, cells were challenged with radiolabeled CB3 at a concentration of 1 × 10$^4$ to 4 × 10$^4$ particles per cell in a volume not exceeding 2% of the total (21). Cells were separated from the reaction mixture by centrifugation and washed twice with phosphate-buffered saline (PBS), and the distribution of label between the supernatants and cell pellets was determined by liquid scintillation.

[$^3$H]leucine incorporation into coxsackievirus-induced proteins. HeLa cells in monolayer cultures were infected with 50 PFU of either CB3 parental or variant strains per cell. After 1 h at room temperature for virus attachment, cells were overlaid with minimal essential medium containing 3% calf serum and 0.02 M HEPES, pH 7.5. Three hours of incubation at 37°C was allowed. The overlay was removed, and monolayers were washed three times with minimal essential medium devoid of serum and amino acids except glutamine (minimal virus infecting medium) and then overlaid with minimal virus infecting medium (2 ml per 60-mm plate) containing 50 μCi of [$^3$H]leucine (Amersham Corp.) per ml. After 2.5 h of incubation at 37°C, cells were removed from plates with trypsin-EDTA (GIBCO Laboratories, Grand Island, N.Y.) and then lysed in 1 ml of 0.5 M urea–1% sodium dodecyl sulfate (SDS)–0.1% 2-mercaptoethanol per 10$^7$ cells. Unincorporated radioactivity was removed by dialysis at 6°C in 1,000 volumes of buffer containing 0.01 M sodium phosphate (pH 7.2), 0.5 M urea, 0.1% SDS, 0.1% 2-mercaptoethanol, and 1 μM sodium azide. Samples for electrophoresis were adjusted to 0.01 M sodium phosphate–1% SDS–0.006% bromphenol blue–10% glycerol and then boiled for 5 min before application to the gel. Proteins were resolved in a continuous SDS-polyacrylamide gel containing 2.5 M urea as described elsewhere (17). Gels were prepared for fluorography (2), and radioactivity was detected on preflashed (18) Kodak XR-5 film (Eastman Kodak Co., Rochester, N.Y.) at −70°C.

## RESULTS

**Relative insensitivity of RD cells to CB and selection of virus variants.** Initial experiments were designed to confirm the observed insensitivity of RD cells to infection by CB. Each parental strain of coxsackievirus was assayed for cytopathic effect and the yield of infectious virus after a 9-h incubation in either RD or HeLa cells. PT2 was included as a positive control of RD cell cytopathology. The data (not shown) revealed that prototype CB strains caused cytopathology in HeLa cells, but not in RD cells, and the yields of coxsackieviruses from RD cells were more than 100 times lower than those from HeLa cells. PT2 caused a pronounced cytopathic effect and yielded comparable amounts of virus in both cell lines.

It was considered possible that the RD cells would select for a variant virus from the parental population. Serial blind passages of the CB parental strains in RD cell monolayers were made to select an RD$^+$ virus population, and the results of virus yields as titrated on RD cells are depicted in Fig. 1. Passage of undiluted and clarified extracts of frozen-thawed cultures yielded RD$^+$ virions from CB1, CB3, CB5, and CB6, respectively. Three to five passages of 24 to 72 h each were usually sufficient to produce a consistent cytopathic effect. Plaques, however, could be produced at low frequency on RD cells by the parental virus inocula at levels representing ca. 10$^{-5}$ to >10$^{-7}$ of the respective titers as

TABLE 1. Comparative characteristics of CB3 strain Nancy and the CB3-RD variant[a]

| CB3 strain | Cytopathic effect | | Plaques on: | | Hema-ggluti-nation | VP1 (mol wt) | Attachment to: | |
|---|---|---|---|---|---|---|---|---|
| | HeLa cells | RD cells | HeLa cells | RD cells | | | HeLa cells | RD cells |
| Nancy | + | − | 3–5 mm, clear | No plaques | − | 34,000 | + | − |
| RD | + | + | 0.5–1 mm, clear | 1–2 mm, cloudy | + | 36,000 | + | + |

[a] Homotypic and cross-neutralization rates were similar by rabbit antiserum against each virus strain.

measured on HeLa cells. Several additional attempts were made to isolate RD+ populations from parental CB2 and CB4, including the large- and small-plaque strains of CB4 (3), without success.

All virus variants could be neutralized by antisera prepared against parental strains and thus were considered to be intratypic host range mutants (1, 16).

**Phenotypic properties of the CB3-RD variant virus.** The CB3-RD variant virus was selected for further study and plaque purified three times by limiting the dilution in RD cell monolayers before characterization. A summary of the comparative phenotypic properties of CB3 and CB3-RD is presented in Table 1. The experiments from which these results were obtained are described below. All observations have been confirmed by utilizing at least one independent strain of the CB prototype and its RD-adapted variant.

(i) **Small-plaque formation.** CB3-RD produced cytopathic effect on both RD and HeLa cells and formed plaques with a similar efficiency on monolayers of either cell line. However, within 2 days, large, cloudy plaques were noted on RD cells, whereas small to pinpoint, clear plaques (SP+) were produced on HeLa cells. The small-plaque formation on HeLa cells clearly distinguished the variant virus populations from their respective large-plaque (SP−)-forming parental strain and probably reflected the acquisition of virus sensitivity to an agar inhibitor (indicated by the enhancing effect of charged polyions on plaque size). The appearance of representative plaques is shown in Fig. 2.

(ii) **Hemagglutination.** The capacity of selected enteroviruses to agglutinate erythrocytes is well recognized, but this property varies within a single serotype (30). Recovery of hemagglutinating (HA+) variants from an HA− strain of echovirus 6 (23) and evidence that the cell type used to propagate enteroviruses can influence its hemagglutination activity (5) prompted the analysis of available coxsackievirus strains to agglutinate human erythrocytes. Virus samples of CB1, CB3, CB5, and CB6 and their RD variants were diluted to give $10^7$ PFU/50 μl (assayed on HeLa cells) and titrated in microtiter plates for hemagglutination activity as described above. The results indicated that, in contrast to the parental viruses, only the RD+ variants were HA+. The titers were as follows: CB1-RD, 256; CB3-RD, 512; CB5-RD, 1,024; and CB6-RD, 256. PT2 failed to hemagglutinate regardless of the cell line used to propagate the virus. Hemagglutination by CB3-RD could be observed over the range of pH values from 5 to 7.8. Pretreatment of erythrocytes by selected enzymes for 1 h at 37°C characterized the hemagglutinin receptor as neuraminidase (1 U, pH 6.5) and trypsin (0.1%, pH 7.0) insensitive but chymotrypsin (0.1%, pH 7.0) sensitive. A similar enzyme sensitivity pattern for erythrocyte receptors (31) and HeLa cell receptors (39) for HA+ and HA− strains of CB3, respectively, has been noted.

(iii) **Altered mobility of VP1.** Comparative analyses by SDS-polyacrylamide gel electrophoresis of [³H]leucine-labeled structural and nonstructural proteins from CB3 and CB3-RD strains in a continuous 10% gel system revealed an

increase in the molecular weight of VP1 for CB3-RD (Fig. 3). Molecular weights of 36,000 and 34,000, respectively, were assigned to CB3-RD VP1 and CB3 VP1, based on the migration of protein standards. The other structural proteins from these two strains comigrated as did the nonstructural proteins. Unlike poliovirus variants, the addition of urea to the gel system did not eliminate the VP1 size discrepancy (17). However, a similar alteration in VP1 was not found for the other RD-adapted strains, suggesting that this change was not unique to RD cell-passaged virus (data not shown). Nevertheless, the results provided evidence for an alteration of a CB3-RD capsid protein.

(iv) **Receptor specificity of CB3 and CB3-RD.** The in vitro markers noted for CB3-RD have been related to alterations in the surface of the virions, which differ from those of the parental CB3 virion population. Since the interaction of the virion with cell surface receptors is a function of the virion surface, comparative attachment kinetics of CB3 and CB3-RD on cultured cells were examined.

Replicate samples of RD cells were suspended in binding medium containing $4 \times 10^6$ PFU of the respective virions, and attachment was monitored as described above. The results (Fig. 4A) indicated a rapid attachment of the CB3-RD strain to RD cells, whereas the attachment of the parental virus was undetected. Neither changes in the pH of the attachment medium (over the pH range of 4 to 8) nor increases in the cell concentration of up to $10^8$ cells per ml significantly increased the binding of parental CB3 to these cells (data not shown). The rapid attachment of the variant
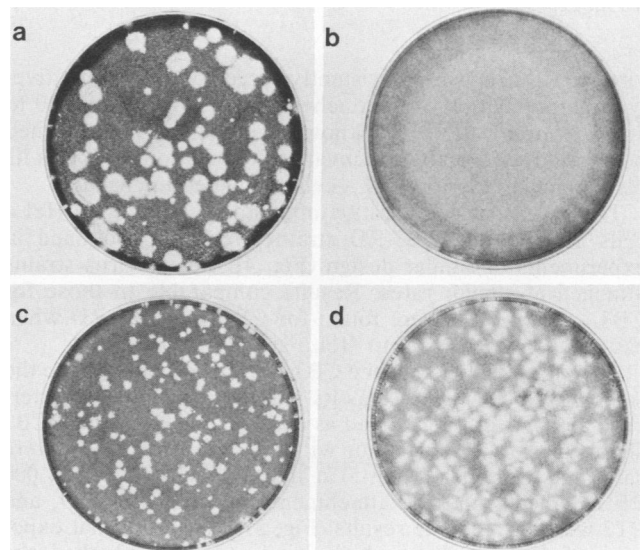


FIG. 2. Representative plaque morphology of the CB3 parental strain on HeLa cells (a) and RD cells (b, no plaques) as compared with the CB3-RD variant strain on HeLa cells (c) and RD cells (d).
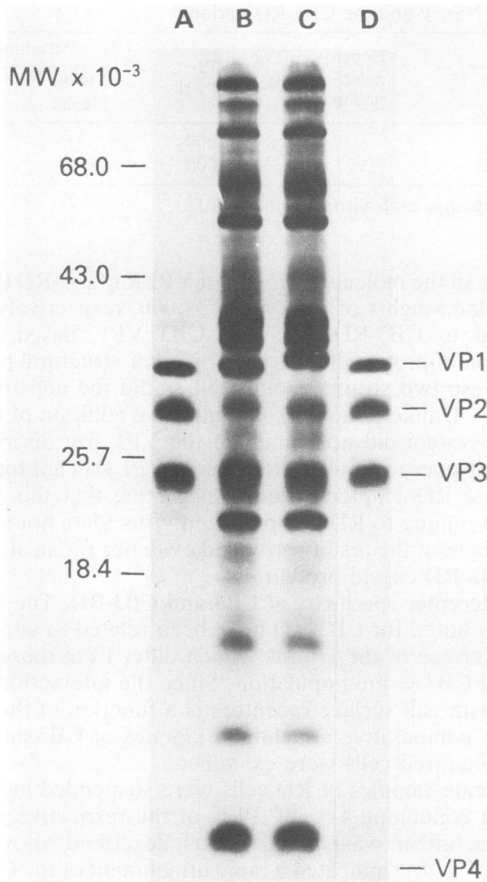
FIG. 3. Fluorogram of the SDS-polyacrylamide gel of structural and nonstructural polypeptides from CB3 strains. Cesium chloride-purified, [$^{35}$S]methionine-labeled CB3 Nancy (parent) virions (lanes A and D) and [$^3$H]leucine-labeled virion-induced polypeptides extracted from HeLa cells infected with CB3 Nancy (lane B) and CB3-RD (lane C) are shown in the figure. The migration of molecular weight (MW) standards, as well as the designation of virion structural polypeptides, is indicated. The specimens shown are the results of a 13-h exposure.



FIG. 4. Attachment of CB3 (●) and CB3-RD (○) to suspensions (10$^7$ cells per ml) of RD (A) and HeLa (B) cells.

despite the addition of CB1, indicating no attachment interference between the poliovirus and coxsackievirus receptors (7).

Titers of CB1-RD (or any other RD variant except CB3-RD) sufficiently high to be used in attachment interference assays could not be obtained. Thus, the reciprocal test, i.e., the blockade of HeLa cell receptors with saturating levels of variant virus, was performed with a different approach. HeLa cells were preincubated with saturating levels (5 × 10$^5$ virions per cell) of purified CB3-RD; after being washed, the cells were tested for the capacity to attach [$^{35}$S]methionine-labeled CB3. The results revealed (Fig. 5B) that less than 10% of the labeled parental virus attached in the presence of the variant virus. Cells incubated with PBS only (as a control) attached ca. 55% of the labeled virus within 15 min. Also, preincubation of cells with PT2, which does not share the same receptor as CB3, allowed attachment of the labeled virus at levels comparable to PBS-treated cells. An additional control confirmed that receptor-saturating amounts of unlabeled CB3 resulted in homologous virus attachment interference. It was concluded that the saturation of receptors on HeLa cells by CB3-RD had blocked the attachment

virus to RD cells was consistently noted, as was the relatively large population of unattached virus (ranging from 20 to 40% of input). It is not known whether this unattached population represented a subvariant population, although its plaquing character on HeLa cells remained unchanged.

The results of comparative attachment kinetics to HeLa cells of CB3 and CB3-RD strains were also obtained in experiments of similar design (Fig. 4B). Both virus strains attached at similar rates. Results comparable to those for CB3 and CB3-RD were found for CB1 and CB1-RD when assayed for attachment to HeLa and RD cells.

To determine whether the CB3-RD variant attached to the same HeLa cell receptor as its parent, attachment interference assays were performed as described above. Since CB1 shares a HeLa cell receptor with CB3 (6), HeLa cells were saturated with CB1 (Conn-5) at an input multiplicity of 1,000 PFU per cell, and the attachments of CB3, CB3-RD, and PT2 were tested. The results (Fig. 5A) indicated that exposure of HeLa cells to high levels of CB1 blocked the attachment of the CB3 parental strain, but not CB3-RD, which attached normally to the CB1 saturated cells. As expected, ca. 60% of the PT2 input attached to HeLa cells
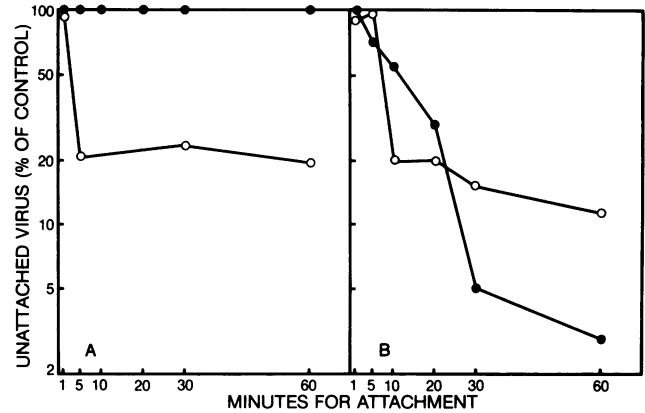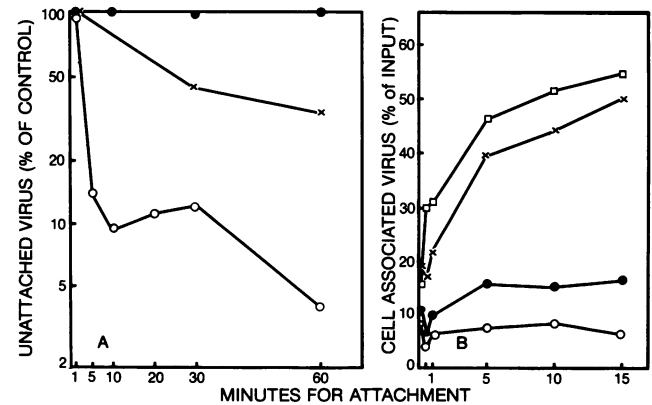


FIG. 5. Reciprocal attachment interference analysis of CB3 strains on HeLa cells. (A) Cells preincubated with CB1 at an input multiplicity of 1,000 PFU per cell before attachment of the CB3 parent (●), CB3-RD (○), and PT2 (×); (B) cells preincubated with either PBS (□) or 3 × 10$^5$ to 5 × 10$^5$ particles of the CB3 parent (●), CB3-RD (○), or PT2 (×) per cell before the attachment of the [$^{35}$S]methionine-labeled CB3 parent.

of parental CB3. Thus, a "unidirectional" attachment interference was found (CB3-RD blocked the attachment of CB3, but CB3 failed to block the attachment of CB3-RD).

## DISCUSSION

The results of this study revealed the presence of naturally occurring particle heterogeneity within coxsackievirus strains. Serial passage through RD cells selectively propagated $RD^+$, $SP^+$, and $HA^+$ variant populations of CB viruses from $RD^-$, $SP^-$, and $HA^-$ parental stocks. The finding of these intratypic variants of coxsackieviruses, however, is not unique. Host range (13, 37), hemagglutinating (31), and small-plaque variants (3) have been reported previously, attesting to the plasticity of phenotypic characteristics of these and other picornaviruses (4, 12, 17). Indeed, Nottay and co-workers (27) provided electrophoretic evidence of a measurable rate of evolution in the genome of both vaccine and wild-type poliovirus T1 during human intestinal passage.

Mutations in virus genomes can result in alterations in virulence, plaque character, antigenicity, and host range. If provided an adequate selective advantage, subpopulations with one or more of these altered characteristics would be replicated preferentially. RD cells, for example, were found to be minimally permissive for the replication of parental CB, the yield observed probably reflecting a few cells in the population being infected initially. Upon serial passage of the viruses, these cells provided a strong selective force for the amplification of small-plaque-forming variants, which hemagglutinated human erythrocytes. Since both the variant and parental CB populations infected HeLa cells, it is reasonable to suggest that the variant $HA^+$ populations persisted and were undetected in the parental strains and that HeLa cells were a poor selective force for the $HA^+$ variants. A limited number of back passages in HeLa cells failed to select revertants to the parental phenotype either because HeLa cells do not select against variant markers or because multiple mutations had occurred. Therefore, even though all CB that were $RD^+$ were also $HA^+$ and $SP^+$, the conclusion that these markers were covariant would be premature. It is significant that no CB2 or CB4 have been found to be $HA^+$.

The mechanism by which $HA^+$ variant viruses are selected by RD cells is of interest since it may mimic other "adaption" processes essential to the alteration in virulence patterns (28). The evidence presented here strongly suggests a role for the early interaction between the virus and cell surface as the major selective force in amplifying variant virus populations. This is based upon the observed alteration of receptor affinity and specificity resulting from RD cell passage. Parental CB3 strains failed to demonstrate detectable binding to RD cells, whereas the CB3-RD variant attached readily. All $RD^+$ CB strains attached to HeLa cells, yet receptor competition studies revealed a unidirectional interference phenomenon.

To explain the unidirectional receptor competition, the parental and variant viruses may attach to different domains of the same multicomponent HeLa cell-receptor complex (8). Alternatively, the RD cells may select for variants with a new virion attachment protein (VAP) on its capsid surface, either as a new domain of the same protein or as a totally unique area. This model assumes that the affinity of variant viruses for the parental receptor site (designated R1) was not altered, and therefore two sites (VAPs) are available for the RD-selected variants to attach to HeLa cells. The second VAP would have an affinity for a second receptor site

(designated R2) found on both HeLa and RD cells. Thus, in this model the RD cells would possess the R2, but not the R1, site.

An analogous virus receptor relationship may occur between parental and variant reoviruses. L cells possess a group receptor for the three reovirus immunotypes (19); however, reovirus type 1 has a distinct histotropism from that of reovirus type 3. This difference is based on the specificity of VAP (sigma 1) for a specific receptor on ependymal cells (34) which differs from that on neurons (36). Thus, in target cells of animals, it appears that a second type of receptor (different from the virus group receptor on L cells) may have important biological significance. Further studies in our laboratory are aimed at distinguishing between the R1 and R2 types of receptors found on HeLa cells. Also, we are determining the relationship between the receptors on RD cells (R2) and the receptors on erythrocytes (R3) for the CB variants which are $RD^+$ and $HA^+$. Preliminary results have revealed that the CB-RD variants have an altered tissue tropism in newborn and adult mice.

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