Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 5883–5887, August 1989
Genetics

Implications of a quasispecies genome structure: Effect of frequent, naturally occurring amino acid substitutions on the antigenicity of foot-and-mouth disease virus

(RNA evolution/epitope/virus neutralization/synthetic peptides)

M. G. MATEU*, M. A. MARTÍNEZ†, E. ROCHA*, D. ANDREU‡, J. PAREJO‡, E. GIRALT‡, F. SOBRINO†, AND E. DOMINGO*§¶

*Centro de Biología Molecular, Universidad Autónoma de Madrid, Canto Blanco, 28049 Madrid, Spain; †Departamento de Sanidad Animal, Instituto Nacional de Investigaciones Agrarias, c. Embajadores 68, 28012 Madrid, Spain; and ‡Departament de Química Orgànica, Universitat de Barcelona, 08028 Barcelona, Spain

Communicated by Manfred Eigen, May 3, 1989

ABSTRACT **We provide evidence that the quasispecies nature (extreme genetic heterogeneity) of foot-and-mouth disease virus is relevant to the virus evading an immune response. A monoclonal antibody neutralizing the viral infectivity (clone SD6) recognizes an epitope located around a highly conserved sequence (amino acid sequence Arg-Gly-Asp-Leu-Ala at positions 141–145) in the capsid protein VP1 of foot-and-mouth disease virus of serotype C_1. The amino acid substitutions Ala-138 → Thr and Leu-147 → Ile (or → Val) reduced 100-fold the binding titer of monoclonal antibody SD6 to virions or to VP1. The effect of those substitutions was quantitatively reproduced with synthetic peptides representing the relevant sequences. This provides evidence that the two chemically conservative amino acid replacements—and not other substitutions present in the virus quasispecies—are responsible for the modified interaction with neutralizing monoclonal antibody SD6. The three substitutions were fixed in the viral capsid during one occurrence of foot-and-mouth disease and, furthermore, they are of a type found frequently among independent foot-and-mouth disease virus isolates. The results implicate the extreme heterogeneity of foot-and-mouth disease virus as an important element of viral pathogenesis.**

Foot-and-mouth disease virus (FMDV) is a picornavirus that causes the economically most important viral disease of cattle and other cloven-hooved animals (1, 2). As for most other RNA genomes, FMDV populations are genetically heterogeneous (3–5) and show the potential for very rapid evolution (6, 7). A direct consequence of the genetic variability of FMDV is its remarkable antigenic diversity (1, 2). Attempts to quantify such diversity and classify FMDV isolates have included the early serological subtyping (8) and the more recent analyses of reactivity with monoclonal antibodies (mAbs) (9–11). Knowledge of the types and frequency of occurrence of amino acid substitutions that lead to variations in viral epitopes is relevant to the design of new synthetic vaccines, whose efficacy may be hampered by the rapid antigenic variation of RNA viruses in nature (7, 10–12). For FMDV, several independent approaches have indicated that a major antigenic determinant involved in neutralization of viral infectivity is located at the carboxyl-terminal half of capsid protein VP1 and, more specifically, around positions 140–160 (13–17). The three-dimensional structure of FMDV of serotype O_1 (18) suggests that the VP1 segment around positions Thr-133 → His-154 of FMDV C-S8c1 (19) is an exposed loop. This VP1 region shows remarkable variation among FMDVs of the same or different serotype (5, 19–23).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

In recent years, we have emphasized that FMDV shows a quasispecies structure (3–5), probably shared by most other RNA genetic elements (6, 7, 12, 24). This concept, which originated in theoretical work by Eigen and his colleagues (25–27), was first shown experimentally to adequately describe populations of phage Qβ (28) and later other RNA genomes as well (reviewed in refs. 6, 7, 12, and 24). According to the quasispecies structure, each FMDV genome population includes one or several "master" sequence(s)—which may nevertheless represent a small proportion of molecules (4, 24, 28)—and a "mutant spectrum" consisting of a distribution of single and multiple residue mutants (4, 12, 24, 27, 28). The proportion of each mutant depends on the frequency with which it arises by mutation and on its competitive growth with all other variants (present and arising) in the replicating population (26–28). Mutant viruses with chemically conservative amino acid substitutions, prone to behave in a quasineutral fashion, are more likely to be represented in a viral quasispecies than variants with mutations that adversely affect fitness even if viable, as documented with phage Qβ (28). In the present study we show that chemically conservative amino acid substitutions fixed at the main antigenic determinant of FMDV C during one epizootic—and that are also found frequently among other FMDV isolates—greatly affected the interaction with one neutralizing mAb (n-mAb), SD6. This antibody recognizes an epitope surrounding a conserved amino acid sequence of VP1, proposed to be a receptor binding site (17, 18, 44). The effect of these naturally occurring amino acid changes has been mimicked with substituted synthetic peptides, which bind n-mAb SD6 to the same extent as complete variant viruses, thus providing an *in vitro* assay for epitopic variation of FMDV.

MATERIALS AND METHODS

Viruses. The origin of the FMDV field isolates (C-S isolates) has been described (19, 29, 30). Plaque-purification was by dilution and plating on BHK-21 cell monolayers as described (4). Each plaque isolate (10^4–10^5 plaque-forming units) was amplified to about 10^8 plaque-forming units by infection of BHK-21 cells, and virions were purified as described (3, 4). The same viral preparation was used for RNA sequence determination and for immunological assays.

mAb SD6-Resistant Mutants. To ensure that each antibody-resistant mutant originated from an independent mutational event, each mutant was selected from virus derived from a

Abbreviations: FMDV, foot-and-mouth disease virus; mAb, monoclonal antibody; n-mAb, neutralizing mAb.

§Present address: University of California, San Diego, Department of Biology C-016, La Jolla, CA 92093.
¶To whom reprint requests should be addressed.

different clone; all clones were from the same plaque-purified FMDV C-S8c1 preparation (4). Virus resuspended from individual plaques was amplified to 10^5–10^6 plaque-forming units by infection of about 10^5 BHK-21 cells, incubated for 2 hr at 4°C with a 1:1 dilution of the supernatant of the hybridoma culture, and plated (4) with a 1:50 dilution of the same supernatant included in the agar overlay. From each plate, virus from one single plaque was isolated, and its resistance to the antibody was tested [no detectable reduction in the number of plaques in the neutralization assay (10)], amplified, and purified as required for immunological assays (10) or for nucleotide sequencing (19).

Synthetic Peptides. A series of peptides representing positions 133–156 (A24 in Fig. 1), 138–156 (A19), 144–156 (A13), 148–156 (A9), and 150–156 (A7) of VP1 of FMDV C-S8c1 were synthesized by the solid-phase method (31) on a phenylacetamidobenzyl-resin (32). Peptides of this same region with selected amino acid substitutions present in variant FMDV C-S isolates (peptides A19I; A19V; A21T; A21T,I; see Fig. 2 for details) were assembled on a phenylacetamidomethyl-resin (33). All peptides had an additional cysteine residue at their carboxyl-terminal position (not indicated in figures) to facilitate conjugation. After hydrofluoric acid cleavage, peptides were purified to homogeneity by medium-pressure liquid chromatography on octadecylsilica, characterized by amino acid analysis and HPLC, and then coupled to keyhole limpet hemocyanin by means of the 3′-maleimidobenzoic acid *N*-hydroxysuccinimide ester procedure (34). Peptide-to-hemocyanin ratios were determined by amino acid analysis and were in the 1000–2000 molar range.

Immunological Assays. Enzyme-linked immunoelectrotransfer blot, enzyme-linked immunodot, and plaque-reduction neutralization assays were as described (10). The same amount of each peptide was used, assessed by reactivity with one or a mixture of mAbs (4G3 7CA8, 7JD1) known to react with different epitopes highly conserved in FMDV C (11).

Nucleotide Sequencing. FMDV VP1 RNA was sequenced by oligodeoxynucleotide primer extension and dideoxy chain-termination, with viral RNA as template, following published procedures (4, 29).

RESULTS

The Epitope Recognized by n-mAb SD6 Involves Two Hypervariable Regions Surrounding a Highly Conserved One. n-mAb SD6, raised against FMDV C-S8c1, reacted with a synthetic peptide representing amino acids 138–156 of VP1 (10). To delimit the site of interaction, the reactivity of this antibody was assayed with an overlapping series of peptides of increasing length (Fig. 1). Although n-mAb SD6 reacted with peptide A19, it did not bind to peptide A13, suggesting that at least part of the epitope recognized by this mAb lies within amino acid segment 138–144. In contrast, n-mAb 4G3 reacted with peptides A19 and A13 (Fig. 1). To define by an independent procedure the VP1 residues involved in the interaction with antibody SD6, 33 mAb SD6-resistant mutants of FMDV C-S8c1 were obtained and their RNA was analyzed by nucleotide sequencing (Table 1). The deduced amino acid sequences show that six different substitutions at position 138, 139, or 146 affected the reactivity of the complete virus or its isolated VP1 with n-mAb SD6 (compare Fig. 1 and Table 1).

Naturally Occurring Amino Acid Substitutions Greatly Affect the Binding of mAb SD6 to FMDV of Serotype C. The availability of a series of epidemiologically closely related, but nonidentical, FMDV field isolates (19, 29) permitted an evaluation of the effect of amino acid substitutions that had been fixed in the course of one occurrence of foot-and-mouth disease on the reactivity of a single epitope, recognized by n-mAb SD6 (Table 2). Binding and neutralization assays were carried out with complete virions, and the former were also carried out with isolated VP1 to exclude a possible effect of substitutions in other capsid proteins on the reactivity. Since substitutions Ala-140 → Thr and Thr-149 → Ala present in FMDV C-S20 and its clonal derivative C-S20c4 had no effect on n-mAb SD6 binding, the results suggest that Ala-138 → Thr or Leu-147 → Ile, or the two together, caused the 100-fold decrease in reactivity seen with most FMDV C-S isolates from 1981–1982 (ref. 10 and Table 2). Such chemically conservative amino acid substitutions are likely to be represented in the mutant spectrum of any FMDV quasispecies (24, 27). Note that Thr-148 → Ala is found in the mutant spectrum of FMDV C-S30, since the corresponding mutation was present in clone C-S30c4, but was undetectable in the uncloned viral population C-S30 (Table 2). Also, the positive reactivity with n-mAb SD6 of FMDV C-S33 remained a puzzle until the clonal derivative C-S33c2 yielded the expected negative n-mAb SD6 binding, suggesting heterogeneity in FMDV C-S33. To derive meaningful correlations between amino acid sequences and immunological reactivities, it was imperative to use the same cloned viral preparation for the two types of analyses. Passage affected the average sequences in some instances. For example, in the previously

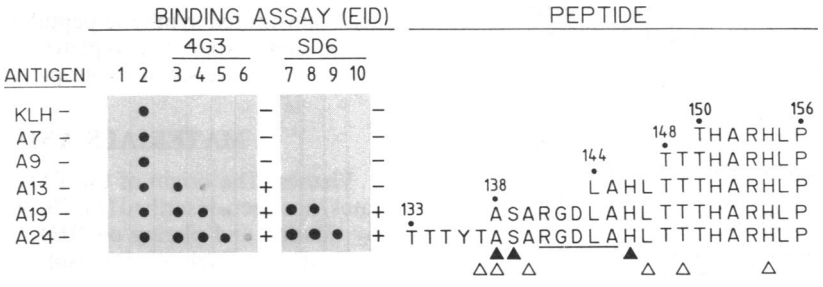

FIG. 1. Reactivity of n-mAbs 4G3 and SD6 with synthetic peptides representing segments of VP1 of FMDV C-S8c1. Keyhole limpet hemocyanin (8 μg) or the indicated peptides A7 to A24 (500 pmol) conjugated to keyhole limpet hemocyanin (8 μg in each assay) were applied to nitrocellulose and allowed to react in an enzyme immunodot (EID) assay as described (10). Lane 1, no first antibody added; lane 2, anti-keyhole limpet hemocyanin serum; lanes 3–6, mAb 4G3 (supernatant of hybridoma culture diluted 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}, respectively); lanes 7–10, mAb SD6 (supernatant of hybridoma culture diluted 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}, respectively). +, Binding above background was obtained by a 100-fold or higher dilution of supernatant of hybridoma culture under the assay conditions (10). −, No signal above background with undiluted supernatant. No signal was observed when no antigen was added to the nitrocellulose sheets. Solid triangles below the amino acid sequence of A24 indicate variant residues in mAb SD6-resistant mutants (compare Table 1). One resistant mutant includes two substitutions (Table 1), and we have no evidence that Thr-149 → Met (not indicated below the sequence) contributed to the resistant phenotype. Open triangles indicate variant positions in the natural FMDVs analyzed (C-S isolates); the substitution Thr-148 → Ala in FMDV C-S35 was found only after further passage of the virus in BHK-21 cells and it is not included (compare Table 2). Underlined residues are conserved in all FMDV isolates of serotype C analyzed to date (19, 29, 30, 35, 36). The single-letter amino acid code is used: A = alanine, D = aspartic acid, G = glycine, H = histidine, L = leucine, P = proline, R = arginine, S = serine, T = threonine, Y = tyrosine.

Table 1. mAb SD6-resistant mutants of FMDV C-S8c1

Mutation	Amino acid substitution	Number of mutants
G (412) → C	Ala-138 → Pro	1
C (413) → A	Ala-138 → Asp	1
A (415) → C	Ser-139 → Arg	2
G (416) → A	Ser-139 → Asn	4
G (416) → T	Ser-139 → Ile	6
T (417) → A	Ser-139 → Arg	2
T (417) → G	Ser-139 → Arg	4
A (437) → G	His-146 → Arg	12
A (437) → G C (446) → T	His-146 → Arg Thr-149 → Met	1

The amino acid substitutions found in each of 33 mAb SD6-resistant mutants, and the corresponding mutations, are indicated. The VP1-coding region of one resistant mutant of each of the groups Ser-139 → Arg, Ser-139 → Asn, Ser-139 → Ile, and His-146 → Arg was sequenced in its entirety. For all other mutants the segment encoding the carboxyl-terminal half of VP1 was sequenced. Nucleotide numbering is from refs. 19 and 29. Amino acid positions are also depicted in Fig. 1.

sequenced FMDV C-S35 (29), position 148 was threonine, whereas in the present preparation derived from the same initial stock, position 148 was alanine (Table 2). Because of these rapid fluctuations in amino acid composition, expected from the quasispecies nature of FMDV (3, 4, 12), and because a positive correlation between reactivity with n-mAb SD6 and some VP1 amino acids outside segment 133–156 was also observed (compare Table 2), it was important to ascertain that those amino acid changes at positions 138 and/or 147—and not other alterations in VP1—were the direct cause of the modified antigenicity.

The Effect of Substitutions on FMDV Antigenicity Is Quantitatively Reproduced with Synthetic Peptides. Synthetic peptides that included the desired amino acid substitutions were used in enzyme immunodot assays. The binding titer of mAb SD6 with FMDV C-S8 peptides incorporating substitution Ala-138 → Thr or Leu-147 → Ile was about 10-fold lower than with unsubstituted peptides. If Ala-138 → Thr and Leu-147 → Ile were both present, as in seven of the FMDV C-S isolates (Table 2), the decrease in titer was >100-fold (Fig. 2), mimicking the effect seen with complete variant viruses (Fig. 2 and ref. 10). Leu-147 → Val had an influence comparable to Leu-147 → Ile (Fig. 2), as expected from the decreased reactivity of FMDV C-S30 (Table 2), suggesting subtle influences of the alkyl groups present in leucine, isoleucine, and valine on the antigen–antibody interaction. Fig. 2 also shows that none of the substitutions tested affected the reactivity of the peptides with n-mAb 4G3, which recognizes a different epitope in segment 144–156 of VP1 (Fig. 1), again paralleling the results with complete virions (compare Fig. 2 and ref. 10).

We conclude that chemically conservative amino acid substitutions that occur frequently in the quasispecies distribution of FMDV genomes (see *Discussion*), and that were fixed in the course of one epizootic, strongly decreased the affinity of the virus for a n-mAb.

DISCUSSION

Difficulties for the development of synthetic vaccines are encountered with most RNA viruses, including retroviruses such as human immunodeficiency virus (reviewed in ref. 12). They all are quasispecies distributions of genomes (3–7, 12, 24–28), and any viral population, whether clonal or not, embodies a "range" of phenotypes (6, 12, 24). Mutations that are neutral or quasineutral with regard to virus viability in the considered environment are likely to be present in higher proportions in a viral quasispecies (24, 27). In the present study we have shown that chemically conservative amino acid substitutions, which have occurred frequently during the spread of FMDV, strongly affected FMDV antigenicity. Alignment of the amino acid sequences of 20 isolates of FMDV C_1 and C_3 shows that there are variations in 40

Table 2. Amino acid sequence of VP1 segment 133–156 of FMDV (isolates C-S) and reactivity of VP1 with n-mAb SD6

FMDV*	Isolation date (month/year) of field isolate	Amino acid sequence of VP1 residues 133–156†	Binding of mAb SD6 to VP1‡
		133 138 140 147 149 156	
C-S8	2/1970	T-T-T-Y-T-A-S-A-R-G-D-L-A-H-L-T-T-T-H-A-R-H-L-P	+
C-S8c1	—		+
C-S9	1/1977	A Y	+
C-S10	1/1979		+
C-S14	12/1979		+
C-S18	2/1980	X	+
C-S20	7/1980	T A	+
C-S20c4	—	T A	+
C-S22	12/1980	T T I A	−
C-S22c2	—	T T I A	−
C-S21	1/1981	T T I A	−
C-S15	1/1981	T T I A	−
C-S15c1	—	T T I A	−
C-S16	1/1981	T T I A	−
C-S17	1/1981	T T I A	−
C-S30	1/1982	T T V A X	−
C-S30c4	—	T T V A A	−
C-S33	1/1982	T T I A X	+
C-S33c2	—	T T I A	−
C-S35	5/1982	T T I A A	−

*Clonal derivatives are indicated with the suffix c. VP1 of the listed C-S viruses differs from C-S8 in the following amino acid substitutions outside segment 133–156: Thr-3 → Ala in all except C-S22 and C-S22c2; Thr-3 → Ser in C-S22 and C-S22c2; Asp-31 → Asn, C-S30, C-S30c4; Val-35 → Ile, C-S22 to C-S35; Asp-46 → Gly, C-S20 to C-S35; Lys-58 → Arg, C-S14, C-S18; Asn-60 → Ser, C-S30, C-S30c4; Asn-99 → Asp, C-S20 to C-S35; Val-112 → Leu, C-S20 to C-S35. (Sequences are from refs. 19, 29, and 30.)

†Symbols for the single-letter amino acid code are given in the legend to Fig. 1; I = isoleucine, V = valine, X = undefined amino acid.

‡In an enzyme immunoelectrotransfer blot assay; +, binding was >70% of that obtained with FMDV C-S8; −, binding was <1% (see also ref. 10).

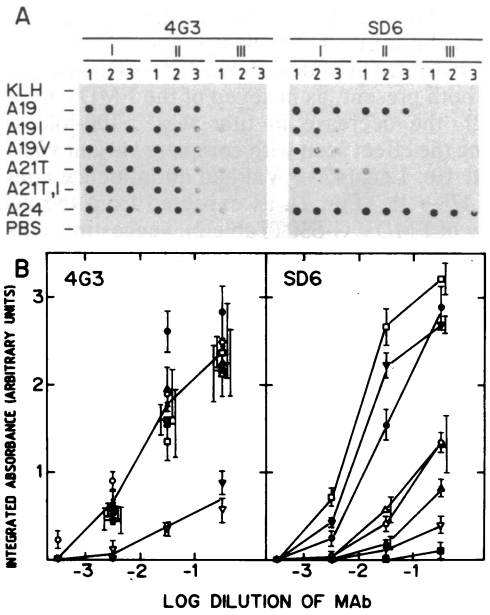

FIG. 2. (*A*) Reactivity of substituted synthetic peptides with n-mAbs 4G3 and SD6 in enzyme immunodot assays (two different experiments are shown in *A* and *B*). The following antigens plus 6 μg of bovine serum albumin in phosphate-buffered saline (PBS) were applied from top to bottom: keyhole limpet hemocyanin (KLH), 1.2 μg (lanes 1), 0.6 μg (lanes 2), and 0.3 μg (lanes 3). Peptides: 40 pmol of each peptide conjugated to 0.25–0.4 μg of hemocyanin (lanes 1), 20 pmol conjugated to 0.12–0.2 μg of hemocyanin (lanes 2), 10 pmol conjugated to 0.06–0.1 μg of hemocyanin (lanes 3). The peptides were as follows: A19 (unsubstituted, sequence in Fig. 1); A19I, A19 with Leu-147 → Ile; A19V, A19 with Leu-147 → Val; A21T, A21 with Ala-138 → Thr; A21T,I, A21 with Ala-138 → Thr, Leu-147 → Ile; A24 (unsubstituted, Fig. 1). PBS: no antigen added. The filters were subjected to an enzyme immunodot assay (10) with either mAb 4G3 or SD6. I, 3-fold dilution of supernatant of hybridoma culture; II, 30-fold dilution; III, 300-fold dilution. (*B*) Antigens used: 40 pmol of each of the following peptides conjugated to 0.25–0.40 μg of keyhole limpet hemocyanin. ●, A19 (unsubstituted); ○, A19I; ▲, A19V; △, A21T; ■, A21T,I; □, A24 (unsubstituted). Viruses (containing 20 pmol of VP1): ▼, FMDV C-S8; ▽, FMDV C-S16. The immunodot assays were performed with 10-fold serial dilutions of mAb 4G3 or SD6 (abscissa) and are plotted against the integrated absorbance from densitometric tracings of each dot (ordinate). Results are the average of five experiments, with standard deviations given on the graphs. For antibody 4G3, the two lines drawn indicate the mean absorbances obtained for each dilution of antibody with the six peptides and the two viruses, respectively. If no mAb was added or if 1.2 μg of keyhole limpet hemocyanin was used as antigen, the absorbance was undetectable.

positions and that in 12 of them the change is either Leu ↔ Ile, Leu ↔ Val, or Ala ↔ Thr (Table 2 and refs. 29, 35, 36; review in ref. 30). The study was possible because the strongly n-mAb SD6 recognized an epitope around VP1 positions 138–147 (Tables 1 and 2), where substitutions during a disease episode (Table 2) had occurred. In the immunodot assay, synthetic peptide 133–156 reacted with n-mAb SD6 to approximately the same extent as the equivalent molar amount of VP1 in complete virus (Fig. 2), suggesting that the epitope is completely included within residues 133–156 of VP1. In addition, since substitutions in peptides accurately reflected their effect on complete virions (Fig. 2), the immunodot test with conjugated peptides constitutes an *in vitro* assay for the effect of amino acid substitutions on FMDV antigenicity.

The analyses of FMDV natural variants and of n-mAb SD6-resistant mutants (Tables 1 and 2) indicate that residues 138, 139, 146, and 147 of VP1, located in two hypervariable regions (29, 35) that flank a conserved sequence, Arg-Gly-Asp-Leu-Ala, at positions 141–145 (compare Fig. 1 and ref. 37), are part of the epitope recognized by n-mAb SD6. This epitope spans at least 10 residues, a length that is greater than usually accepted for a linear epitope and agrees with the length of another epitope on FMDV of serotype A_{10} (38). Because of its resemblance to sequences involved in the interaction of fibronectin with cells, it has been proposed that sequence Arg-Gly-Asp may serve as the attachment site of FMDV to cells (17, 18, 44). If this were the case, the neutralizing activity of mAb SD6 could be explained by a blockade of the virion–cell interaction. We have also found that other n-mAbs produced against distant FMDVs of serotype C recognize nonidentical epitopes, each involving the hypervariable regions flanking the sequence Arg-Gly-Asp-Leu-Ala (unpublished results). The effect of amino acid substitutions Ala-138 → Thr and Leu-147 → Ile or Leu-147 → Val on recognition by n-mAb SD6 may be the result of the restriction of local mobility imposed by the additional bulky radicals on the β-carbon of threonine, isoleucine, and valine. This could reduce any induced fit involved in the antigen–antibody recognition. The structural differences between alanine and threonine or between leucine and isoleucine or valine are also evidenced by the fact that alanine and leucine are most frequently found in α-helices, whereas threonine, isoleucine, and valine are most frequent in β-sheets (39). Residues 138 and 147 may also directly interact with n-mAb SD6, the alkyl group of leucine being part of the active site. Thus, neutralization of FMDV appears to be modulated by very subtle mechanisms critically dependent on amino acid substitutions that occur with high frequency during the natural evolution of FMDV (19, 29, 35, 36).

As suggested by others (7) and by us (10), the rapidly emerging evidence of heterogeneity in the epitopes involved in the neutralization of FMDV (10, 11) and of the antigenic variation of pathogenic RNA viruses in general (reviewed in refs. 6, 7, 12, and 40) requires reconsidering strategies for the development of synthetic vaccines. Formulations based on limited numbers of epitopes prone to the type of variations documented in the present study are unlikely to provide successful protection against quasispecies distributions of infecting viruses (7, 10, 11). The presently available evidence suggests the following requirements for vaccine development: (*i*) to assess statistically the composition and frequency of the relevant epitopic sequences represented in viruses that have circulated in recent years and are cocirculating at present, to delimit the portion of "sequence space" (27) to be considered (5); (*ii*) to study cross-reactivities of each of the relevant epitopic regions with monoclonal and polyclonal sera; (*iii*) to provide multiple epitopes in a vaccine formulation—preferentially to trigger both humoral and cellular responses (41)—to decrease the probability of selecting antibody-resistant variant viruses. Success of such approaches will depend on the extent of genetic and phenotypic flexibility of the virus, a matter largely unexplored.

It may be argued that the effects of conservative substitutions on FMDV antigenicity are merely a particular occurrence, unlikely to be extensible to other systems. However, it has recently been shown that even one conservative amino acid substitution in protein gp120 can drastically reduce recognition of human immunodeficiency virus-infected cells by T-cell clones (42). Furthermore, the interactions between amino acids of the antigen and those of the antibody, as elucidated by crystallographic analyses of antigen–antibody complexes (43), are such that effects of substitutions such as those reported here with mAb SD6 and VP1 will be frequent. All of the above observations implicate quasispecies as an important element of viral pathogenesis.

We are indebted to M. Dávila for technical assistance. Work at the Centro de Biología Molecular was supported by the Comisión

Asesora para la Investigación Científica y Técnica, Fondo de Investigaciones Sanitarias, Consejo Superior de Investigaciones Científicas (Spain), and Fundación Ramón Areces. Work at the Instituto Nacional de Investigaciones Agrarias was supported by foundations of this institute and by the U.S.–Spanish Joint Committee for Scientific and Technological Cooperation. Work at the University of Barcelona was supported by the Comisión Interministerial de Ciencia y Tecnología and by the U.S.–Spanish Joint Committee for Scientific and Technological Cooperation.

1. Bachrach, H. L. (1968) *Annu. Rev. Microbiol.* **22,** 201–244.
2. Pereira, H. G. (1981) in *Virus Diseases of Food Animals*, ed. Gibbs, E. P. J. (Academic, New York), Vol. 2, pp. 333–363.
3. Domingo, E., Dávila, M. & Ortín, J. (1980) *Gene* **11,** 333–346.
4. Sobrino, F., Dávila, M., Ortín, J. & Domingo, E. (1983) *Virology* **128,** 310–318.
5. Dopazo, J., Sobrino, F., Palma, E. L., Domingo, E. & Moya, A. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 6811–6815.
6. Holland, J. J., Spindler, K., Horodyski, E., Grabau, E., Nichol, S. & VandePol, S. (1982) *Science* **215,** 1577–1585.
7. Steinhauer, D. & Holland, J. J. (1987) *Annu. Rev. Microbiol.* **41,** 409–433.
8. Pereira, H. G. (1977) *Developments in Biological Standardization* (Karger, Basal), Vol. 35, pp. 167–174.
9. Grubman, M. J. & Morgan, D. O. (1986) *Virus Res.* **6,** 33–43.
10. Mateu, M. G., Rocha, E., Vicente, O., Vayreda, F., Navalpotro, C., Andreu, D., Pedroso, E., Giralt, E., Enjuanes, L. & Domingo, E. (1987) *Virus Res.* **8,** 261–274.
11. Mateu, M. G., da Silva, J. L., Rocha, E., de Brum, D. L., Alonso, A., Enjuanes, L., Domingo, E. & Barahona, H. (1988) *Virology* **167,** 113–124.
12. Domingo, E. & Holland, J. J. (1988) in *RNA Genetics*, eds. Domingo, E., Holland, J. & Ahlquist, P. (CRC, Boca Raton, FL), Vol. 3, pp. 3–36.
13. Bachrach, H. L., Moore, D. M., McKercher, P. D. & Polatnick, J. (1975) *J. Immunol.* **115,** 1636–1641.
14. Strohmaier, K., Franze, R. & Adam, K. H. (1982) *J. Gen. Virol.* **59,** 295–306.
15. Bittle, J. L., Houghton, R. A., Alexander, H., Shinnick, T. M., Sutcliffe, J. G., Lerner, R. A., Rowlands, D. J. & Brown, F. (1982) *Nature (London)* **298,** 30–33.
16. Pfaff, E., Mussgay, M., Boehm, H. O., Schulz, G. E. & Schaller, H. (1982) *EMBO J.* **1,** 869–874.
17. Geysen, H. M., Barteling, S. J. & Meloen, R. H. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 178–182.
18. Acharya, R., Fry, E., Stuart, D., Fox, G., Rowlands, D. & Brown, F. (1989) *Nature (London)* **337,** 709–716.
19. Sobrino, F., Palma, E. L., Beck, E., Dávila, M., de la Torre, J. C., Negro, P., Villanueva, N., Ortín, J. & Domingo, E. (1986) *Gene* **50,** 149–159.
20. Rowlands, D. J., Clarke, B. E., Carroll, A. R., Brown, F., Nicholson, B. H., Bittle, J. L., Houghten, R. A. & Lerner, R. A. (1983) *Nature (London)* **306,** 694–697.
21. Beck, E. & Strohmaier, K. (1987) *J. Virol.* **61,** 1621–1629.
22. Stave, J. W., Card, J. L., Morgan, D. O. & Vakharia, V. N. (1988) *Virology* **162,** 21–29.
23. Pfaff, E., Thiel, H. J., Beck, E., Strohmaier, K. & Schaller, H. (1988) *J. Virol.* **62,** 2033–2040.
24. Domingo, E., Martínez-Salas, E., Sobrino, F., de la Torre, J. C., Portela, A., Ortín, J., López-Galindez, C., Pérez-Breña, C., Villanueva, N., Nájera, R., VandePol, S., Steinhauer, D., DePolo, N. & Holland, J. (1985) *Gene* **40,** 1–8.
25. Eigen, M. (1971) *Naturwissenschaften* **58,** 465–523.
26. Eigen, M. & Schuster, P. (1979) *The Hypercycle: A Principle of Natural Self-Organization* (Springer, Berlin).
27. Eigen, M. & Biebricher, C. K. (1988) in *RNA Genetics*, eds. Domingo, E., Holland, J. & Ahlquist, P. (CRC, Boca Raton, FL), Vol. 3, pp. 211–245.
28. Domingo, E., Sabo, D., Taniguchi, T. & Weissmann, C. (1978) *Cell* **13,** 735–744.
29. Martínez, M. A., Carrillo, C., Plana, J., Mascarella, R., Bergada, J., Palma, E. L., Domingo, E. & Sobrino, F. (1988) *Gene* **62,** 75–84.
30. Domingo, E., Mateu, M. G., Martínez, M. A., Dopazo, J., Moya, A. & Sobrino, F. (1989) in *Applied Virology Research*, eds. Kurstak, E., Marusyk, R. G., Murphy, F. A. & Regenmortel, N. H. V. (Plenum, New York), Vol. 2, in press.
31. Barany, G. & Merrifield, R. B. (1980) in *The Peptides*, eds. Gross, E. & Meienhofer, J. (Academic, New York), Vol. 2, pp. 1–284.
32. Giralt, E., Andreu, D., Pons, M. & Pedroso, E. (1981) *Tetrahedron* **37,** 2007–2010.
33. Mitchell, A. R., Kent, S. B. H., Engelhard, M. & Merrifield, R. B. (1978) *J. Org. Chem.* **43,** 2845–2852.
34. Kitagawa, T. & Aikawa, T. (1976) *J. Biochem.* **79,** 233–236.
35. Piccone, M. E., Kaplan, G., Giavedoni, L., Domingo, E. & Palma, E. L. (1988) *J. Virol.* **62,** 1469–1473.
36. Gebauer, F., de la Torre, J. C., Gomes, I., Mateu, M. G., Barahona, H., Tiraboschi, B., Bergmann, I., Augé de Mello, P. & Domingo, E. (1988) *J. Virol.* **62,** 2041–2049.
37. Beck, E. & Strohmaier, K. (1987) *J. Virol.* **61,** 1621–1629.
38. Meloen, R. H., Puyk, W. C., Posthumus, W. P. A., Lankhof, H., Thomas, S. A. & Schaaper, W. M. M. (1988) in *Vaccines 88*, eds. Ginsberg, H., Brown, F., Lerner, R. A. & Chanock, R. M. (Cold Spring Harbor Lab., Cold Spring Harbor, NY), pp. 35–40.
39. Levitt, M. (1978) *Biochemistry* **17,** 4277–4285.
40. Skehel, J. J. & Wiley, D. C. (1988) in *RNA Genetics*, eds. Domingo, E., Holland, J. & Ahlquist, P. (CRC, Boca Raton, FL), Vol. 3, pp. 139–146.
41. Francis, M. J., Hastings, G. Z., Syred, A. D., McGinn, B., Brown, F. & Rowlands, D. J. (1987) *Nature (London)* **300,** 168–170.
42. Siliciano, R. F., Lawton, T., Knall, C., Karr, R. W., Berman, P., Gregory, T. & Reinherz, E. L. (1988) *Cell* **54,** 561–575.
43. Mariuzza, R. A., Phillips, S. E. V. & Poljak, R. J. (1987) *Annu. Rev. Biophys. Chem.* **16,** 139–159.
44. Fox, G., Parry, N. R., Barnett, P. V., McGinn, B., Rowlands, D. J. & Brown, F. (1989) *J. Gen. Virol.* **70,** 625–637.