Antigenic analysis of SAT 2 serotype foot-and-mouth disease virus isolates from Zimbabwe using monoclonal antibodies

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SUMMARY

This paper compares strains of foot-and-mouth disease (FMD) serotype SAT (South African Territories) 2 viruses isolated from Zimbabwe and other African countries using monoclonal antibodies (MAb). A sandwich-ELISA was used to examine the relative binding of anti-SAT 2 MAb to the various viruses. The MAb-binding profiles of viruses isolated from field samples were compared using hierarchical cluster analysis. Viruses were obtained from game animals, mainly African buffalo (*Syncerus caffer*) which is the natural host and reservoir for SAT serotypes in Africa, and from cattle showing clinical signs of FMD, as well as from animals suspected of carrying the virus subclinically. Some isolates have been adapted for use as vaccine strains. The results showed that most of the Zimbabwe isolates collected between 1989 and 1992 were an antigenically closely-related group. Although differences were observed between Zimbabwe isolates collected between 1989 and 1992 and those collected in 1987, there was no correlation with the different MAb binding patterns within the 1987 group and the epidemiological information received from the field. Similar profiles were observed for many SAT 2 viruses, including viruses isolated over a 50-year period and from geographically distant areas. This indicates an inherent stability in antigenic profiles of SAT 2 viruses. The MAb panel was capable of assessing antigenic variation, since very different profiles were obtained for some isolates. The work also allowed comparison and characterization of anti-type SAT 2 MAb from different laboratories. The findings are discussed with reference to selection of vaccine strains.

INTRODUCTION

The SAT 2 serotype of foot-and-mouth disease virus (FMDV) is restricted to Africa although it has been isolated from cattle from an abbatoir in the Arab Republic of Yemen where it was probably introduced by trade animals from Sudan. The SAT 2 viruses have been implicated in 52% of FMD outbreaks in cattle in Zimbabwe since 1931 where, historically, primary foci of infection have been confined to areas where cattle and buffalo have occurred together [1]. On the

assumption that buffalo can spread FMD to cattle, strict control measures have been initiated to keep cattle separated from FMD infected buffalo. This is done by regionalization into 'a clear zone' and 'control zones'. The control zones surround wildlife areas where game fences separate wild animals from domestic stock. The control area encompasses both a vaccination zone next to the game fence and a buffer zone between the vaccination zone and FMD-free non-vaccinating areas, where cattle are not vaccinated but are kept under constant surveillance for FMD. International recognition of an established disease-free zone has made Zimbabwe eligible to supply beef to European Union markets and the maintenance of this trade depends on the ability of the veterinary authorities to limit FMD to designated wildlife areas. The disease-free status of the clear zone depends on strict movement control of animals and their products between zones, continual surveillance of the control zones and effective vaccination of animals in the vaccination zone.

Between April–September 1989, SAT 2 outbreaks occurred in a disease-free zone. Cattle on infected premises were vaccinated and held under quarantine for 4·5 years and cattle on surrounding farms were vaccinated and quarantined for 6 months. Cattle born after the outbreak were permitted off infected properties after 2 years. FMD virus continued to be isolated from oesopho-pharyngeal scrapings (probang samples) from cattle on previously infected farms for up to 3 years and nucleotide sequencing of the virus isolates provided evidence that these carrier cattle were responsible for initiating a further outbreak of FMD type SAT 2 in 1991 (N. J. Knowles, unpublished data).

There is a need to differentiate antigenically FMDV isolates from clinically affected cattle, from cattle carrying virus following an outbreak, and from carrier buffalo in game areas adjacent to farming areas, in order to compare these with each other and against isolates adopted as vaccine strains. This is important to ensure that vaccine strains share antigens with the potential outbreak strains and therefore induce immunity through the stimulation of cross-reactive antibodies. The primary objective of this study was to examine epitopes shared by viruses using monoclonal antibodies (MAb) in an attempt to assess the likely protection afforded by SAT 2 vaccine strains against viruses currently circulating in Zimbabwe including those carried by buffalo in game areas. A second objective was to assess the method for identifying suitable FMD virus strains for incorporating into future vaccines for use throughout Africa and in the European Vaccine Bank.

FMD viruses can be compared using MAb which react with different epitopes. A panel of neutralizing MAb against FMD RHO 1/48, has been characterized by amino acid sequencing of MAb escape mutants [2]. This study defined three distinct epitopes centering on the capsid VP1 loop region. One epitope was linear and highly conserved in field isolates, a second epitope was conformational and not as conserved and a third epitope was conformational and entirely specific for the virus used to elicit the MAb. The last epitope was found in a region of extremely high amino acid sequence variation in the VP1 loop, associated with extra amino acid sequence found in SAT 2 viruses as compared to serotypes O, A and C. Other MAb against SAT 2 viruses were obtained from the FMD laboratory at Onderstepoort in South Africa.

MATERIALS AND METHODS

Viruses

Most of the original isolations of viruses from samples submitted to the World Reference Laboratory for FMD, IAH Pirbright, were made in cell culture using primary bovine thyroid cells (BTY). Cell culture fluid containing virus was obtained after removal of cell debris by low speed centrifugation. Isolates were then stored directly at −70 °C or in 50 % glycerol at −20 °C. Isolates have been amplified by passage (different passage levels) in a continuous cell line of IB-RS-2 cells. Samples had been submitted either as epithelium from clinical cases of FMD or as scrapings from the oesopho-pharyngeal region (probang samples) of cattle and buffalo suspected of asymptomatically carrying live FMD virus following previous clinical or subclinical infection, 'carrier animals' [3]. Several samples, including those from impala (*Aepyceros melampus*) were received as cell culture fluids from the Foot-and-Mouth Disease Laboratory, Onderstepoort, South Africa.

Sixty-two SAT 2 virus isolates were examined, 37 were isolated from samples collected in Zimbabwe between 1987 and 1992, the remaining 25 came from samples collected in Zimbabwe before 1987 and other African countries between 1948 and 1990. Some of the isolates have been adopted as vaccine strains, one isolate was derived from the same outbreak farm and has been shown by genetic sequence analysis [4] to be very closely related to a vaccine strain currently used in Zimbabwe.

Monoclonal antibodies

A panel of 21 mouse MAb produced against type SAT 2 strains of FMD virus was used in this study. Nine neutralizing MAb were prepared against the SAT 2 isolate Rhodesia 1/48 (RHO 1/48), and have been characterized by Crowther and colleagues [2]. The remaining 12 MAb were produced by the Foot-and-Mouth Disease Laboratory, Onderstepoort; 7 against SAT 2 isolate SAR 16/83, 3 against ZIM 5/83 and 2 against BOT 3/77. Five were reported as non-neutralizing.

ELISA characterization of viruses using MAb

The binding of the individual MAb to the different virus isolates was measured in a sandwich-ELISA as previously described [5]. Briefly, rabbit and guinea-pig polyclonal antisera were prepared as described elsewhere [6]. Wells of microtitre ELISA plates (Nunc Maxisorb) were coated with 50 μl of a pre-titrated dilution of a mixture of rabbit polyclonal anti-SAT 2 sera diluted in 0·05 M carbonate/bicarbonate buffer, pH 9·6 and incubated overnight at 4 °C. The mixture consisted of antibodies against 5 SAT 2 strains (ZIM 5/81, BOT 3/77, NIG 6/81, KEN 65/82, ZAM 3/81) to produce a broad spectrum capture reagent capable of binding all SAT 2 viruses at the IAH, Pirbright. Plates were then washed by flooding and emptying wells four times with phosphate buffered saline (PBS). After blotting away most of the residual washing fluid, virus samples (50 μl/well) were added at a constant dilution in PBS containing 5 % skimmed milk powder (Marvel) and 0·1 % Tween 20 (blocking buffer). Plates were incubated on a rotary shaker for 1 h at 37 °C then washed. MAb (50 μl/well) were then added, each at a constant
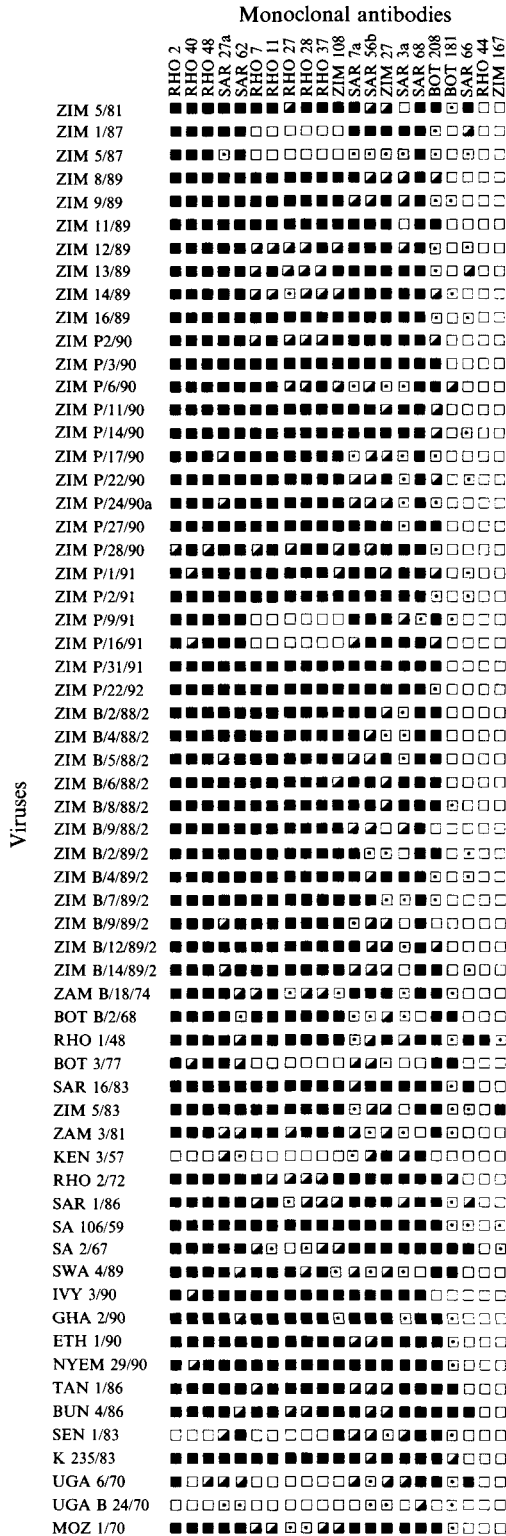
F. L. DAVIDSON AND OTHERS

Fig. 1. Analysis of type SAT 2 FMD viruses using MAb. Binding of MAb as compared to homologous reaction. □, 0–20 %; ▣, 21–45 %; ◪, 46–75 %; ■, 76–100 %.

Fig. 2. Differentiation of SAT 2 isolates using MAb. Measure: squared euclid; method: complete linkage.

dilution in blocking buffer, representing an excess of antibody as determined from titration curves for each MAb using the homologous virus as antigen in the sandwich-ELISA described here. Each MAb was used at the last dilution which gave the maximum colour in that system. Polyclonal guinea-pig sera (same mixture as for rabbit) were added at a pre-titrated dilution in blocking buffer, in order to assess the amount of each virus captured. Plates were uncubated at 37 °C on a rotary shaker for 1 h and then washed. The relevant antispecies conjugates were then added (50 μl/well) diluted in blocking buffer. Plates were incubated at 37 °C for 1 h as above and washed. The test was developed by the addition of 50 μl/well of OPD/H_2O_2 and stopped by the addition of 50 μl/well of 1 M-H_2SO_4 after 15 min. The colour was quantified using a multichannel spectrophotometer (Titertek Multiscan), reading at 492 nm.

Processing of Data

The mean OD values for each MAb/virus reaction were first corrected by subtracting the background value obtained from wells which contained MAb without virus. The adjusted OD values for each MAb were then expressed as a percentage of the mean OD value obtained using the polyclonal guinea-pig serum for each virus. This value was then expressed as a percentage of the value obtained for the particular MAb against the homologous virus.

Characterization of Viruses

The results for the binding of MAb to all the viruses are shown in Figure 1 as boxes representing four percentage ranges 0–20, 21–45, 46–75 and 76–100 %. The ranges were based on the criteria previously established [3]. More detailed identification of the inherent groupings in the data was made using multivariate statistics by hierarchical cluster analyses (Unistat Statistical Package for MS Windows, Unistat Ltd, London, UK). A dendogram showing results for all 62 viruses and all MAb is shown in Figure 2. The distance measured, which represents the similarity between viruses, was analysed using the Squared Euclid formula, computing the distance between groups using complete linkage. The relationships for mainly Zimbabwean isolates collected since 1980 were further examined by separating the cattle and buffalo samples.

Characterization of MAb

The MAb were compared according to their reactions with the 62 virus isolates, using multivariate statistics by hierarchial cluster analyses as described above. The binding of the South African MAb with selected RHO 1/48 MAb escape mutants, produced as described elsewhere [2], was also measured using the sandwich ELISA.

RESULTS

Analysis of viruses

The dendrogram following cluster analysis for the relationships between all the viruses (Fig. 2) indicates that ten isolates formed a distinct group: ZIM P 9/91, ZIM P 16/91, ZIM 1/87, ZIM 5/87, BOT 3/77, SA 2/67, SEN 1/83, KEN 3/57, UGA 6/70 and UGA 24/70. These isolates were differentiated because of their lack of binding to MAb RHO 7, 11, 27, 28 and 37. There were large differences between some of the isolates within this cluster. Viruses SEN 1/83, KEN 3/57, UGA 24/70, and to a lesser extent UGA 6/70, did not bind with MAb RHO 2, 40 and 48, which reacted strongly with all other SAT 2 viruses analysed to date. Viruses ZIM P 9/91 and 16/91 were isolated from probang samples collected from the same animal at different times and there is no known epidemiological connection between these two isolates and the similarly reacting ZIM 1/87. Isolates ZIM 1/87 and ZIM 5/87 were taken from separate, geographically distant outbreaks in 1987. Bot 3/77 could be differentiated due to the lack of reaction with MAb SAR 68, which recognized all the Zimbabwean isolates.

The remaining 52 viruses formed a second broad cluster where they were generally closely related to each other and with the exception of three viruses, (BOT B 2/68, ZAM 3/81 and SWA 4/89) which did not bind with MAb SAR 68, all bound the MAb: RHO 2, 40 and 48, 7, 11, 27, 28, 37, ZIM 108 and SAR 68.

Figure 3 shows in more detail the relationships of the Zimbabwean and neighbouring Botswana isolates. Figure 4 shows the buffalo viruses and Zimbabwean cattle viruses separately. The Hwange buffalo isolates were antigenically similar to each other, to recent Zimbabwean cattle isolates and to probang isolates from carrier cattle. The isolates from buffalo in Botswana-
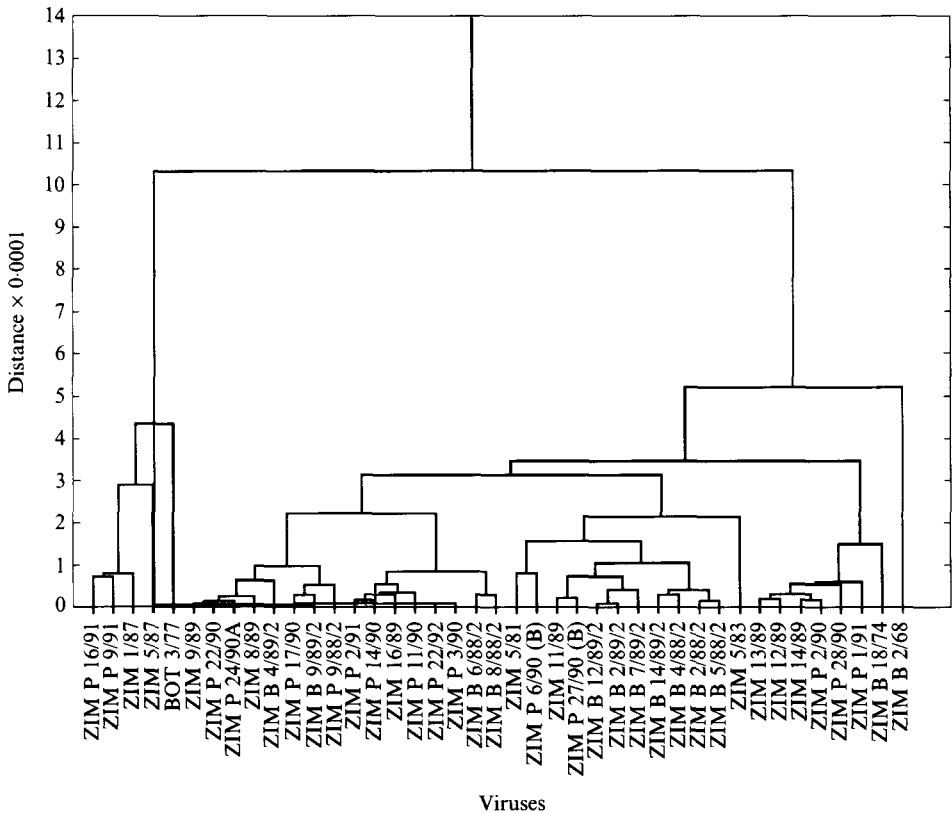
Distance × 0·0001

Viruses

Fig. 3. Hierarchical cluster analysis of Zimbabwean isolates from buffalo and cattle. Measure: squared euclid; method: complete linkage.

BOT B 2/68 and Zambia-ZAM B 18/74, as well as the cattle isolate BOT 3/77, were clearly different from the Zimbabwean buffalo isolates. The isolate ZIM 5/83 which was isolated from the same outbreak farm as the vaccine strain ZIM 7/83 currently used in Zimbabwe, was antigenically related to the majority of Zimbabwe isolates. No correlation could be made between the antigenic subgroupings of the isolates shown in Figures 2, 3 and 4 with the present understanding of the epidemiology based on sequence analysis and field information.

Analysis of MAb

The cluster analysis of the relative binding of the various MAb to all viruses is shown in the dendrogram in Figure 5. Analysis of MAb was also made with reference to the studies made on the binding of the MAb to selected RHO 1/48 MAb escape mutants as described previously [2] and to the virus neutralization activity of the MAb. These data are summarized below the cluster analysis in Figure 5 and allow the initial characterization of the MAb from Onderstepoort, in terms of the MAb produced at Pirbright. Boxes A to E, illustrate our assessment of epitope recognition by the MAb. Five major clusters for MAb reactions were
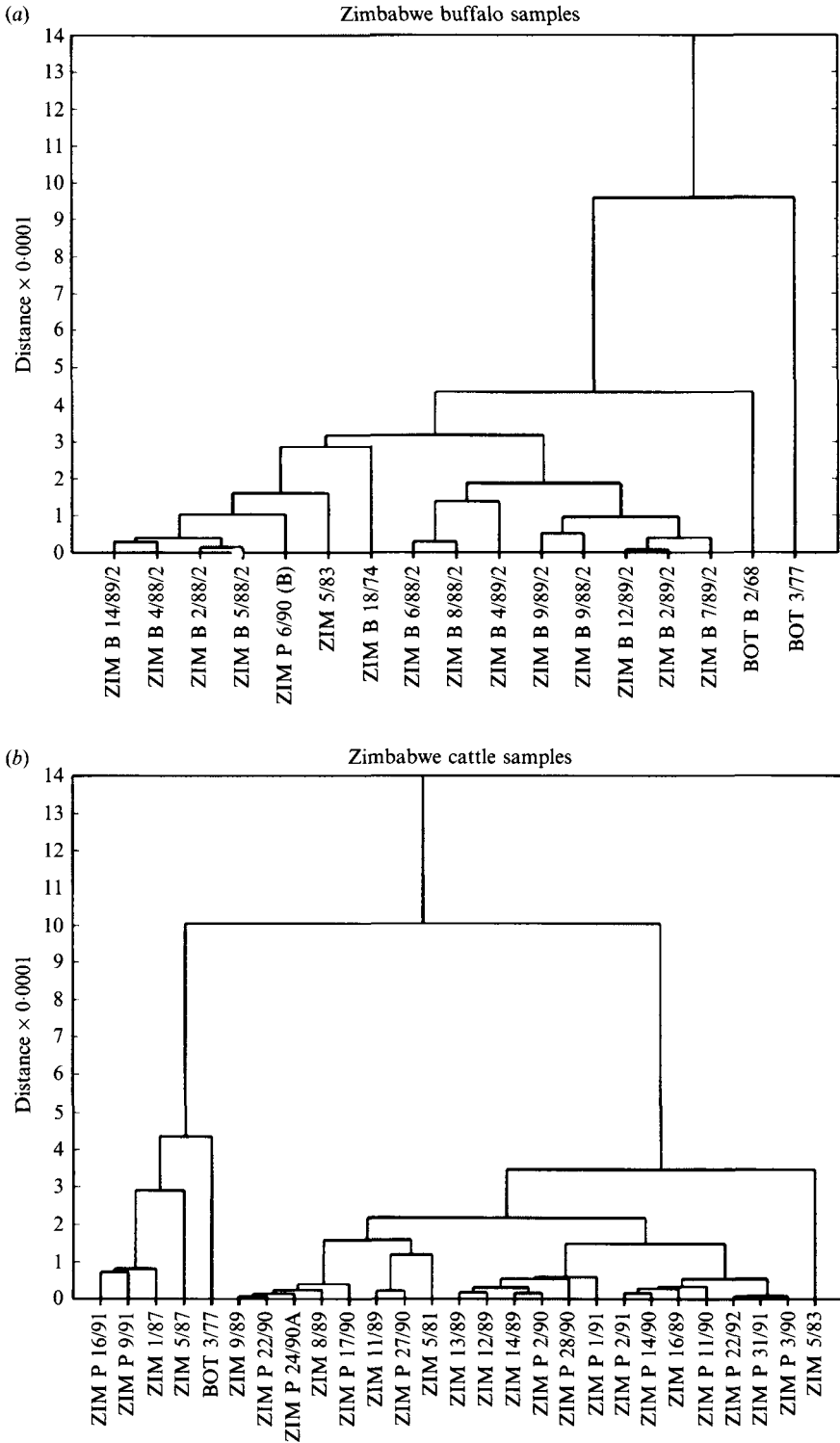
(a)

(b)

Fig. 4. Hierarchical cluster analysis of Zimbabwe isolates from buffalo and cattle.
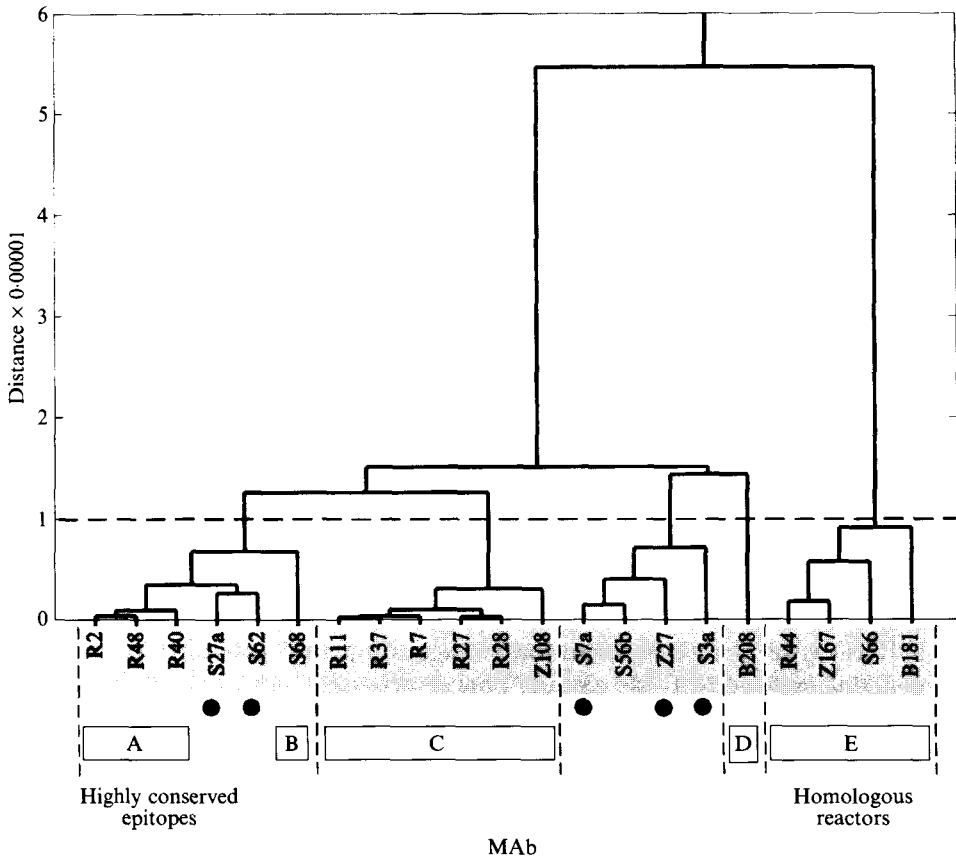Measure: squared euclid; method: complete linkage.

Fig. 5. Cluster analysis of MAb with reference to 62 virus isolates and assessment of their epitype recognition. ▨, Five clusters defined from dendrogram > 1; ●, non-neutralizing MAb (as reported by South African laboratory); □, A–E, epitype assessment using all MAb data.

defined from the analysis of the virus binding studies. MAb Rho 2, 40, 48, SAR 27a, 62 and 68, respectively, formed a cluster. Crowther and colleagues [2] established that MAb RHO 2, 40 and 48 recognize the same linear epitope. This epitope was highly conserved, Figure 1 illustrates that there is strong binding (equivalent to that with the homologous virus) between these MAb and 58 (93 %) of the isolates. MAb SAR 27a and 62 were reported as being non-neutralizing and bound to all the isolates studied. This is consistent with previous work in the IAH, Pirbright Laboratory where non-neutralizing FMD MAb have been shown not to discriminate between viruses. MAb SAR 68 also bound strongly with 58 isolates but there were significant differences in reactivity between MAb RHO 2, 40, 48 and MAb SAR 68 with 8 isolates, indicating that MAb SAR 68 may be recognizing a different highly conserved epitope. This was confirmed by studies using escape mutants.

The MAb RHO 7, 11, 27, 28, 37 and ZIM 108 formed another group. Crowther and colleagues [2] showed that MAb RHO 7, 27, 28 and 37 react with the same

conformational epitope and it is likely that ZIM 108, a neutralizing MAb, also reacts at this site; this was strongly indicated in the MAb escape mutant study where ZIM 108 did not bind to escape mutants produced by MAb Rho 1/48, 7, 11, 27, 28 or 37, but bound to all other mutants. Fifty-three (85%) of the viruses in the study bound MAb from this group.

A cluster was formed by reportedly non-neutralizing MAb SAR 3a, 7a, and ZIM 27 and MAb SAR 56b (reportedly neutralizing). However, MAb 3a differentiated SAT 2 isolates and based on previous studies (as indicated above), might have been expected to neutralize viruses whereas MAb 56b did not discriminate viruses and was reportedly neutralizing. This is shown in Figure 1 where MAb SAR 7a, ZIM 27 and SAR 56b reacted with 62 or 63 of the isolates whereas MAb SAR 3a only bound to 53 of the viruses. All these MAb and the non-neutralizing MAb SAR 27a and 62 bound with all the Rho 1/48 MAb escape mutants. Virus neutralization tests will be made on the MAb in this cluster to confirm their true neutralizing status.

Bot 208 is a neutralizing MAb which reacted independently and discriminated between viruses, binding weakly or not at all with 27% of the isolates. The epitope recognized by BOT 208 has not been characterized and was not conserved between Zimbabwean isolates, with little or no binding in 35% of cases. The MAb bound to all RHO 1/48 MAb escape mutants, suggesting that this MAb identified an independent epitope to those already characterised.

The clustering of MAb BOT 181, RHO 44, ZIM 167 and SAR 66 is midleading since they had highly specific binding profiles and failed to react with the majority of the isolates. RHO 44 has been characterized (2) and shown to bind only with a conformational epitope on RHO 1/48 virus. The epitope exists on the loop in a region of high amino acid variation and it is suspected that MAb RHO 167, BOT 181 and ZIM 167 may be acting at a similar conformational site.

The dendrogram data were highly reproducible with the same relationships being established for virus and MAb, in three repeat exercises.

DISCUSSION

The use of a relatively simple sandwich-ELISA provided a rapid method for the measurement of differences in binding of specific MAb with a large number of viruses. Most of the isolates from Zimbabwe between 1989 and 1992 showed very similar binding profiles and reacted with MAb characterizing at least three epitopes. The epitopes identified were shared by some viruses from other parts of Africa and many vaccine strains. These profiles were observed for viruses from infected cattle, carrier cattle and buffalo in Zimbabwe and this provides evidence of antigenic links between these populations. No previously identified epidemiological link could be found between the clearly different 1987 Zimbabwean viruses and the similarly profiled carrier viruses (ZIM P 9/91 and ZIM P 16/91) isolated from probang samples collected in 1991. These viruses were different from other Zimbabwean isolates because they failed to bind MAb RHO 7, 27, 28 and 37, which identify a conformational site (2). ZIM P 9/91, ZIM P 16/91 and ZIM P

24/90 (data not shown) were collected from the same carrier animal over a period of 6 months. Isolates collected over the same period from other carrier cattle, including those on the same farm, gave MAb profiles similar to Zimbabwean isolates collected between 1988 and 1992. A possible explanation is the co-existence of minor sub-populations of viruses with altered binding at this conformational site. Selection of sub-populations with changes at this epitope may have occurred and been maintained in the carrier animal, thus providing the virus with a means of escaping immune mechanisms. If this epitope plays a dominant role in post-vaccination immune response, then the efficacy of SAT 2 vaccination could be reduced.

It is interesting that similar profiles were observed for other SAT 2 viruses isolated over a 50-year period and from a wide geographical area, including all 1990 isolates examined from the Ivory Coast, Ghana, Ethiopia and North Yemen. This indicates that the SAT 2 viruses have a similar antigenicity but it must be restated that this MAb panel was capable of assessing antigenic variation, since very different profiles were obtained for some isolates.

Evidence that the MAb in the panel react with different epitopes comes from the analysis of the binding data against 62 viruses (where there is variation in results), the analysis of the binding of the South African MAb with the RHO 1/48 MAb escape mutants and previous work [2], where three epitopes were defined. Strictly the definition of an epitype should be put forward when dealing with MAb binding. We define an epitype as the topographical region on the virus capsid which is identified by one or more MAb with variable affinity according to the exact nature of the MAb interaction at that region. Thus distinct epitypes can be regarded as being equivalent to the rather loose description 'antigenic sites' from tests based on polyclonal antibody binding.

This study confirmed that the MAb binding with a linear site epitype namely RHO 2, 40 and 48, gave the same profiles for the majority of viruses examined indicating that this site was highly conserved among SAT 2 isolates.

A second epitype was associated with a conformational site [2] and was bound by RHO 1/48 MAb 7, 11, 27, 28 and 37. This study was able to associate one of the MAb from the South African laboratory with this epitype (ZIM 108).

A third epitype was identified by MAb having a specificity only for the virus used to produce them. These are characterized by MAb RHO 44 which was shown to identify a conformational epitope within VP1 protein of the capsid in a region where there are extra amino acids as compared to type O, A and C viruses from 155 to 161 [2]. The region 198–206 of VP1 is also highly variable in SAT 2 viruses and specific changes here correlate with those observed in the 155–161 region. In contrast, the sequence variation observed in types O, A and C from 198 to 206 of VP1 is very low. Examination of the sequence data from 113 SAT 2 isolates indicates that viruses from the same outbreak have a unique pair of amino acid motifs in the 155–161 and 198–206 regions of VP1 and that epidemiological links between strains correlated with these motifs (work to be published). Thus viruses sharing epitopes identified by all the MAb other than RHO 44, can be identified through their unique 'signature' of amino acids at 155–161 and 198–206 on VP1. MAb ZIM 167, SAR 66 and BOT 181 probably identify similar epitopes and the prospect that this is a major immunogenic region is disturbing. If this site were

'dominant', i.e. the majority of polyclonal antibodies were directed against it, then protection would be afforded mainly through such antibodies. Any changes in this region in a field strain would possibly allow the virus to escape vaccine mediated immunity. Work to examine this hypothesis using MAb 44 escape mutants is progressing.

A fourth and previously unidentified epitype was identified by MAb SAR 68; this was highly conserved. The MAb BOT 208 is possibly recognizing a further epitype but additional work is needed to confirm this. The problem of characterization of the MAb is being addressed through the production and sequencing of various MAb escape mutants.

The relative importance of these epitopes to cattle is not known, however, most of the MAb used in this study neutralized SAT 2 viruses and there is evidence from Crowther and colleagues [7], that mouse MAb recognize the same type O FMD virus epitopes as polyclonal antibodies from cattle antisera. Neither the immunogenicity of the epitopes identified in this study nor their relevance to the protective response in animals can be predicted. Logically, it might be expected that where epitopes are shared there will be a better chance that cross-reactive antibodies will be produced so that the greater the degree of similarity in epitope profile, the greater the possibility that cross-protection will be induced. As most of the SAT 2 isolates submitted to the World Reference Laboratory since 1989 and many vaccine strains showed similar binding with MAb characterizing at least three epitopes it could be expected that a potent vaccine showing similar MAb profiling should give adequate protection against currently circulating SAT 2 viruses. This presumes that MAb identify all 'important' epitopes, which can only be assessed through increasing the production of MAb against a variety of SAT 2 isolates and characterizing the 'spectrum' of epitopes they identify and their relative importance to the immune response of the animal.

Examples of poor antibody responses following vaccination with SAT 2 vaccines have frequently been reported in the field [8]. It is a practical irrelevancy, however, if all the required epitopes are present but antibody response in the animal is poor even after booster vaccinations. The factors such as ability to grow to high titre in tissue culture, antigenicity, stability, etc. are equally if not more important when considering a field isolate as a candidate for adaption to a vaccine seed and cannot be assessed from MAb profiling. However, the use of the MAb profiling method to assess viruses after adaption and immediately before vaccine formulation is to be highly recommended since there are often antigenic changes observed by selection during the adaption process.

The converse situation, where strains with a poor antigenic match, but high immunogenicity, has also been reported in the field. For example KEN 3/57 produced a vaccine of consistent high potency which evoked a high antibody response against the homologous strain. This vaccine was used to control an outbreak even though the r_1 value against the field challenge isolate was 0·04, as measured by cross neutralization, indicating that it was antigenically very different [8]. The use of MAb may identify the epitope(s) involved in the cross-protection afforded by Ken 3/57 and lead to better vaccine formulation.

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