



Figure 5. *a* and *b* Optical textures of 2-(4'-cyanophenyl)-5-n-heptyl-1,3-dioxane at different temperatures.

in Figures 4 and 5*a* and *b*. The compound OS exhibits a highly ordered smectic B phase, as seen from the mosaic (oblong platelets) texture shown in the figure on the cover. It is evident from Figure 5*b* that the compound PDX exhibits a schlieren texture, which is a characteristic of smectic C phase. The compound PDX is biaxial in nature. The molecular packing obtained by crystal structure analysis of the compound PDX supports the existence of smectic C phase⁸.

1. de Gennes, P. G., *Physics of Liquid Crystals*, Clarendon, Oxford, England, 1974
2. Chandrasekhar, S., *Liquid Crystals*, Cambridge University, Cambridge, England, 1977.
3. Neugebauer, H. E. J., *Can. J. Phys.*, 1950, 18, 292.
4. Neugebauer, H. E. J., *Can. J. Phys.*, 1954, 32, 1
5. Lippincott, E. R., *J. Chem. Phys.*, 1955, 23, 603; 1957, 26, 1678
6. Lippincott, E. R. and Stutman, J. M., *J. Phys. Chem.*, 1964, 68, 2926
7. Lippincott, E. R., Nagarajan, G. and Stutman, J. M., *J. Phys. Chem.*, 1966, 70, 78
8. Bellad, S. B., et al., *Z. Kristallogr.*, 1992 (in press).

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Production and characterization of monoclonal antibodies to foot-and-mouth disease virus subtype A22

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Monoclonal antibodies (MAbs) were produced by fusion of mouse myeloma cells and splenocytes of BALB/c mice immunized by infection with live purified (146S particles) foot-and-mouth disease virus subtype A22 or by inoculation with inactivated 146S particles. Different experimental protocols such as varying methods of hyperimmunization of mice, propagation of hybridomas and cell fusion parameters were critically examined. The use of splenocytes collected from each individual mouse separately for fusion entailed higher yields of positive hybridomas, than when splenocytes collected from more than one mouse were pooled. Further, immunization by one injection with live virus and intrasplenic final booster inoculation enabled the production of higher number of positive hybridomas. It was also noted that hybridomas could be propagated with ease and maintained equally well in media supplemented with colostrum-deprived newborn calf serum or horse serum as those containing foetal calf serum (FCS). The four virus-specific MAbs produced were characterized by various tests. In particular, they were tested for their capacity to neutralize viral infectivity and capacity to bind to isolated viral proteins (VP1, VP2, VP3). In enzyme immuno-transfer blot assay (EITB), there was evidence that one of the four MAbs recognized an epitope on two separated viral proteins. The other three MAbs recognized conformation-independent epitopes on separated VP1.

PROTECTION of domestic animals against foot-and-mouth disease virus (FMDV) is directly related to the presence in their sera of type-specific antibodies capable of neutralizing virus infectivity¹. The intact virion is composed of 60 copies each of the four structural capsid proteins designated VP1 to VP4 (ref. 2). Evidence from several lines of investigation centering on the analysis of the isolated capsid proteins and the protease treatment of VP1 molecule³ has suggested that the latter is the major immunogenic protein in the virion and is the most exposed on viral surface. Immunization of animals with isolated VP1 and its

fragments had shown that this protein carries antigenic determinants which induce neutralizing antibodies⁴ and confer protection^{5,6}.

Disruption of FMDV *in vitro* results in the release of the RNA genome and 12S protein subunits containing equimolar amounts of VP1, VP2 and VP3 proteins⁷. It was shown by Morgan *et al.*⁸, that 12S protein subunits are immunogenic and abrogate *in toto* the neutralizing capacity of anti-VP1 antibodies. On the contrary, adsorption of polyclonal antiviral antibodies with isolated 12S protein subunits caused the removal of only a portion of the neutralizing activity^{9,10}.

Neutralizing and nonneutralizing monoclonal antibodies (MAbs) to FMDV have been produced which react with intact particles or with 146S and 12S particles or with 146S, 12S and VP1. Moreover, there is evidence that there are multiple conformation-dependent and conformation-independent neutralization epitopes on FMDV types 'O' and 'A' strains¹¹⁻¹⁴. Rapid and efficient protocols for production, selection and propagation of FMDV-specific hybridomas have been described by McCullough *et al.*¹⁵

The present communication reports on the production and characterization of MAbs of FMDV subtype A22 and the antigenic relationships of FMDV subtypes A22 and A5/10 demonstrated by the MAbs. In addition the results of examination of varying experimental protocols to obtain optimum hybridomas are described.

Cells and media. The mouse myeloma cell line SP2/0 Ag14 was used for fusion experiments. The BHK21 C13 cell line was used for virus cultivation and for plaque reduction (PR) and microneutralization tests (MNT).

RPMI-1640 medium was used for production of MAbs unless otherwise stated and Eagle's medium (MEM) was used for propagation of BHK-21 cells and the virus. The media contained glutamine (2 mM), sodium bicarbonate (5.5%), benzyl penicillin (125 IU/ml) and dihydrostreptomycin sulphate (187 IU/ml) and were supplemented with foetal calf serum (FCS) or colostrum-deprived newborn calf serum (NBCS) or horse serum (HS) as the case may be.

In separate experiments, positive hybridomas were propagated in each of the two media supplemented with one of the above sera. Glass prescription bottles were seeded with a cell density of 2×10^4 cells/ml in 12 ml of each given medium and incubated at 37°C. Bottles were examined for 5 days to check growth rate and percentage of viable cells.

Virus. FMDV subtype A22 vaccine strain grown in BHK-21 cell cultures was used for production of MAbs. FMDV types O, A, C and Asia-1 were used to determine the type specificity or cross-reactivity of the MAbs. The virus was harvested from infected cell cultures, clarified by low-speed centrifugation. The virus

was then precipitated by polyethylene glycol (PEG) treatment¹⁶ and subjected to sucrose density gradient centrifugation¹⁷ and the purified intact virions (146S) were quantified by optical density. The virus aliquots were inactivated with bromoethylenimine (3 mM) when required according to the procedure of Bahnmann¹⁸.

Production of monoclonal antibodies. Four cell fusions were carried out. The immunization protocol in respect of the first two consisted of inoculation of BALB/c mice with inactivated virus according to standard procedures. The final injection was given intravenously. In case of third and fourth fusions, multiple injections with live virus were given with intrasplenic terminal injection.

In the first and second experiments, fusion was carried out with a mixture of splenocytes obtained from groups of two mice each. In the third and fourth experiments, splenocytes harvested from individual mice were used. The splenocytes were hybridized with SP2/0 cells as per the procedure used by Kohler and Milstein¹⁹ in general. Briefly, aliquots of 10^8 splenocytes were mixed with 10^7 myeloma cells in exponential phase. The mixture was centrifuged in 10 ml of serum-free RPMI-1640 medium at 500g for 8 min. The cell pellet was gently suspended in one ml of warm PEG (50% w/v PEG 1450 in RPMI-1640) and left for a minute after which the fusion mixture was diluted in the medium. After low-speed centrifugation, the cell pellet was suspended in 100 ml of the appropriate growth medium and distributed in one ml amounts into each well of 24-well plates. The cultures were incubated at 37°C in an atmosphere of 8% CO₂. The following day, one ml of the growth medium supplemented with HAT was added to each well.

The growth of hybridomas was monitored by frequent inspection and replenishment of the spent medium. Culture supernates were screened for specific antibodies by ELISA and MNT. The positive hybridomas were cloned at least twice, by the limiting dilution technique. Four monoclonals secreting virus infectivity neutralizing MAbs were selected for characterization.

Characterization of monoclonal antibodies. The indirect ELISA reported by Abu-Elzein and Crowther²⁰ was used to detect the MAbs. The wells of ELISA plates (Dynatech) coated with purified 146S virus particles (8 µg/ml) were used in this assay, whereas the triple antibody sandwich ELISA described by Butchiah and Rao²¹ was applied to determine the FMDV type specificity of the MAbs. An indirect fluorescent antibody test (FAT) using FITC-conjugated anti-mouse immunoglobulin subclass specific sera was carried out to determine the isotype of the MAbs according to standard procedures. The agar gel immunodiffusion

RESEARCH COMMUNICATIONS

(AGID) test described by Meloen and Briarie²² was followed using 146S particles of FMDV to determine the virus precipitating ability of MAbs.

The MAbs were tested for their virus infectivity neutralizing ability in both microneutralization and plaque reduction assays. The microneutralization described by Crowther *et al.*²³ was followed. In brief, the two-fold dilution series of the MAbs preparation was tested against 100 TCID₅₀ of virus using BHK-21 monolayer cells in 96-well tissue culture plates. The log₁₀ titre of the MAbs at 50% end point was determined according to established procedures. Similarly, the MAbs were also tested by varying log₁₀ virus dilutions and constant antibody dilution (1/4) method.

The plaque reduction assay was carried out by testing a two-fold dilution series of the MAb against 100 pfu of virus in BHK-21 monolayer cell cultures formed in petri dishes, using agar overlay medium containing 0.9% Noble agar in Eagle's medium with 0.15% tryptose phosphate broth (TPB) and 2% bovine serum albumin (BSA). After incubation for 48–60 hours at 37°C under 5% CO₂ tension, the plaques were stained with crystal violet and the antibody titre was expressed as log₁₀ PRNT₅₀.

The MAbs were also tested for their ability to protect mice against challenge with virulent virus. Groups of five-to-six-day-old mice were given 50 µl of two-fold dilution series of MAbs intramuscularly. After 24 hours, they were challenged along with control mice with 50 µl of 100 mouse LD₅₀ dose of the virus given I/M. The end-points of protection were determined as per standard statistical procedures.

The MAbs were further tested for their ability to bind separated viral proteins in an enzyme-linked immunoelectrotransfer blot (EITB) assay. Briefly, the 146S virus particles were disrupted by treatment at 90°C for 3 min in Tris-HCl buffer (80 mM; pH 6.3) containing 10% SDS, 8 M urea, 2-mercaptoethanol (1–2 M), glycerol (18%) and bromophenol blue (0.02%). The proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane and allowed to react with each of the four MAbs according to the method described by Towbin *et al.*²⁴. The transfer was monitored by staining a nitrocellulose strip with amido black and gels with Coomassie brilliant blue. After the MAbs were allowed to react with the nitrocellulose strips containing separated viral proteins either at 4°C for 12–14 hours or at room temperature for 2 hours, the strips were treated with a standard preparation of goat anti-mouse IgG-HRPO conjugate for one hour at room temperature and washed. The enzyme reaction mixture comprised 0.015% H₂O₂ and 4-chloro-1-naphthol (0.5 mg/ml) and 20% methanol in PBS. Colour development was arrested by thorough washing in distilled water.

The four fusion experiments yielded 46, 45, 67 and 69 hybridomas respectively. Three of the 46 hybridomas

from the first fusion produced virus-specific antibodies as detected by ELISA. Two of them had virus infectivity neutralizing activity as well. Corresponding figures were 5 and 4 hybridomas in the second fusion, 7 and 5 in the third and 8 each in the fourth fusion (Table 1). Five of the 8 positive hybridomas obtained in the fourth fusion were cloned over thymocyte feeder cells by the limiting dilution method. One of the clones (II/C-1) grew very slowly and was therefore not included in further characterization.

In the third and fourth fusions, splenocytes from individual mice were fused with myeloma cells. Significantly higher number of hybridomas resulted from the third and fourth fusion experiments (Table 1). Similarly, the number of positive hybridomas obtained from the third and fourth fusions was higher in which live immunogen was used than those of first and second. A short schedule of immunization by infection with live virus appears to be the preferred method over that of inactivated immunogen for obtaining higher number of positive hybridomas.

Butchaiah and Rao²¹ used a modification in the commonly used protocol and prolonged the immunization schedule of mice to 3–4 months with inactivated virus and introduced intrasplenic booster inoculation. This resulted in some improvement in obtaining a greater number of positive hybridomas. But an inherent risk of this prolonged immunization procedure is the mortality of sensitized mice due to other causes. This could be obviated by using a large number of mice. In the current study an average of eight served as a good population size and ensured an adequate number of survivors which were available as fusion partners.

No significant difference in the rate of cell growth and percentage of viable cells was observed when hybridomas were grown in FCS-supplemented RPMI-1640 or MEM. Similarly the substitution of FCS with NBCS or HS gave comparable results in the present study. This finding is in harmony with the previous observations of McCullough *et al.*¹⁵. This finding that hybridomas could be generated and maintained in medium enriched with NBCS or HS has a practical value since FCS is very expensive and has to be imported.

The tested clones (II/A4 and ID/4) were found to

Table 1. Production of polyclonal hybridomas secreting antibodies to FMDV subtype A22

Cell fusion number	Number of wells seeded	Number of hybridomas produced		
		Total	ELISA-positive	Virus neutralization-positive
1	96	46	3	2
2	96	45	5	4
3	96	67	7	5
4	72	69	8	8

secrete MAbs of IgG3 subclass (see Table 5). This finding supports the consensus that extended immunization schedule typically results in an IgG response. All the four monoclonals produced MAbs which precipitated both FMDV serotypes A22 and A5/10 (Table 5). Pontes de Carvalho²⁵ opined that a molecular requirement for the development of a precipitation matrix in antigen-antibody reaction is the recognition by IgG molecule of at least three epitopes on the antigen. Accepting this view, one would expect that a MAb-antigen complex is likely to precipitate only when a similar or identical epitope is repeated three or more times on the antigen molecule. In the present study, whole intact virus (146S) containing multiple copies of the immunogenic protein was used, thus fulfilling the molecular requirement of epitope polyvalency.

In this study, all the four MAbs gave positive reactions with both virus subtypes A22 and A5/10 in sandwich ELISA. However, only one of them (MAb I/D4) cross-reacted also with type C virus (Table 2). Similarly all the four MAbs neutralized the infectivity of subtypes A22 and A5/10 in both MNT and PR assays (Tables 2,3,4,5). Surprisingly, the heterologous subtype A5/10 was neutralized better than the homologous subtype A22 by one (III/A4) of the four MAbs (Table 4). This may be explained by the higher frequency of repetition of the epitope recognized by MAb III/A4 on subtype A5/10 rather than A22. To some extent, this further explains the immunodominance of subtype A5/10. All the four neutralizing MAbs conferred passive protection in mice (Table 5). In the present study, none of the heterologous types, O, C and Asia-1, were neutralized by any of the MAbs (Table 3) although the MAb I/D4 showed some binding activity with type C virus in ELISA (Table 2).

The varying degrees on cross-reactivity between subtypes A22 and A5/10 shown by the MAbs in ELISA and MNT was quite expected and was in conformity with reported occurrence of such cross-reactions between subtypes. This phenomenon may be due to sharing of sets of epitopes between closely related subtypes. Our finding suggests that at least some epitopes on the subtype A22 are conserved amongst the galaxy of A subtypes.

Table 2. FMDV type specificity of MAbs determined by ELISA

FMDV type	Monoclonal antibody			
	III/A4	III/A6	I/B4	I/D4
A22	0.22*	0.27	0.21	0.47
A5/10	0.16	0.18	0.15	0.37
O	0.07	0.06	0.06	0.09
C	0.09	0.05	0.05	0.15
Asia-1	0.04	0.03	0.04	0.03

*Absorbance values at 495 nm.

Table 3. Infectivity neutralization of different types of FMDV by monoclonal antibodies

FMDV type	Monoclonal antibody			
	III/A4	III/A6	I/B4	I/D4
A22	1.95*	1.66	1.66	1.95
A5/10	1.66	1.35	1.35	1.35
O	0	0	0	0
C	0	0	0	0
Asia-1	0	0	0	0

*Log₁₀ SN₅₀ titres in varying-antibody, constant-virus dilution method.

Table 4. Infectivity neutralization of FMDV subtype A22 and A5/10 by monoclonal antibodies

FMDV type	Virus infectivity titre in absence of MAbs	Virus infectivity titre in presence of MAbs			
		III/A4	III/A6	I/B4	I/D4
A22	6.5	1.5*	1.5	1.5	1.5
A5/10	6.5	0.5	4.5	4.5	1.5

*Log₁₀ TCID₅₀ titres in varying-virus, constant-antibody dilution method.

Cross-reactivity was not generally evident between the type-specific sera and different types of FMDV except in very low dilutions. However, it is reasonable to assume that the cross-reactivity revealed by MAb I/D4 only in ELISA in the present study may be related to the occurrence of a common sequence of the epitope at least partly on the two types namely A and C. Alternatively, it is indicative of low-grade reaction which is normally suppressed by the high-affinity interactions that occur when potent polyclonal sera react with the virus type suspensions²⁶. Although such

Table 5. Characterization of MAbs and their reactivities against homologous virus subtype A22

Clone	Immunoglobulin class/subclass	AGID	Mouse protection assay	EITB		
				VP1	VP2	VP3
III/A4	IgG3	+	+	+	-	+
III/A6	ND	+	+	+	-	-
I/B4	ND	+	+	+	-	-
I/D4	IgG3	+	+	-	-	-

+, Positive; -, negative, ND, not done.

AGID, Agar gel immunodiffusion, EITB, enzyme-linked immunoelectrotransfer blot

cross-binding of MAbs with heterologous types of FMDV was observed, so far cross-neutralization of MAbs with heterologous types has not been reported.

MAbs III/A4, III/A6 and I/B4 reacted with separated VP1 of A22, whereas MAb I/D4 did not react with any of the separated VPs (Table 5). An interesting observation was that MAb III/A4 bound to separated VP3 in addition to VP1. These results proved the presence of at least three distinct neutralization epitopes on the surface of FMDV subtype A22. One of them is probably conformation-dependent present on the intact 146S particle and best defined by MAb I/D4, while the second was perhaps conformation-independent present on separated VP1 recognized by the other MAbs III/A6 and I/B4. The binding of MAb III/A4 with two different isolated viral proteins may suggest that a particular epitope or at least part of the epitope sequence is repeated on both VP1 and VP3. It is thus possible that the epitope recognized by this MAb III/A4 is different from the other two epitopes defined above. This finding however needs to be further confirmed by adsorption studies as well as further recloning.

Subunit vaccines consisting of FMDV type A VP1-specific immunodominant domain have been effective in protecting cattle and pigs against challenge infection^{27,28}. This is presumably due to the generation of antibody specific to the neutralization epitopes on the VP1 structure. However, inducing protective immunity in these animals with a VP1 preparation derived from type O virus was not successful²⁹. The latter observation underscores the need of development of strategies for synthesizing conformational as well as sequential epitopes as protective immunogens of FMDV.

11. Melen, R. H., Briar, J., Woortmeyer, R. J. and Vanzaane, D. J., *J. Gen. Virol.*, 1983, **64**, 1193.
12. Baxt, B., Morgan, D. O., Robertson, B. H. and Timpone, C. A., *J. Virol.*, 1984, **51**, 298.
13. Morgan, D. O., Robertson, B. H., Moore, D. M., Timpone, C. A. and McKercher, P. D., in *Control of Virus Diseases* (eds. Kurstak, E. and Marusyk, R. G.), New York, 1984, p. 135.
14. Ouldridge, E. J., Barnett, P. V., Parry, N. R., Syred, A., Head, M. and Rweyemamu, M. M., *J. Gen. Virol.*, 1984, **65**, 203.
15. McCullough, K. C., Butcher, R. N. and Parkinson, D., *J. Biol. Stand.*, 1983, **11**, 171.
16. Wagner, G. G., Card, J. L. and Cowan, K. M., *Arch. Gesamte Virusforsch.*, 1970, **30**, 343.
17. Grubman, M. J., Baxt, B. and Bachrach, H. L., *Virology*, 1979, **97**, 22.
18. Bahnemann, H. G., *Arch. Virol.*, 1975, **47**, 47.
19. Kohler, G. and Milstein, C., *Nature*, 1975, **256**, 495.
20. Abu-Elzein, E. M. E. and Crowther, J. R., *J. Hyg.*, 1978, **80**, 391.
21. Butchiah, G. and Rao, B. U., Indian Veterinary Research Institute Annual Report, 1986.
22. Melen, R. H., and Briar, J., *J. Gen. Virol.*, 1980, **51**, 107.
23. Crowther, J. R., McCullough, K. C., Brocchi, E. and De Simone F., Sess. Res. Group. Stand. Tech. Comm. Eur. Commission Cont. FMD, Brescia, Italy, FAO, Rome, 1984, p. 40.
24. Towbin, H., Stachelin, T. and Gordon, J., *Proc. Natl. Acad. Sci. USA*, 1979, **76**, 4350.
25. Pontes de Carvalho, L. C., in *Genes and Antigens of Parasites*, 2nd edn. (Proceedings of a course held at the Oswaldo Cruz Institute, Dec. 1983), 1983.
26. Duchesne, M., Cartwright, T., Crespo, A., Boucher, F. and Fallourd, E., *J. Gen. Virol.*, 1984, **65**, 1559.
27. Kleid, D. G., Yansura, D., Small, B., Dowbenko, D., Moore, D. M., Grubman, M. J., McKercher, P. D., Morgan, D. O., Robertson, B. H. and Bachrach, H. L., *Science*, 1981, **214**, 1125.
28. McKercher, P. D., Moore, D. M., Morgan, D. O., Robertson, B. H., Callis, J. J., Kleid, D. G., Shires, S. J., Yansura, D. G., Dowbenko, D. and Small, B., *Am. J. Vet. Res.*, 1985, **46**, 587.
29. Dimarchi, R., Brooke, G., Gale, C., Cracknell, V., Doel, T. and Mowat, N., *Science*, 1986, **232**, 639.

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1. Pay, T. W. F., Hingley, P. H., Radlett, P. J., Black, L. and O'Reilly, K. J., Sess. Re. Group. Stand. Tech. Comm. Eur. Commission. Cont. FMD. Lelystad, Netherlands, FAO, Rome, 1983, p. 52.
2. Bachrach, H. L., in *Virology in Agriculture*, (Beltsville symposium in Agricultural Research I, eds. Romberger, J. A. and Montclair, N. J.), Allanheld, Osmuch & Co., 1977, pp. 3-22.
3. Strohmaier, K., Franze, R. and Adam, K. H., *J. Gen. Virol.*, 1982, **59**, 295.
4. Kaaden, O. R., Adam, K. H. and Strohmaier, K., *J. Gen. Virol.*, 1977, **34**, 327.
5. Bachrach, H. L., Moore, D. M., McKercher, P. D. and Polatnick, J., *J. Immunol.*, 1975, **115**, 1136.
6. Bachrach, H. L., Morgan, D. O. and Moore, D. M., *Interuirology*, 1979, **12**, 65.
7. Van de Woude, G. F., Swaney, J. B. and Bachrach, H. L., *Biochem. Biophys. Res. Commun.*, 1972, **48**, 1222.
8. Morgan, D. O., Moore, D. M. and McKercher, P. D., in *New Developments with Human and Veterinary Vaccines* (eds. Mizrahi, A., Hartman, I., Klingberg, M. A. and Kohn, A.), New York, 1980, p. 169.
9. Cowan, K. M., *J. Immunol.*, 1968, **101**, 1183.
10. Brown, F. and Smale, C. J., *J. Gen. Virol.*, 1970, **7**, 115.

***Acanthocheilonema viteae* in *Mastomys natalensis*: Evaluation of efficacy of microfilarial vaccine**

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The efficacy of microfilarial antigens derived from *Acanthocheilonema viteae* has been evaluated in normal as well as exposed animals simulating human population in filaria-endemic area. The crude antigen (CAG) or one of its Sephadex fractions (Fr. I) offered only microfilarial-stage specific resistance against challenge made with either infective larvae, microfilariae or adult parasites. Subsequent study with CAG showed better protection, including significant effect on adult parasite population, if the recipients were already carrying a prepatent infection, or displaying patent microfilaraemia or even