The precise mechanism of neutralization of viruses by antibody is at present only poorly understood. In most instances it has been shown that neutralizing antibodies interact with surface "capsid" polypeptides (1–8). Examples of specific capsid peptides that have been shown to be responsible for the induction of neutralizing antibody include hexon and fiber polypeptides for adenovirus (7, 8) and hemagglutinin for influenza (6). In general, these results have been obtained by purifying individual viral proteins or a group of proteins and then preparing antisera to them (1–5, 7, 8). For reovirus, we have examined this question using a genetic approach.

Reovirus contains 10 segments of double-stranded RNA (dsRNA) surrounded by a double capsid shell; the segments are named according to size classes: three large segments (L1, L2, and L3) with mol wt of ≈2.3–2.5 × 10⁶, three medium segments (M1, M2, and M3) with mol wt of ≈1.4–1.6 × 10⁶, and four small segments (S1, S2, S3, and S4) with mol wt of ≈0.6–0.9 × 10⁶. The outer capsid consists of three polypeptides, σ1, σ3, and μ2 (9). The σ1 and σ3 polypeptides are derived from genome segments S1 and S4, respectively (10). In the nomenclature contained in this paper, we have assigned the μ2 polypeptide as being a product of the M1 gene. This assignment is based on migration of dsRNA segments on gel systems containing Tris-glycine buffers. We have found that what we are defining as the M1 segment migrates as the M2 segment in systems containing Tris-acetate buffers. Thus, our assignment of the μ2 capsid polypeptide to the M1 segment is identical to Joklik's assignment of the μ2 polypeptide to the M2 segment (10). There are three serotypes of mammalian reovirus (types 1, 2, and 3) defined on the basis of neutralization antibodies (11). Using hybrid recombinant clones consisting of genome segments derived from type 1 or 2 reovirus mixed with genome segments derived from type 3 reovirus, we have determined the gene segregating with, and responsible for, neutralization and type specificity of reovirus. The results below show that the S1 genome segment which codes for σ1, the minor outer capsid polypeptide...
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(10), is the genome segment responsible for type specificity. Thus the σ1 polypeptide is the type-specific antigen.

Materials and Methods

Cells. Mouse L cells were maintained in suspension culture in Joklik's modified Eagle's minimal essential medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 5% fetal calf serum (International Biological Laboratories, Inc., [IBL], Rockville, Md.). Monkey CV-1 cells were maintained in monolayers in "improved" minimal essential media supplemented with zinc, insulin, and Hepes buffer (IMEMZO) (IBL) supplemented with 10% fetal calf serum (IBL).

Virus. Reovirus type 1 (Lang strain), type 2 (Jones strain), and type 3 (Deering strain) were the same as previously described (11). Hybrid recombinant clones were prepared by mixedly infecting L cells with equal multiplicities of temperature-sensitive (TS) mutants of type 3 reovirus (12) and clones derived from either type 1 or type 2. A large number of progeny clones were collected at 39°C (a nonpermissive temperature for the TS mutants) and shown to be recombinants. Eight such clones were utilized in the present analysis (Fig. 1 and Table I).

Neutralization Assay. Antisera against the three reovirus serotypes were obtained from the National Institute of Allergy and Infectious Diseases, Bethesda, Md., catalog numbers V-701-511-570 (anti-1), V-702-501-570 (anti-2), and V-703-501-570 (anti-3). These antisera were prepared in geese. Approximately 100 plaque-forming units of the three serotypes and hybrids were incubated with various dilutions of antisera for 45 min at 37°C, then titrated at 31°C, on L-cell monolayers as previously described (12). Greater than 80% reduction of plaques was considered neutralization of virus.

Analysis of Viral RNA. Analysis of viral RNA was performed as previously described (13). Briefly, the three serotypes of reovirus and the hybrid clones were grown at 31°C on CV-1 monolayers at a multiplicity of infection of 10 in 60 × 15 mm plastic Falcon tissue culture dishes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) in 5 ml of IMEMZO. At 2.5 h postinfection, media containing 0.25 mCi 32P was added and at 48 h, infected monolayers were washed with an isotonic buffer [0.14 M NaCl, 0.01 M Tris-hydrochloride (pH 7.4), 0.0015 M MgCl2 (Buffer A)] and cells were collected. The cells were treated with 0.5% Nonidet P-40 in Buffer A, nuclei were removed by centrifugation, and RNA was precipitated at -20°C in ethanol. The precipitates were pelleted by high speed centrifugation, dried, dissolved in gel sample buffer, and applied to slab gels (10% acrylamide, 0.267% bis-acrylamide) as described by Laemmli (14). After electrophoresis, gels were fixed and autoradiography performed.

Results

Migration Pattern of dsRNA Segments. In Fig. 1 the migration patterns of dsRNA from reovirus types 1, 2, and 3 and from eight hybrid clones are shown, accompanied by a diagrammatic representation of these patterns. It can be seen that the serotype of origin for each genome segment can be determined for the hybrid clones by comparison to the three serotypes. Clones A–F represent hybrid clones between serotypes 1 and 3, while clones G and H represent hybrid clones between serotypes 2 and 3. For example, clone C contains L1, L3, and S1 segments from type 1 and the remainder of segments from type 3. The genome segment most easily distinguished is the S1 segment, which migrates much faster for types 1 and 2 than it does for type 3. We have been unable to reproducibly distinguish the S2 segment of type 1 from that of type 3, but the S2 segment of type 2 is easily distinguishable from the homologous type 3 segment (Fig. 1, clones G and H). In terms of comparing the genome RNA patterns between types 2 and 3, it has previously been shown that the middle

2 Sharpe, A. H., R. F. Ramig, T. A. Mustoe, and B. N. Fields. A genetic map of reovirus. I. Correlation of genome RNAs between serotypes 1, 2, and 3. Virology. In press.
Fig. 1. (A) Autoradiograms of reovirus dsRNA segments from serotypes 1, 2, and 3 (I, II, and III) and eight hybrid clones (A–H). Clones A–F represent hybrid clones between serotypes 1 and 3, and clones G and H represent hybrid clones between serotypes 2 and 3. (B) Diagrammatic representation of the migration patterns shown in Fig. 1 A. The 10 dsRNA segments for reovirus consist of three large segments (L1, L2, and L3), three medium segments (M1, M2, and M3), and four small segments (S1, S2, S3, and S4). The segments from types 1 and 2 are represented by thick bars, and those from type 3 by thin bars. Dashed lines (S2 segments from clones A–F) represent segments that cannot be assigned to either type 1 or type 3.
Neutralization of Hybrid Clones of Reovirus with Type-Specific Antibody

| Number of times | 1  | 2  | 3 | Neutralization type |
|----------------|----|----|---|---------------------|
| A              | 1  | 1  | 3 | M1                   |
| B              | 3  | 3  | 1 | L1                   |
| C              | 1  | 1  | 1 | L2                   |
| D              | 3  | 3  | 3 | L3                   |
| E              | 1  | 3  | 3 | M2                   |
| F              | 3  | 3  | 3 | M3                   |
| G              | 3  | 3  | 3 | S4                   |
| H              | 3  | 3  | 3 | S3                   |

| Gene coding for: | M1 | S1 | S4 | L1 | L2 | S2 | M2 | S3 | L3 | M3 |
|-----------------|----|----|----|----|----|----|----|----|----|----|
| Antibody titer* | 1,280 | <20 | 1,280 | 1,280 | 1,280 | <20 | 1,280 | <20 | 1,280 | <20 |

* Reciprocal maximum antiserum dilution resulting in >80% plaque reduction.

Neutralization of Reovirus

Table I

Neutralization of Hybrid Clones of Reovirus with Type-Specific Antibody

and small size class RNA segments of type 2 migrate in an order that does not correspond to the order of these segments in reovirus type 3.2 Specifically, the M2 segment from type 2 migrates slower than the type 2 M1 segment and the S4 segment from type 2 migrates slower than the type 2 S3 segment. Thus, clone G (Fig. 1) contains a type 3 M2 segment and a type 2 M1 segment; similarly, clone H (Fig. 1) contains a type 2 S4 segment and a type 3 S3 segment.

Neutralization of Hybrid Clones by Type-Specific Antibody. Antibody prepared against type 1 reovirus neutralized the homologous strain at a dilution of 1:1,280 while showing no effect on type 3 at a serum dilution of 1:20. Antibody prepared against type 2 had a titer of 1:1,280 against type 2 and 1:20 against type 3. Type 3 antibody neutralized type 3 virus at a dilution of 1:160-320 while neutralizing type 1 or 2 at dilution of 1:20.

When the hybrid clones were examined for neutralization by type-specific antibody, they all behaved as either type 1 or 3 (Table I). No clones behaved as type 2. The titer needed for neutralization of hybrid clones always corresponded to the titer needed to neutralize the parental reovirus serotypes; the titers were never intermediate. In every hybrid clone studied the type as determined by neutralization testing, correlated solely with the S1 gene product; i.e., when S1 is derived from type 1 the hybrid is neutralized by type 1 antiserum while every clone containing S1 from type 3 is neutralized by type 3 antiserum. Both of the other outer capsid peptides (coded for by the M1 and S4 genome segments) did not correlate with the neutralization serotype in almost half the hybrids examined. In a similar manner, all of the other segments could be excluded as being responsible for type specificity.

The type-specific immune sera used in these experiments do not contain antibody predominantly to the σ1 protein, but are reactive with all the structural polypeptides of the virion. This has been shown by direct immune precipitation of all viral polypeptides at dilutions of antisera from 1:20 to 1:5,000 (T. A. Mustoe and B. N. Fields, unpublished observations). Thus, antibodies directed against the other two outer capsid proteins (σ3 and μ2) do not neutralize the virus.
Discussion

These studies demonstrate the feasibility of using a genetic approach to define the biologic properties of a virus. In this instance, we have shown that the S1 gene codes for the viral neutralization antigen, the $\sigma$1 polypeptide. As with most viruses, the reovirus protein responsible for neutralization is a capsid protein. The $\sigma$1 polypeptide, despite its prominent biologic role as the neutralization antigen, represents only 1% of the virion's polypeptides and less than 2% of the total mass of the outer capsid (9). The two major outer capsid proteins, $\mu$2 and $\sigma$3 comprise 64% of the total virion and over 98% of the outer capsid (9). There are approximately 550 and 900 molecules of $\mu$2 and $\sigma$3, respectively, per virion but only 30 molecules of $\sigma$1 (9). Thus, the relatively minor $\sigma$1 polypeptide must occupy a strategic position to be the sole determinant of type specificity and be the target of neutralizing antibody. When the outer shell of reovirus is progressively digested by chymotrypsin, $\sigma$3 is removed first, followed by $\mu$2 and finally $\sigma$1 (15). It has been speculated that $\sigma$1 is associated with the $\lambda$2 core spikes that penetrate into the outer capsid shell of the intact virion. It is through these spikes that viral transcriptase may be activated and single-stranded messenger RNA released (16). Perhaps binding of the $\sigma$1 polypeptide by antibody prevents this crucial event in viral replication. In addition, binding of the $\sigma$1 polypeptide may interfere with adsorption of the virus into the cell or uncoating of the virus.

One particularly significant advantage of this approach in determining the biologic properties of the individual virion components is that potential artifacts that can occur by chemical extraction of individual viral components are avoided. It is easy to imagine that the chemical extraction of polypeptides might alter the natural configuration of the protein or expose regions of the polypeptides that are normally hidden. Antibody produced to proteins isolated in this manner might play no role in natural infections. This approach is currently being used to study other biologic properties of reovirus virions and viral-host interactions. We have recently found that the $\sigma$1 polypeptide is also the hemagglutinin polypeptide of reovirus (H. Weiner, unpublished observations).

Summary

The S1 genome segment of reovirus is linked to type specificity as determined by neutralization antibody. This gene segment codes for a minor outer capsid polypeptide ($\sigma$1). Therefore, $\sigma$1 is the peptide responsible for induction of neutralization antibody and confers type specificity. This biologic property of reovirus was defined using hybrid recombinants clones between reovirus types 1 and 3 and 2 and 3.

Note Added in Proof. The dsRNA segment designated as M1 in this publication (defined on the basis of migration in Tris-glycine buffer systems) is identical to the dsRNA segment previously designated M2 (defined on the basis of migration in Tris-acetate buffer systems). To be consistent with the earlier designation, in future publications from our laboratory we will refer to this dsRNA as M2.

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