ESCAPE MUTANTS OF FOOT-AND-MOUTH DISEASE VIRUS SELECTED BY MONOCLONAL ANTIBODIES DIRECTED TO A TRYPSIN-SENSITIVE NEUTRALIZATION EPITOPE

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Summary. – Five monoclonal antibodies (MoAbs) against Indian reference/vaccine strain of foot-and-mouth disease (FMD) virus subtype A22 (IND17/77) and a guinea pig antibody against a synthetic peptide representing amino acids (aa) 136-151 of VP1 polypeptide of A22 virus were used in the study. All the antibodies either failed to react or showed a reduced reactivity with trypsin-treated (TT)-146 S virus particles in enzyme-linked immunosorbent assay (ELISA), and could neutralize the infectivity of the reference virus. The antibodies were hence identified as specific to a trypsin-sensitive neutralizable antigenic site of the virus. Using the antibodies we isolated mutants which showed either no or reduced reactivity with the homologous as well as heterologous antibodies in ELISA. The mutants could not be neutralized with the respective antibodies but were efficiently neutralized with the serum from vaccinated cattle (BVS). These results indicated that the antibodies elicited in cattle following vaccination protected them adequately against the mutants selected and that the trypsin-sensitive neutralizable antigenic site of FMD A22 virus as identified by the MoAbs may not be dominant in eliciting a neutralizing antibody response in vaccinated cattle.

Key words: foot-and-mouth disease virus subtype A22; monoclonal antibody; anti-peptide antibody; neutralization-escape mutant; bovine vaccinate serum

Introduction

FMD is an acute, systemic viral disease affecting both domestic and wild animals, such as cattle, swine, sheep, goat, elephant etc. The causative agent, FMD virus, belongs to the *Aphthovirus* genus of the family *Picornaviridae* and exists in seven distinct serotypes (O, A, C, Asia 1, SAT1, SAT2 and SAT3). FMD is one of the most troublesome viral diseases of farm livestock because of its antigenic variability along with a constant emergence of mutants/variants, a relatively short duration of immunity to a given serotype and an establishment of carrier status in recovered animals. During replication, FMD virus genomic RNA undergoes a remarkable genetic variation, a salient property of RNA viruses (Domingo *et al.*, 1990).

The occurence of the disease due to FMD type A virus assumes significance due to a tendency of this serotype to undergo antigenic variation frequently as evidenced by the existence of 30 or more subtypes (Pereira, 1981; Rweyemamu, 1984). One of the major problems in controlling the disease in endemic countries is antigenic variation within the serotype/subtype and emergence of variants. The antigenic variation may be so great that an immunity against

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Abbreviations: aa = amino acid; BVS = bovine vaccinate serum; ELISA = enzyme-linked immunosorbent assay; FMD = foot-and-mouth disease; HCS = healthy calf serum; HRP = horseradish peroxidase; HRS = healthy rabbit serum; LAH = lactalalbumin hydrolysate; MoAb = monoclonal antibody; NI = neutralization index; PBS = phosphate-buffered saline; s.c. = subcutaneously; TT = trypsin-treated

a particular strain of virus within a serotype/subtype need not necessarily ensure protection against infection with other viruses within that serotype/subtype (Rowlands *et al.*, 1983). Such variation is attributed to selection of (antigenically different) mutants/variants produced under the influence of several factors operating in the field including selective pressure exerted by the host antibodies or other immune mechanisms (Crowther, 1986; Mateu, 1995). Such viruses may avoid a protective humoral antibody which has been induced after infection or vaccination with the original member of the serotype/subtype (Hyslop and Fagg, 1965; Crowther, 1993). The variation can be identified in the laboratory also through the virus passage in different tissue culture cells in the presence/absence of neutralizing antibodies (Sobrino *et al.*, 1983; Schild *et al.*, 1983; Carrillo *et al.*, 1989; Diez *et al.*, 1989).

The objective of the present investigation was to isolate mutants (neutralization-escape mutants) of FMD A22 virus employing MoAbs and an anti-peptide guinea pig serum (against the aa 136-151 region of VP1 polypeptide of A22 virus) and to characterize these mutants in *in vitro* neutralization test and ELISA using BVS so as to assess whether the immune response elicited in cattle following vaccination (with the reference virus/strain) is capable of providing an adequate protection against the mutants thus selected.

Materials and Methods

Reference virus. FMD virus subtype A22 (IND17/77), used for the vaccine production by the Indian Veterinary Research Institute, was used as reference in the study. The virus was adapted to BHK-21 cell line. Complete virus particles (146 S antigen) were prepared by polyethylene glycol (PEG 6000) precipitation of the infected cell culture fluid followed by ultracentrifugation in discontinuous CsCl gradient (Bachrach *et al.*, 1964; Wagner *et al.*, 1970). The concentration of 146 S particles was estimated using the extinction coefficient (E) of 76 at 260 nm for 1% solution (Bachrach *et al.*, 1964). Trypsin treatment of 146 S particles was done according to Bartelling *et al.* (1979) with some modifications. One mg of trypsin was added to 1 mg of 146 S particles and the mixture was incubated for 15 mins at 37°C. Then 1 mg of soybean trypsin inhibitor (Sigma) was added and the 146 S particles were repurified by CsCl density gradient centrifugation. Virus preparations were stored at -70°C in small aliquots.

Reference serum. Anti-146 S serum against the reference virus (A22) was raised in guinea pigs and rabbits by a procedure described earlier (Ferris and Donaldson, 1984).

Anti-peptide antibody against a synthetic peptide representing aa 136-151 of VP1 (1D) polypeptide of FMD A22 virus was raised in guinea pigs. This peptide was obtained from Dr. T. Andronova of the M.M. Shemyakin Institute of Bioorganic Chemistry, Academy of Sciences, Moscow, Russia. Guinea pigs were immunized subcutaneously (s.c.) with 25 µg of the peptide per animal in complete Freund's adjuvant and bled on day 21 post immunization for collection of the serum. The vaccinated guinea pigs withstood a live virus challenge (intradermal inoculation into hind pads) given on day 28 post vaccination.

Bovine vaccinate serum (BVS) was obtained from FMD-free cattle vaccinated s.c. with A22 monovalent alhydrogel vaccine (2.5 ml per animal) on day 21 post vaccination.

MoAbs. Five neutralizing MoAbs against the reference virus, prepared at this laboratory, were used in the study.

Isolation of neutralization-escape mutants to the MoAbs and the anti-peptide antibody. The mutants were isolated by growing the parental virus (IND17/77) in BHK-21 cell monolayer in the presence of the antibody. Briefly, 1 ml of the parental virus (10^7 $TCID_{50}$) was mixed with 150 µl of the undiluted antibody and the mixture was incubated at 37°C for 30 mins with intermittent shaking. Ten ml of serum-free maintenance medium (Glasgow modification of Eagle's Minimum Essential Medium), containing the particular antibody at the dilution of 1/50 (v/v), was added to a preformed confluent monolayer of BHK-21 cells in a 25 cm^2 culture flask and the culture was inoculated with the pre-incubated virus-antibody mixture. The infected culture flasks were incubated at 37°C and after complete cytopathic effect was observed the cell culture fluid was collected and clarified by low-speed centrifugation to remove cell debris. The resulting virus in the form of infectious culture fluid was tested in sandwich ELISA (see below) for its reactivity with the selecting antibody. The binding reactivity of the virus with the antibody was calculated and interpreted according to Samuel *et al.* (1991). A reduction in the binding reactivity of the virus grown under the antibody pressure in relation to the parental virus indicated selection of a neutralization-escape mutant population. If required, an additional passage was made in the presence of the antibody and again the virus was tested as described before.

Plaque purification of mutants. A procedure, described earlier for plaque purification of virus (Dulbecco, 1952), was followed with some modifications. Serial 10-fold dilutions (10^{-1} to 10^{-11}) of the virus were prepared in the serum-free maintenance medium. Preformed monolayers of BHK-21 cells grown in flat bottom 6-well cell culture plates (Linbro) were washed with the prewarmed maintenance medium and three wells were infected (0.2 ml per well) with each dilution of the virus. The virus adsorption proceeded for 15 mins at 37°C and the cell sheets were then washed with the maintenance medium and overlaid with 2.5 ml of 1% Noble agar (39 – 40°C), prepared in the maintenance medium supplemented with 3% foetal calf serum, 250 µg/ml DEAE-dextran and 10 mmol/l Hepes buffer pH 7.4. After the agar gel solidified, the culture plates were covered and kept in an incubator with 5% CO_2 atmosphere. After about 20 hrs, a number of discrete plaques in each well were identified and the agar layers above the plaques were collected individually into 2-ml sterile tubes containing 0.5 ml of the maintenance medium and frozen at -70°C. The virus from each plaque was amplified in BHK-21 cells grown in flat bottom 24-well culture plates. The infected cell culture fluid was collected, clarified by a low-speed centrifugation and stored at -70°C in small aliquots for subsequent use. The reactivity of each plaque population with the antibodies was determined in sandwich ELISA.

Micro-neutralization test. An *in vitro* micro-neutralization test using BHK-21 cells was carried out according to Rweyemamu *et al.* (1978), and log values of neutralization indices (NI) of the

antibodies against different virus populations were calculated (Reed and Muench, 1938).

Sandwich ELISA (McCullough *et al.*, 1985) was employed to assess the reactivity of the antibodies with intact as well as TT-146 S virus particles and different mutant populations. The test was performed in 96-well flat bottom plates (Greiner). Briefly, wells of the plates were coated with 50 µl per well of anti-146 S A22 rabbit serum diluted to 1:4000 (pretitrated dilution) in carbonate-bicarbonate buffer. The coating was effected at 37°C for 1 hr. The wells were washed (4x) with phosphate-buffered saline pH 7.2 (PBS) supplemented with 0.1% Tween-20 (washing buffer). To asses the reactivity of the antibodies with TT-146 S virus particles versus native ones, the virus preparations were used at a concentration of 2 µg/ml (50 µl/well). To analyze the reactivity of mutant virus populations, infected cell culture fluids (undiluted, 50 µl/well) were used as the source of virus. The trapping of the virus was allowed to take place at 37°C for 1 hr. The wells were washed as before and antibodies at pretitrated dilutions were added to the wells (2 wells for each antibody, 50 µl/well). The MoAbs and anti-mouse conjugate were diluted in 1% (w/v) skimmed milk powder prepared in washing buffer, and the guinea pig antibodies and anti-guinea pig conjugate were diluted in washing buffer containing 3% (w/v) lactalalbumine hydrolysate (LAH), 5% (v/v) healthy rabbit serum (HRS) and 5% (v/v) healthy calf serum (HCS). The antibodies were allowed to react for 1 hr at 37°C and the wells were then washed. Anti-mouse horseradish peroxidase (HRP) conjugate (A-4416, Sigma) diluted to 1:1000 and an anti-guinea pig HRP conjugate (P141, Dakopatts) diluted to 1:2000 were dispensed in 50 µl volumes into corresponding wells. The plates were incubated for 1 hr at 37°C, washed and again incubated with 50 µl per well of o-phenylenediamine dihydrochloride solution for 10 mins at 37°C. The reaction was stopped by adding 50 µl of 1 mol/l H_2SO_4 to each well. Background controls for each antibody were included in each plate. The absorbance at 492 nm (A_{492}) of individual wells was read. Corrected A_{492} values were obtained by subtracting the background A_{492} values from the sample A_{492} values.

To identify the trypsin sensitivity of an antibody binding site on the virus, the A_{492} values obtained for each antibody with native and TT-146 S virus particles (5 tests per antibody) were compared and the percent reduction in the reactivity was calculated.

The level of relationship of a mutant virus population to the parental virus was estimated and interpreted according to Samuel *et al.* (1991). The % values (relationships) were qualified by 3 ranges of the reaction, namely, values of 76 – 100% reflected reaction equal to the parental virus, values of 20 – 75% reflected reduced affinity/reactivity, and values below 20% reflected no reaction.

Results

Reactivity of antibodies

All the 5 MoAbs showed a reduction to 54 – 83% in their reactivity with TT-146 S particles as compared to native ones in ELISA (Table 1). The MoAbs neutralized the infectivity of the reference virus to various extent (log NI 2.11-3.48). The anti-peptide guinea pig serum also neutralized the parental virus (log NI 3.0) and failed to react with TT-146 S particles in ELISA.

Selection of mutant virus populations

The MoAbs and the anti-peptide guinea pig serum were used to isolate the mutants. The mutants were identified on the basis of their reactivity with the respective antibodies in sandwich ELISA. The results in terms of percent reactivity values are presented in Table 2. It was observed that the virus populations selected in the presence of MoAbs 22, 27, 34 and 37S showed a reactivity reduced to 38 – 46% with the respective MoAbs as compared to the parental virus and were hence identified as mutants. The mutants also showed a reduced reactivity with the heterologous MoAbs as well as with the anti-peptide antibody. But the virus populations selected in the presence of MoAb 37A and the anti-peptide antibody did not show reduced reactivity with the respective antibodies and hence it was difficult to assess whether any mutant population at all was selected. It was also observed that the mutants which were isolated after 2 passages in the presence of MoAbs 27 and 37S ("the mutants of MoAbs 27 and 37S"), upon subsequent passages in the absence of the antibody pressure, showed an increased reactivity with the respective MoAbs (Table 3). This observation indicated outnumbering of the mutant populations by the antibody-reactive virus during replication in the absence of the antibody. However, the mutants of MoAbs 22 and 34 maintained their low reactivity with the respective antibodies even after subsequent passages in the absence of the antibody pressure indicating that these two mutant populations were stable.

Plaque purification of mutants

As the reactivity pattern of the mutants selected with MoAbs 22 and 34 was maintained after subsequent passages in the absence of the selecting antibodies, these mutants were not plaque purified. The mutants selected with MoAbs 27, 37S and 37A, and with the anti-peptide antibody, were plaque purified in order to isolate stable mutant populations. A number of plaques were picked up at random from each of these mutant populations (22, 30, 16 and 22 plaques from mutants of MoAbs 27, 37S, 37A and the anti-peptide antibody, respectively). The virus from each plaque was tested for its reactivity with the respective antibody. All but one of the plaques picked up from the MoAb 27 mutant showed a reactivity reduced to 18 – 48% with the homologous antibody (MoAb 27) and hence were identified as mutant populations of MoAb 27. All the plaques picked up from the mutant population of MoAb 37S showed a reactivity reduced

Table 1. Characteristics of the antibodies used in the study

Antibody (dilution)	Reactivity with antigen in sandwich ELISA (A_{492})			log NI
	Cell culture fluid	146 S particles	TT-146 S particles[a]	
MoAb 22 (undiluted)	1.26	1.34	0.60(56%)	3.2
MoAb 27 (1:10)	1.20	1.41	0.27(81%)	3.48
MoAb 34 (undiluted)	1.21	1.36	0.57(58%)	3.1
MoAb 37S (1:10)	1.37	1.41	0.24(83%)	3.2
MoAb 37A (1:5)	1.13	1.05	0.49(54%)	2.11
Anti-petide serum (1:10)	0.76	0.73	0.11(85%)	3.0

Antigens used at concentration of 2 µg/ml.
[a]Values in parentheses indicate percent reduction in the reactivity compared to that with native 146 S particles.

Table 2. ELISA reactivity of the mutant populations selected following the antibody presure with homologous and heterologous antibodies

Virus/mutant	Reactivity with antibody (%)					
	MoAb 22	MoAb 27	MoAb 34	MoAb 37S	MoAb 37A	Anti-peptide serum
Parental A 22[a]	100	100	100	100	100	100
MoAb 22[b]	44	27	52	30	36	43
MoAb 27[b]	45	38	43	40	55	34
MoAb 34[b]	45	26	46	30	34	39
MoAb 37S[b]	44	39	43	42	53	30
MoAb 37A[b]	128	116	125	120	130	91
Anti-peptide serum	92	79	88	76	94	141

[a]Reference strain at passage No. 6 in BHK-21 cells. [b]Mutant virus populations obtained after 2 passages in BHK-21 cells in the presence of respective MoAb.

Table 3. ELISA reactivity of the mutant populations selected after passaging in the presence and then in the absence of the selecting antibody

Antibody	Reactivity of mutants (%)[a]		
	Passage No. 1 (+Ab)	Passage No. 2 (+Ab)	Passage No. 3 (–Ab)
MoAb 22	71	44	48
MoAb 27	74	38	81
MoAb 34	70	45	53
MoAb 37S	76	42	84

[a]Reactivity of parental virus = 100%. (+Ab) = in the presence of antibody; (–Ab) = in the absence of antibody.

to 13 – 39% with the homologous MoAb. Of the 16 plaques isolated from the mutant population of MoAb 37A, all but 3 of the plaques showed a reactivity reduced to 28 – 42% with the homologous MoAb. Only 3 of the 22 plaques isolated from the virus population obtained after a passage in the presence of the anti-peptide antibody showed a reactivity reduced to 28 – 42% with the antibody.

Characterization of mutants

Selected plaque purified mutants of MoAbs 27, 37S and 37A, and of the anti-peptide antibody (9, 11, 5 and 3 plaques, respectively), and uncloned mutants of MoAbs 22 and 34 were analyzed for their reactivity with the homologous as well as heterologous antibodies and BVS in sandwich ELISA. The results are shown in Table 4. All the mutant populations showed either no (<20%) or reduced (<50%) reactivity with all the MoAbs as well as with the anti-peptide antibody. However, all the mutant populations showed strong reactivity (>76%) with BVS.

Two mutant plaques of MoAb 27 (plaques No. 2 and 8), 3 mutant plaques of MoAb 37S (plaques No. 3, 10 and 25), 2 mutant plaques of MoAb 37A (plaques No. 6 and 16), 3 mutant plaques of the anti-peptide antibody (plaques No. 3, 12 and 30) and the uncloned mutant populations of MoAbs 22 and 34 were subjected to micro-neutralization test using respective antibodies and BVS. It may be seen from the results (Table 5) that the mutant populations isolated with the MoAbs could not be neutralized with the

Table 4. ELISA reactivity of the mutant populations with different antibodies

Virus/ mutant	Reactivity with antibody (%)						
	MoAb 22 serum	MoAb 27	MoAb 34	MoAb 37S	MoAb 37A	Anti-peptide	BVS
Parental A22	100	100	100	100	100	100	100
MoAb 22[a]	44	27	45	24	37	28	150
MoAb 34[a]	48	32	46	34	42	36	148
Mutant plaque of MoAb 27							
No. 2	29	9	27	8	29	32	113
5	40	25	42	25	28	30	127
7	37	12	34	13	24	22	124
8	36	15	32	14	28	24	119
11	41	21	40	22	13	20	113
13	44	26	43	25	13	20	113
14	29	11	28	9	24	10	142
16	30	12	28	10	17	8	119
21	45	27	44	28	23	33	117
Mutant plaque of MoAb 37S							
No. 3	31	15	30	13	26	20	132
10	28	13	29	13	25	22	121
11	29	10	27	8	19	14	121
14	30	12	28	11	19	12	124
16	33	16	31	14	17	12	123
17	31	12	29	11	21	14	123
20	28	10	27	9	17	12	126
21	28	10	29	10	23	14	136
22	29	11	28	11	21	21	135
23	30	12	28	10	26	16	119
25	32	17	31	15	28	22	162
Mutant plaque of MoAb 37A							
No. 4	29	11	28	11	24	20	134
6	31	13	27	11	28	20	134
7	30	13	28	11	30	24	127
15	32	16	30	16	36	16	121
16	40	23	41	23	39	26	130
Mutant plaque of anti-peptide serum							
No. 3	40	21	42	22	39	24	132
12	46	36	47	37	34	22	132
30	45	32	46	32	38	37	131

[a]Uncloned mutants.

respective antibodies. These mutants could be neutralized with the anti-peptide serum at a lower dilution (1:16) but not at a dilution equal to or below 1:50. However, the parental (reference) virus was neutralized with both these dilutions of the anti-peptide serum (log NI >3.0). Similarly, the mutant plaques of the anti-peptide antibody could be neutralized in the presence of excess of the antibody (log NI <2.5 at the serum dilution of 1:16) but failed to be neutralized effectively (log NI <1.2) when the serum was diluted to 1:50. BVS against the reference virus could effectively neutralize all the mutants (log NI >3.0 at the serum dilution of 1:64).

Table 5. Neutralization of mutant populations with MoAbs, anti–peptide serum and BVS

Virus/ mutant/ plaque	Antibody, dilution			
	Homologous MoAb undiluted/1:2[a]	Anti–peptide 1:16	serum 1:50	BVS 1:64
Parental A22	+	+	+	+
Mutant plaque of MoAb 27				
No. 2	–	+	–	+
8	–	+	–	+
Mutant plaque of MoAb 37S				
No. 3	–	+	–	+
10	–	+	–	+
25	–	+	–	+
Mutant plaque of MoAb 37A				
No. 6	–	+	–	+
16	–	+	–	+
Uncloned mutants				
MoAb 22	–	+	–	+
MoAb 34	–	+	+/–	+
Mutant plaque of anti–peptide serum				
No. 3	ND	+	–	+
12	ND	+	+/–	+
30	ND	+	–	+

[a]MoAb 37A used at the dilution of 1:2, other MoAbs used undiluted. (+) = log NI ≥2.5; (±) = log NI <1.2; (–) = no neutralization.

Discussion

FMD still remains one of the most troublesome and economically important diseases of livestock. The occurrence of the disease in vaccinated populations lead sometimes to a doubt that the emergence/evolution of variants/mutants in the field may be the cause of the outbreak. The evolution of antigenic variants of FMD virus in partially immune populations (Fagg and Hyslop, 1966), persistently infected animals (Gebauer *et al.*, 1988) and cell cultures in the presence and absence of neutralizing antibodies (Sobrino *et al.*, 1983; Carrillo *et al.*, 1989; Diez *et al.*, 1989; Rieder-Rojas *et al.*, 1992) have been observed. The host immune response is considered one of the major selective factors leading to antigenic variation of viruses during their replication in the animal population in the field (Mateu, 1995).

With the application of MoAbs it has become possible to identify different neutralizable antigenic sites/epitopes on different strains of FMD virus, and the subsequent selection and analysis of neutralization-escape mutants to MoAbs against specific epitopes have helped in understanding the implication of such mutants in vaccinal immunity (Crowther *et al.*, 1993). The present investigation aimed at isolating neutralization-escape mutants, using MoAbs to FMD A22 virus and the antibody to a synthetic peptide representing an important immunogenic region (VP1, aa 136-151) of the virus, and analyzing their susceptibility to neutralization by BVS.

The MoAbs used in the study were capable of neutralizing the infectivity of the parental virus in micro-neutralization test and showed reduced reactivity with the TT-146 S virus particles in ELISA. This observation showed that the MoAbs were directed against a trypsin-sensitive, neutralizable antigenic site on A22 virus. The MoAbs varied in their reactivity with the TT-146 S particles. Maximum reduction in the reactivity (>80%) was shown by the MoAbs 27 and 37S, whereas the other 3 MoAbs led to a lesser (<60%) reduction as compared to the native virus particles. This difference in the reactivity showed that the MoAbs identified more than one epitope on the trypsin-sensitive site of A22 virus.

Two trypsin-sensitive, neutralizable antigenic sites have been identified in VP1 polypeptide of FMD A10 virus; one was located within aa 140-160 and the other at the C-terminus (aa 200-213) (Thomas *et al.*, 1988). It has been shown that out of the 4 structural polypeptides (VP1 to VP4) of the FMD virus, VP1 carries an important antigenic determinant of virus neutralization, and synthetic peptides representing the 2 trypsin-sensitive areas of the virus were capable of inducing neutralizing antibodies in experimental animals (Strohmaier *et al.*, 1982). Experiments with neutralizing MoAbs, synthetic peptides, and MoAb-resistant mutants (neutralization-escape mutants) have identified the VP1 aa 140-160 region (trypsin-sensitive) as a site of interaction of neutralizing antibodies with FMD virus of serotypes A, O, and C (Domingo *et al.*, 1990). Recently, it has been shown that in type C virus, on average about 57% and 27% of the virus neutralizing activity and about 35% and 12% of the virus-binding activity in convalescent and vaccinated swine sera, respectively, were directed against the major site A which was located within aa 138-150 of VP1 (Mateu *et al.*, 1995). All these observations have revealed that the region of aa 141-160 of VP1 forms an important antigenic site. This region in A22 Iraq 24/64 virus occurs as a linear epitope on the virus particle (Bolwell *et al.*, 1989). The peptide representing aa 136-151 of VP1 of A22 virus, against which the guinea pig serum was raised and used in the present study, elicited high level of neutralizing antibodies in cattle and the vaccinated cattle were protected

against virulent challenge (Venkataramanan *et al.*, 1994). The anti-peptide guinea pig serum showed strong virus neutralizing activity against the reference virus (log NI 3.0) but failed to react with the TT-146 S particles in ELISA. This observation indicated that the region of aa 136-151 of VP1 of A22 virus harbours a trypsin-sensitive neutralizable epitope(s) against which antibodies were produced in guinea pigs inoculated with the peptide.

It has been established that FMD virus populations do not exist as single molecular species but are heterogenous (Domingo *et al.*, 1992). When these are subjected to neutralizing antibody pressure, a majority of the population becomes neutralized but some viral subpopulations escape neutralization (neutralization-escape populations/mutants). Different mutant populations, which are already present in the original virus, are merely enhanced in the presence of neutralizing antibody (Baxt *et al.*, 1989; Carrillo *et al.*, 1989; Rieder-Rojas *et al.*, 1992). The escape of picornavirus from neutralization with MoAbs is mediated by substitutions of very few, defined amino acids of the capsid, genereally located on the tip of some surface exposed loops (Mateu, 1995). In the present study, mutant virus populations could be isolated with the MoAbs as well as the anti-peptide antibody. From the reactivity pattern in ELISA it is clear that the mutants had either a reduced or no reactivity with the homologous as well as heterologous antibodies. MoAbs 27, 37S and 37A and the anti-peptide antibody showed no reactivity (<20%) with 20, 20, 7 and 10 plaques. MoAbs 27, 37S and 37A and the anti-peptide antibody showed a reactivity reduced to 20-42% with 8, 8, 21 and 18 plaques, and with the uncloned mutants of MoAbs 22 and 34. MoAbs 22 and 34 showed a reactivity reduced to 27 – 48% with all the mutants. No reactivity indicates that the epitope(s) recognized by the antibodies is/are changed, and a reduced reactivity denotes that the epitope(s) recognized by the antibodies on the mutants have some similarity (not identity) to that/those of the reference virus (Samuel *et al.*, 1991). The results of ELISA are in agreement with those obtained in the neutralization test. The mutants failed to be neutralized by respective selecting antibodies. The failure of neutralization of the mutants with the anti-peptide antibody at a higher dilution (lower concentration) showed that the affinity of the neutralizing antibodies in the anti-peptide serum to the mutants was lower as compared to that to the parental virus.

The strong reactivity (>76%) of all the mutants with BVS in ELISA and also the efficient neutralization (log NI >3.0) of the mutants with BVS indicates that there are neutralizable antigenic sites/epitopes on the virus other than those defined by the MoAbs/anti-peptide antibody used in the present study and hence the mutants were still recognized by other neutralizing antibody populations present in the serum (BVS). Our results show that changes in the trypsin-sensitive neutralizable antigenic site of FMD A22 virus are of not much significance as the neutralizing antibody response elicited in vaccinated cattle was not restricted to this site alone. Brocchi *et al.* (1987) have reported that some of the isolates of FMD type A virus which showed variations with MoAbs against the trypsin-sensitive region of FMD A5 Parma virus could be efficiently neutralized with BVS raised against the reference virus. Moreover, it has been recently observed (Mateu *et al.*, 1995) that the neutralizing and virus-binding activities against FMD type C virus in both vaccinated and convalescent swine sera are not restricted to the major antigenic site A comprising aa 138-150 of VP1.

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