Four Independent Antigenic Determinants on the Capsid Polypeptides of Aphthovirus

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SUMMARY

Four independent antigenic determinants in the virus capsid have been defined in radioimmunoassay tests using antisera prepared against isolated structural polypeptides VP 1, 2 and 3. Only one determinant was detected on the intact particle and it was contained within VP1 (called the VP1-A determinant). Three determinants were found on the 12S subparticle, two contained within VP1 (VP1-A and VP1-B determinants) and one contained within VP2 (VP2 determinant). The fourth determinant was contained within VP3 (VP3 determinant) and could only be detected when the virus was disrupted to individual polypeptides. It is concluded that the VP1-A determinant is likely to be of epidemiological importance since (i) it was present on the intact virion, (ii) variation at this determinant was detected with heterologous field strains, (iii) it contained a determinant responsible for the induction of neutralizing antibodies and (iv) it was an antigenic component of the trypsin-sensitive region of VP1 which has been shown previously by several workers to be of critical importance in the immunogenicity of the virus.

INTRODUCTION

Aphthovirus, or foot-and-mouth disease virus (FMDV), is a picornavirus which shows extensive antigenic variation. Seven distinct serotypes and numerous subtypes have been defined by serological analysis (Brooksby 1958). From studies on antisera raised against the purified isolated polypeptides, the major immunizing determinant for virus neutralization has been assigned to the specific virus protein, VP1 (Laporte et al., 1973; Bachrach et al., 1975; Kaaden et al., 1977; Meloen et al., 1979). Furthermore, Strohmaier et al. (1982) have suggested two immunizing sites for neutralizing antibody within the carboxy-terminal third of the polypeptide based on studies using cyanogen bromide-cleaved fragments. The primary sequence of VP1 has now been determined for several serotypes and subtypes (Kurz et al., 1981; Kleid et al., 1981; Boothroyd et al., 1982) and from such evidence Beck et al. (1983) have concluded that there are two highly variable regions in the polypeptide (amino acid residues 40 to 60 and 130 to 160) which could represent two antigenic sites. Interestingly, the latter sequence contains one of the sites proposed by Strohmaier et al. (1982) and in recent studies with short synthetic peptides only that region was capable of inducing good titres of neutralizing antibody (Bittle et al., 1982; Pfaff et al., 1982). Peptides based on residues 9 to 41 gave no such activity but Bittle et al. (1982) did find some slight activity in a peptide representing the second site proposed by Strohmaier et al. (1982). Inspection of the polypeptide sequence shows that the more immunogenic determinant overlaps the position of the exposed trypsin-sensitive sites in VP1 in the intact particle (Strohmaier et al., 1982). This is consistent with the fact that for many strains of FMDV, including that used in this study, trypsin treatment of virus particles drastically reduces their infectivity (Wild et al., 1969), their ability to induce neutralizing antibody (Rowlands et al., 1971; Cavanagh et al., 1977; Table 2 of this paper) and, more significantly, their ability to

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absorb neutralizing antibodies from antisera to whole virus (Wild et al., 1969). However, in recent work with 15 neutralizing monoclonal antibodies, Meloen et al. (1983) found that 12 antibodies reacted equally well with intact and trypsin-treated virus particles and three reacted less well with trypsinized virus (1 % of intact). Therefore, for their strain of virus there must be at least two determinants involved in virus neutralization, only one of which is sensitive to trypsin. There may be still other antigenic determinants on the virus particle which are not affected by trypsin, since electron microscopic studies on virus antibody complexes have shown at least three binding sites, only one of which was destroyed by trypsin (Brown & Smale, 1970). Nothing is known about these other sites except that they are probably in VP1, since most evidence indicates that it is the major component of the virus surface (Rowlands et al., 1975; Bachrach et al., 1975).

When the virus particle is treated with acid or heat, it is degraded to 12S subparticles (Talbot & Brown, 1972). Unlike previous workers such as Bachrach et al. (1975), Meloen & Briaire (1980) found that the 12S subparticles still reacted with anti-VP1 sera but whether this was due to new determinants or to intact particle determinants surviving the disruption procedure is unknown. In addition, several workers have shown that 12S particles also react with anti-VP2 and anti-VP3 sera, unlike the intact particle, but apart from the demonstration by Meloen & Briaire (1980) that such determinants were broadly cross-reactive, nothing more is known.

The work on VP1 has elegantly defined one important determinant responsible for the induction of neutralizing antibodies, which is present on the exterior of the particle and closely associated with the trypsin-sensitive region. However, as reviewed above, it may not necessarily be the only or the most important antigenic determinant. In the present study, we have attempted to identify antigenic determinants by their reaction with antibody rather than by their ability to induce antibodies. In all, four antigenic determinants have been identified and variation has been shown to occur at three of the sites in heterologous viruses of the same serotype.

METHODS

Virus strains. The principal viruses used in this study were Pacheco (originally a 1964 South American field strain, type O subtype I) and mutants isolated from it (McCahon et al., 1977). In addition, three heterologous type O viruses were used, BFS 1860 (a 1967 British field strain, subtype 1) and two older field strains that had been passaged repeatedly in the laboratory, either principally in tissue culture (V1, subtype 6) or the suckling mouse (M11, subtype 1).

Preparation of antigens. Viruses were grown in BHK cells and purified as described by King & Newman (1980). Similarly, the labelling of the virus with [35S]methionine was as described by these authors. All preparations were stored as sucrose gradient materials at −20 °C.

Trypsin treatment of virus and 12S subparticles was as described by King & Newman (1980). Where necessary, electrofocusing analysis, as described by these authors, was used to confirm that cleavage of the appropriate polypeptides had occurred in a quantitative fashion.

12S subparticles were prepared by incubating sucrose gradient fractions of purified virus with an equal volume of 0.1 M-phosphate buffer (pH 6.2) at 55 °C for 30 min. From analysis on sucrose gradients with proteins of known sedimentation coefficient (7.5S and 10S) it was estimated that at least 99% of radioactively labelled 146S particles were converted to 12S subparticles by this procedure.

For complete disruption of 126S particles into their constituent polypeptides (for detection of VP3 antigenic determinant) the gradient fractions containing virus were centrifuged to produce a virus pellet which was then resuspended in a lysis buffer (50 mM- Tris, 5% (v/v) 2-mercaptoethanol, 0.01 M-EDTA, 0.125 M-Tris, 0.1 M-HCl, and incubating at room temperature (approx. 20 °C) for 2 h. About 30% of the polypeptide present in the gel was eluted by this technique. Where necessary, the pH of polypeptide preparations, whether isolated polypeptides or disrupted virus, was adjusted to approximately 7.5 before being used in radioimmunoassay competition tests to avoid disruption of the competing antigens.

Preparation of antisera. For preparation of polypeptide-specific antisera the three major polypeptides of the virus (VP1, 2 and 3) were isolated from electrofocusing gels (see above). Two rabbits were used to raise antisera against each of the three polypeptides. Each rabbit received approx. 200 µg of polypeptide suspended in a modified non-ulcerative Freund’s adjuvant (kindly provided by Dr B. A. Morris, University of Surrey). This
emulsion was given intradermally at multiple sites on the back. A second dose of approx. 150 µg was given in aqueous phase intravenously to the ear 35 days after the first inoculation. In every case the serum taken at 7 days after the second inoculation was used.

For native antigen antisera three antigens were used: (i) whole virus purified as described above; (ii) purified virus treated with trypsin as described above and purified in a second sucrose gradient; (iii) 12S subparticles prepared from purified whole virus as described above and purified in a second sucrose gradient. Two rabbits were used to prepare antisera to each of these three antigens. Each rabbit was given 100 µg of freshly prepared whole virus or its equivalent in 12S or trypsin-treated virus. The antigen was suspended in incomplete Freud's adjuvant and given as a single dose intramuscularly in the rear leg. A second dose of freshly prepared antigen (100 µg of whole virus or its equivalent) was administered in aqueous phase intravenously to the ear at 42 days after the first inoculation.

For trypsin site-specific-absorbed serum preparation one of the anti-whole virus sera described above was diluted 1:500 in Dulbecco's phosphate-buffered saline containing 0.5% fraction V bovine serum albumin, pH 7.2 (PBS-BSA), containing 0.4% normal rabbit serum. Then 500 µl diluted serum was mixed with 5, 16, 0.5 or 0 µg of freshly prepared trypsin-treated virus purified on a second sucrose gradient (see above). After incubation at 37 °C for 1 h followed by 4 °C for 72 h, the antigen–antibody complexes plus any excess antigen were removed by centrifugation (45000 rev/min for 90 min in a Beckman SW50.1 rotor). The supernatant fluids were titrated by the radioimmunoprecipitation test (RIP) against both untreated and trypsin-treated virus to determine the proportion of antibodies that had been removed by absorption. Only the sample which had been absorbed with 5 µg trypsin-treated virus failed to react with trypsin-treated virus in the RIP test and therefore was subsequently used as it was specific for the sites destroyed by trypsin.

Neutralization assay. This was performed as a plaque assay on 90 mm diam. BHK monolayers as described by McCahon & Slade (1981). Twofold dilutions of antisera were mixed with sufficient Pacheco virus (parental t°) to give 250 to 300 p.f.u. per 0.4 ml of mixture, when no neutralization occurred. The mixtures were incubated for 1 h at 37 °C (0.4 ml mixture per plate, 2 plates per serum dilution). The neutralization titre was taken as that dilution which allowed 50% survival of virus.

Radioimmunoprecipitation (RIP) tests. Twofold dilutions of antisera were prepared in PBS-BSA buffer containing 0.4% normal rabbit serum, and 50 µl volumes were transferred to the wells of a conical-bottomed microtitre plate. Radioactively labelled virus antigen diluted in PBS-BSA buffer was then added (50 µl containing 2000 to 4000 cts/min). Finally, 50 µl of PBS-BSA buffer was added to each well and the plates incubated for 1 h at 37 °C. To precipitate the antigen–antibody complexes, 25 µl of goat anti-rabbit serum (Wellcome Reagents, Beckenham, Kent, U.K.) diluted 1:25 in PBS-BSA buffer was added to each well and the resulting mixtures were then centrifuged for 1 h at 37 °C followed by 18 h at 4 °C. The plates were then centrifuged for 30 min at 1800 rev/min and the amount of radioactivity in the precipitates was estimated indirectly by measuring the radioactivity remaining in the supernatant fluid (100 µl on filter paper discs counted in a liquid scintillation counter).

Simultaneous competition radioimmunoassay (RIA) tests. This test was used to examine the ability of different antigens or different forms of antigen to compete with radioactively labelled antigen for antibody. Routinely, 1-fold dilutions of unlabelled competing antigen in PBS-BSA buffer were mixed with an equal volume (50 µl) of labelled antigen in the same diluent in conical-bottomed microtitre plate. A 50 µl volume of antisera diluted in PBS-BSA buffer containing 0.4% normal rabbit serum was then added (sufficient to precipitate between 70 and 80% of the labelled antigen in the absence of unlabelled competing antigen, estimated in RIP test). After incubating the mixtures for 1 h at 37 °C, the antigen–antibody complexes were precipitated by the use of anti-rabbit serum and the radioactivity in the precipitates estimated as for the RIP test. However, in tests using labelled disrupted virus as antigen (e.g. detection of VP3 antigenic determinant) it was necessary to measure directly the label associated with the immune precipitate because of the high background radioactivity due to the other labelled but non-reacting polypeptides (VP1, 2 and 4) in the antigen. In such tests the immune precipitates were drained and washed twice with 250 µl volumes of PBS-BSA buffer per well. Finally, each precipitate was dissolved in 100 µl lysis buffer as used for preparation of disrupted virus (see above) and counted for radioactivity.

The results were used to estimate the percentage inhibition of the binding of labelled antigen to antibody by varying concentrations of labelled competing antigen. The amount of radioactively representing 0% inhibition of binding was estimated from controls in which competing antigen was absent, and that representing 100% inhibition estimated from controls with antibody absent. The percentage inhibition was plotted against logₐ concentration of unlabelled competing antigen and expressed in µg/ml protein.

**RESULTS**

**Specificity and reactivity of polypeptide antisera**

For simplicity in presentation the results are scored in Table 1 as (+) or (−) but are based on RIA tests as shown in Fig. 3 or RIP tests as shown in Fig. 1. Each antiserum was shown to be
Two antigenic determinants on VP1 have been identified using the anti-VP1 serum (Fig. 1). The major one (called the VP1-A determinant) was present on the intact virus capsid and was specific for the antigen against which it was raised by an RIA test in which isolated VP1, 2 and 3 were allowed to compete with labelled disrupted virus for antiserum. The three types of antisera differed in their ability to react with different forms of antigen, e.g. only the anti-VP1 sera were able to react with intact virus particles, which is in agreement with the results of Bachrach et al. (1975) and Meloen et al. (1979), both groups having used type A viruses. Since, in each case, both antisera raised against a polypeptide gave the same result, only one antiserum to each polypeptide was used in subsequent work. Using these three sera, four different antigenic determinants were defined.

**Antigenic determinants on VP1**

Two antigenic determinants on VP1 have been identified using the anti-VP1 serum (Fig. 1). The major one (called the VP1-A determinant) was present on the intact virus capsid and was...
FMD virus antigenic determinants

Table 2. Ability of various antigens to induce neutralizing antibodies

<table>
<thead>
<tr>
<th>Immunogen*</th>
<th>Rabbit</th>
<th>Neutralization titre (log_{10})† after</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>First dose</td>
</tr>
<tr>
<td>146S particles</td>
<td>1</td>
<td>5.2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>ND</td>
</tr>
<tr>
<td>146S trypsin-treated</td>
<td>1</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3.4</td>
</tr>
<tr>
<td>12S subparticles</td>
<td>1</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4.6</td>
</tr>
<tr>
<td>Isolated VP1</td>
<td>1</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.3</td>
</tr>
<tr>
<td>Isolated VP2</td>
<td>1</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Isolated VP3</td>
<td>1</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>&lt;1.0</td>
</tr>
</tbody>
</table>

* Purified materials either from sucrose gradients or electrofocusing gels.
† Reciprocal of dilution required to give 50% neutralization of 250 to 300 p.f.u. Rabbits were given two inoculations of antigen separated by approx. 35 days. Sera were examined 21 to 28 days after the first inoculation and 14 days after the second inoculation.
‡ ND, Not done.

destroyed by trypsin treatment of purified virions. The effect of trypsin appeared to be total in that there was no detectable precipitation of the treated virus even at serum concentrations 300 times higher than that required to precipitate 50% of the untreated virus. When the treated virus was disrupted to 12S subparticles a minor antigenic determinant (called the VP1-B determinant) was exposed. Unlike the VP1-A determinant, this new determinant was not destroyed by trypsin treatment of the 12S subparticles (data not shown). From RIP tests on five independently prepared radioactively labelled antigens, this anti-VP1 serum was shown to contain at least three times as much antibody to the VP1-A determinant as to the VP1-B determinant. Therefore, in this rabbit the VP1-B determinant was clearly less immunogenic than the VP1-A determinant and the majority of antibodies in the serum were directed against the VP1-A determinant.

The VP1-A determinant contains a determinant involved in virus neutralization

The VP1-A determinant is defined as the determinant on the intact particle that reacts with the anti-VP1 serum. As had been found previously with other aphthovirus strains (Laporte et al., 1973; Bachrach et al., 1975; Kaaden et al., 1977; Meloen et al., 1979) this anti-VP1 serum was able to neutralize virus infectivity (Table 2). Since infectivity is a function of the intact particle, it follows that the VP1-A determinant must contain at least one determinant involved in the neutralization of virus infectivity. Consistent with this idea is the observation that trypsin treatment destroyed both the reactivity of the VP1-A determinant (Fig. 1) and the ability of the virus particle to induce neutralizing antibodies (Table 2).

Antibodies to VP1-A are present in large amounts in anti-whole virus sera

Further evidence of the importance of the VP1-A determinant in the immunogenicity of the virus particles was provided by examination of sera prepared against intact virus particles. Six sera (three serum samples from each of two rabbits) were examined for their reactivity with untreated and trypsin-treated virus in an RIP test. All the sera possessed some reactivity against trypsin-treated virus and the titres varied between 25 and 70% of the titre seen for the same serum with untreated virus. Therefore, it would appear that a large proportion of the antibodies against whole virus were recognized by the trypsin-sensitive region showing that it contains an important immunogen for the virus. To examine further the specificity of such antibodies, a small sample of one of these sera was absorbed with an excess of trypsin-treated virus as described in Methods and used in simultaneous competition RIA tests (Fig. 2). The serum was
now specific for trypsin-sensitive sites on the intact virus (Fig. 2a). Of the three polypeptides only VP1 inhibited binding and the inhibition reached 80% with the maximum amount of VP1 used (Fig. 2b). Therefore, most, if not all, of the antigenic sites that induced such antibodies were contained within VP1. However, VP1 was obviously less efficient than virus particles at inhibiting binding (63 and 0.9 µg/ml of VP1 protein in isolated VP1 and in virus particles respectively, gave 50% inhibition). In addition, this absorbed serum was shown to have the same antigen specificity as the anti-VP1 serum, in that it was able to discriminate between closely related heterologous viruses (compare Fig. 2c with Fig. 5). Therefore, we conclude that antibodies to VP1-A are present in significant amounts in anti-whole virus sera which is further evidence of the importance of this determinant.

**VP1-A determinant is present on 12S and isolated VP1**

Simultaneous competition RIA experiments showed that the VP1-A determinant was present on 12S and isolated VP1 and was able to compete efficiently with intact virus particles (146S) (Fig. 3). Neither the slopes of the dose–response curves nor the protein concentrations required to inhibit 50% of binding differed significantly at the 5% level (Student’s t-test). Therefore, it appears that no significant alteration in the number or reactivity of VP1-A determinants occurred when the 146S particles were broken down to 12S subparticles. This interpretation is
supported by the fact that 12S particles were quite efficient at inducing neutralizing antibody (Table 2).

Examination of laboratory mutants for alterations in the VP1-A determinant

Forty-six mutants derived from Pacheco were screened for difference at the VP1-A determinant using a simultaneous competition RIA experiment (Fig. 4a, c) in which mutant virus was allowed to compete with radioactively labelled parental virus for anti-VP1 serum. The majority of the mutants were temperature-sensitive (ts) and all had been shown either by electrofocusing (King & Newman, 1980; King et al., 1980) or by recombination studies (McCahon et al., 1977) to be located in or near the region of the genome coding for the capsid polypeptides. No mutants with altered reactivity were found.

The VP1-B determinant is a single antigenic site

This conclusion was derived from examining 39 of the laboratory mutants as described for the VP1-A determinant but using trypsin-treated 12S particles as the competing antigen. Most of the mutants had normal VP1-B activity; these included one mutant ts28 which was known to have a ts lesion in VP3 (King et al., 1980). However, two ts+ revertants of ts28 (R28 and R30) carried an extragenic suppression mutation in VP1 which affected their reactivity at the VP1-B determinant (Fig. 4b, d). The VP1 of these two revertants differed from the VP1 of their parent virus, ts28, by an electrophoretic charge that corresponded to a single amino acid substitution (King et al., 1980). Since a single amino acid substitution was able to render the antigen unrecognizable to the serum, it follows that the VP1-B determinant is a single antigenic determinant on the VP1 molecule.
Fig. 4. Examination of laboratory mutants for antigenic change in VP1. Purified 146S preparations of mutant virus were allowed to compete with radioactively labelled parental virus for anti-VP1 serum in simultaneous competition RIA tests. VP1-A determinant: labelled parental virus versus mutant ts28 (a) or ts28 revertant R30 (c). VP1-B determinant: 12S prepared from trypsin-treated labelled parental virus versus mutant ts28 (b) or ts28 revertant R30 (d).

Table 3. Effect of proteolytic cleavage of VP1 and VP2 on their antigenic sites

<table>
<thead>
<tr>
<th>Antigen*</th>
<th>Anti-VP1 serum</th>
<th>Anti-VP2 serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>12S subparticle (VP1 intact, VP2 intact)</td>
<td>1:11000</td>
<td>1:2900</td>
</tr>
<tr>
<td>12S subparticle from trypsin-treated virus (VP1 cleaved, VP2 intact)</td>
<td>1:1200</td>
<td>1:2300</td>
</tr>
<tr>
<td>Trypsin-treated 12S subparticle (VP1 cleaved, VP2 cleaved)</td>
<td>1:950</td>
<td>1:70</td>
</tr>
</tbody>
</table>

* Antigens were analysed on electrofocusing gels to confirm proteolytic cleavage.
† Dilutions of antiserum required to precipitate 70% of different forms of antigen from the same labelled virus preparation.

Antigenic determinants on VP2

VP2 antisera only reacted with 12S subparticles and completely disrupted virus (Table 1). VP2 is not cleaved by trypsin treatment of the intact particle but it is cleaved when 12S subparticles are exposed to trypsin (King & Newman, 1980). This cleavage of VP2 drastically reduced the ability of the 12S particle to react with anti-VP2 serum (Table 3). The residual activity of the anti-VP2 serum (1/70) was probably not due to another antigenic determinant but rather to the presence of a small amount of uncleaved VP2 which could be seen when this trypsin-treated 12S preparation was analysed on electrofocusing gels (not shown). Simultaneous competition RIA experiments similar to those shown in Fig. 3 demonstrated that isolated VP2 was similar to the 12S subparticle in competing for antibody (3·3 μg/ml of isolated VP2 versus a calculated 1·0 μg/ml VP2 in 12S subparticles was required to produce 50% inhibition of binding). Therefore, using this serum, there appears to be a single antigenic determinant (called the VP2 determinant) on VP2 which is fully exposed on 12S subparticles and is trypsin-sensitive.

Thirty-three structural protein mutants were screened for differences in the VP2 antigenic determinant (as described for VP1 determinants) but no differences were found.
**Antigenic determinants on VP3**

Only one determinant was demonstrated on VP3. It was only detectable when the virus was completely disrupted (see Table 1) or on isolated VP3. No attempt was made to screen structural protein mutants for changes in the VP3 antigenic determinant.

**Examination of naturally occurring strains for variation in the antigenic determinants**

Simultaneous competition RIA tests were used to examine three heterologous type O strains for variation at each of the four antigenic determinants defined for the Pacheco strain. By serological tests, such as complement fixation and neutralization of virus infectivity, BFS 1860 was shown to be most closely related to Pacheco, and M11 was the next most closely related (J. R. Lake & D. McCahon, unpublished results). The third heterologous virus, VI, is the most different from Pacheco, and is a different serological subtype (subtype 6) from the other three viruses (subtype 1). Antigenic variation was observed in determinants VP1-A, VP1-B and VP2 but not in the VP3 determinant (Fig. 5).

**DISCUSSION**

Four antigenic determinants have been identified in the virus capsid but only one, the VP1-A determinant on VP1, is likely to be of epidemiological importance. This is because it was the only determinant detected on the intact virus particle which is the predominant form of antigen used in the serological identification of virus strains. This VP1-A determinant is present in the critical area of the capsid which is trypsin-sensitive and in the accompanying paper (Haresnape et al., 1983) evidence is presented showing that it consists of a single antigenic determinant which is located in a region of VP1 that is uniquely sensitive to several proteases. The antigenic importance of the trypsin-sensitive region has been reviewed in the Introduction and is further substantiated here for this virus by the demonstration that it is important for the induction of neutralizing antibodies (Table 2), and that a significant proportion of the antiviral antibodies present in whole virus antisera are directed against that region. That the VP1-A determinant is
an antigenically important part of the trypsin-sensitive region was shown by the fact that (i) the VP1 serum contained neutralizing antibody, (ii) the antibody left in a whole virus antiserum after absorption with trypsin-treated virus had the same specificity as the VP1 antiserum and (iii) variation at this site occurred in heterologous viruses. Therefore, we conclude that the VP1-A determinant is an important antigen on the surface of the virus particle.

The demonstration that the VP1-A determinant was not reduced in activity or number when the virus particle was disrupted to 12S subparticles was surprising if VP1-A were an important antigenic determinant, since the general consensus is that a drastic reduction in immunogenicity occurs under those conditions (Doel & Chong, 1982; Cartwright et al., 1982). Both these groups of workers found that a single dose of 12S subparticles failed to induce any neutralizing antibodies in guinea-pigs whereas an equivalent dose of 146S particles gave titres of $10^{2.8}$ to $10^{2.9}$ per 0.15 ml serum. By contrast, in this study using a different strain, a single dose of 12S subparticles in rabbits was able to induce good neutralizing antibody titres comparable to those produced by whole virus (see Table 2). There are two possible reasons for these conflicting results. (i) The VP1-A determinant is important but unusually stable in this strain, or (ii) there is a trivial reason such as the species of animal used to produce the antibody.

It is interesting that of the three other antigenic determinants, only the two which were on the surface of the 12S subparticle (VP1-B and VP2) were variable. Since they are internal sites in the intact virion it is unlikely that they reflect the selection of antigenic variants under the influence of antibody. A more likely explanation is that, like the VP1-A determinant in the intact particle, they are in regions on the surface of the subparticle where mutation can occur without seriously affecting the function(s) of the intact virus particle. This would be consistent with the failure to detect antigenic variation in the $ts$ mutants at any of these sites, since in order to produce their pronounced biological effect, the $ts$ mutations would need to be in regions that are critical to the function(s) of the particle. Since VP1-B is clearly variable among field isolates, it is interesting to speculate that perhaps that determinant corresponds to the second highly variable site (amino acid positions 40 to 60) defined from comparative sequence studies by Beck et al. (1983).

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