Differentiation of convalescent animals from those vaccinated against foot-and-mouth disease by a peptide ELISA

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Abstract

We have identified continuous antigenic determinants within the amino acid sequences of the conserved nonstructural region containing proteins 2C and 3ABC of foot-and-mouth disease virus which can distinguish between the sera from vaccinated and infected animals. An ELISA based on a 3B peptide gave a positive reaction with sera from cattle, pigs, sheep and guinea pigs infected with all seven serotypes of the virus, but not with sera from vaccinated animals. In experiments with cattle and pigs to determine the duration of the antibody response, positive reactions were obtained as late as one year after infection. The advantages of using peptides from the nonstructural viral proteins instead of recombinant proteins for differentiating vaccinees from infected animals include their exquisite specificity, nonreactivity with antibodies against host cell-derived proteins (e.g. *E. coli* and insect cell proteins), and their ease of preparation. © 1999 Elsevier Science Ltd. All rights reserved.

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Identifying animals that have been infected with foot-and-mouth disease virus (FMDV) is of considerable importance because it is well established that infected cattle and sheep frequently become carriers of the virus and consequently may become the source of new outbreaks of the disease. This situation is compromised by the difficulty in distinguishing infected animals from those that have been vaccinated against the disease since both groups contain neutralizing antibodies in their sera. Moreover, asymptomatic carrier animals can be found in vaccinated herds.

Consequently, several groups have sought to devise tests that can distinguish infected animals from those that have been vaccinated. The approach has been to identify antibodies against virus-specific proteins that are present only in infected animals. The virus infection associated antigen (VIAA), now recognised as the RNA polymerase 3D [1], was long regarded as a marker of infection but the presence of antibodies against this antigen occurred too frequently in vaccinated animals for it to be a reliable differential serological marker [2]. More recently, nonstructural protein 2C and the nonstructural 3ABC polyprotein and derivatives have been identified which apparently meet this criterion [3–10]. These virus-specific proteins have been produced either in recombinant *E. coli* [3–7] or in insect cells infected by appropriately engineered baculoviruses [6,8,10]. One of the problems associated with the use of these products, however, is the presence of antibodies against expression vector antigens that copurify with the recombinant products. Antibodies to *E. coli* are commonly found in the sera of uninfected and unvaccinated animals [6,8]. We have recently made a similar observation with antibodies to insect proteins [8]. Also, the diverse array of continuous and discontinuous antigenic sites presented by long recombinant FMDV proteins can result in significant nonspecific reactions, including cross-reactivities to other picornaviruses [3], that make interpretation of test results difficult. These specificity problems limit a

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Table 1
Serum Panel 1 for mapping continuous antigenic sites

Serum Panel 1	Type/Strain
GP[a]-R00023*	A/5 Westerwald
GP-R00059	A/22 Turkey
GP-R00071	A/27 Columbia 67
GP-R00079*	O/1 Campos
GP-R00100	C/3 Resende
GP-R00101	C/4 Tierra del Fuego
GP-R00105	Asia 1/1 Pakistan
GP-R00116	SAT1/4 S. Rho 58
GP-R00124	SAT2/1 Rho 48
Normal guinea pig serum	—
BOV[b]-ROO690	Weak VIAA+ reference[c]
BOV-R01053[d]*	A/5 Westerwald
	O/1 Kaufbeuren
	SAT2/1 Rho 48
Normal bovine serum	—

[a] Guinea pig sera.

[b] Bovine sera.

[c] Produced by diluting AGID-reactive serum with normal bovine serum to produce a weakly reactive reference sample.

[d] Steer was sequentially inoculated by the indicated strains on days 1, 29, and 84, respectively.

* Sera used for second round of epitope mapping.

differential nonstructural protein assay to a cumbersome immunoblot format [4,5]. Accordingly, we have sought to identify continuous immunodominant sites on 2C and 3ABC, which only recognize antibodies against these proteins, and produce such recognition sites as inexpensive and site-specific synthetic peptides.

Our approach to the mapping of continuous viral determinants has been by the solid-phase synthesis of overlapping peptides, followed by analysis of their reactivities to well-characterized serum samples [11,12]. Overlapping peptides from the 2C and 3ABC candidate regions were adapted to the simple indirect ELISA format and examined for serological reactivities to panels of well-characterized serum samples. Continuous determinants for detection of antibodies evoked by exposure to infectious virus were identified by means of these analytical enzyme immunoassays, and an ELISA which consists of a synthetic peptide taken from the nonstructural region as solid-phase immunoabsorbent was designed as a differential serological test. The synthetic peptide antigen detects evidence of exposure to infectious virus with equally high sensitivity and specificity both in the presence and absence of vaccination, irrespective of the serotype.

1. Materials and methods

1.1. Peptide synthesis

Peptides corresponding to the highly conserved nonstructural proteins 2C and 3ABC were synthesized according to the sequence for A12. Each peptide was produced by an independent synthesis on a solid-phase support using an Applied Biosystems Peptide Synthesizer Model 430A, and Fmoc protection for the α-NH$_2$ terminus and side chain protecting groups of trifunctional amino acids. Completed peptides were cleaved from the solid support and side chain protecting groups removed by 90% trifluoroacetic acid. Synthetic peptide preparations were characterized for correct composition by Matrix-Assisted Laser Desorption Time-of-Flight Mass Spectrometry using a PerSeptive Biosystems/Vestec LaserTec Benchtop 11 Mass Spectrometer, and by Reverse Phase HPLC.

1.2. Serum samples

The guinea pig and bovine sera of Panel 1 (Table 1) were obtained from animals infected with the indicated viruses in the course of previous studies in biocontainment facilities at the USDA Plum Island Animal Disease Center (PIADC), Greenport, NY, at Merial Animal Health Ltd Biological Laboratory, Pirbright, UK, and at the Institute for Animal Science and Health, Lelystad, the Netherlands. The samples of Serum Panel 2 (Table 2) from cattle infected or vaccinated with diverse types and subtypes of FMDV 21 days previously were supplied by Merial Animal Health, and the sera from naive animals were drawn from the reagent repository at the Foreign Animal Disease Diagnostic Laboratory, PIADC. The bovine and swine seroconversion panels (Figs. 3–6) were serial bleeds collected following the in-contact infections or vaccinations reported previously [9], except for swine ♯16 and ♯19 which were infected by contact in 1997 at PIADC with O Taiwan. Abattoirs in the USA, an FMD-free country, provided the normal swine and bovine specimens for determination of the reactivity distributions of naive animals.

1.3. ELISA method

96-well microtitre plates were coated by 1 h incubation at 37°C with either mixtures of two or three 2C, 3ABC peptides or individual peptides at the concentrations indicated in the Results, using 100 μl per well in 10 mM NaHCO$_3$ buffer, pH 9.5. The peptide-coated wells were incubated with 250 μl of 3% by weight of gelatin in phosphate-buffered saline (PBS) at 37°C for 1 h to block nonspecific protein binding sites, washed three times with PBS containing surfactant (UBI™ WASH BUFFER, United Biomedical Inc., Hauppauge, NY) and then dried. Test samples were diluted with a HEPES-buffered solution containing surfactant, inactivated goat serum, and carrier proteins

Table 2
Serum Panel 2: bovine samples for serological validation of a non-structural peptide ELISA

Sample ID	Description[a]	Infecting or vaccine virus	Absorbance[b]		
			3A	3B	3A + 3B
1	VS	O1 BFS 1860	0.236	0.049	0.217
2	CS	O1 BFS 1860	>2.000	>2.000	>2.000
3	VS	O India 53/79	0.253	0.071	0.298
4	CS	O India 53/79	>2.000	>2.000	>2.000
5	VS	O Turkey 1/78	0.218	0.048	0.212
6	CS	O Turkey 1/78	>2.000	>2.000	>2.000
7	VS	O1 Campos	0.179	0.132	0.233
8	CS	O1 Campos	>2.000	>2.000	>2.000
9	NEG		0.135	0.075	0.145
10	NEG		0.163	0.033	0.151
11	NEG		0.126	0.082	0.185
12	NEG		0.054	0.031	0.069
13	NEG		0.143	0.070	0.279
14	VS	A22 Iraq 24/64	0.262	0.051	0.242
15	CS	A22 Iraq 24/64	>2.000	>2.000	>2.000
16	VS	A Kenya 42/66	0.249	0.096	0.219
17	CS	A Kenya 42/66	>2.000	>2.000	>2.000
18	VS	A24 Cruzeiro	0.495	0.058	0.558
19	CS	A24 Cruzeiro	>2.000	>2.000	>2.000
20	NEG		0.249	0.161	0.226
21	NEG		0.079	0.053	0.102
22	NEG		0.047	0.031	0.070
23	NEG		0.113	0.112	0.132
24	NEG		0.095	0.039	0.114
25	VS	C Pando	0.575	0.196	0.610
26	CS	C Pando	>2.000	>2.000	>2.000
27	VS	C Noville	0.949	0.031	0.862
28	CS	C Noville	>2.000	>2.000	>2.000
29	NEG		0.036	0.164	0.223
30	NEG		0.086	0.074	0.090
31	NEG		0.083	0.047	0.082
32	NEG		0.168	0.123	0.152
33	NEG		0.092	0.089	0.093
34	VS	SAT 1 Bot 1/68	0.294	0.113	0.291
35	CS	SAT 1 Bot 1/68	1.949	0.639	>2.000
36	VS	SAT 2 K183/74	0.398	0.118	0.401
37	CS	SAT 2 K183/74	>2.000	>2.000	>2.000
38	VS	SAT 3 BEC 1/65	0.234	0.101	0.242
39	CS	SAT 3 BEC 1/65	1.984	1.172	2.000
40	NEG		0.178	0.052	0.175
41	NEG		0.063	0.044	0.075
42	NEG		0.280	0.214	0.343
43	NEG		0.206	0.126	0.182
44	NEG		0.378	0.044	0.344
45	VS	Asia 1 India	0.272	0.123	0.287
46	CS	Asia 1 India	>2.000	>2.000	>2.000
Pos[c]			1.638	>2.000	>2.000
Neg[c]			0.134	0.049	0.134

[a] CS, bovine convalescent serum; VS, bovine vaccinee serum; NEG, bovine negative sera.

[b] Absorbance at 492 nm on experimental ELISAs for ruminants. Serum samples were diluted 1:20 with UBI SPECIMEN DILUENT BUFFER (UBI, Hauppauge, NY).

[c] Reference samples, positive control was infected guinea pig serum, negative control was normal goat serum.

(UBI™ SPECIMEN DILUENT BUFFER, United Biomedical Inc.) at dilutions of 1:20, 1:50, or 1:100 (v/v) as indicated. 100 µl of the diluted sample were added to each of the wells and allowed to react for 1 h at 37°C. The wells were then washed six times with UBI™ WASH BUFFER (United Biomedical Inc.). The standardized preparation of anti-swine IgG antibody labelled with horseradish peroxidase (HRP) for
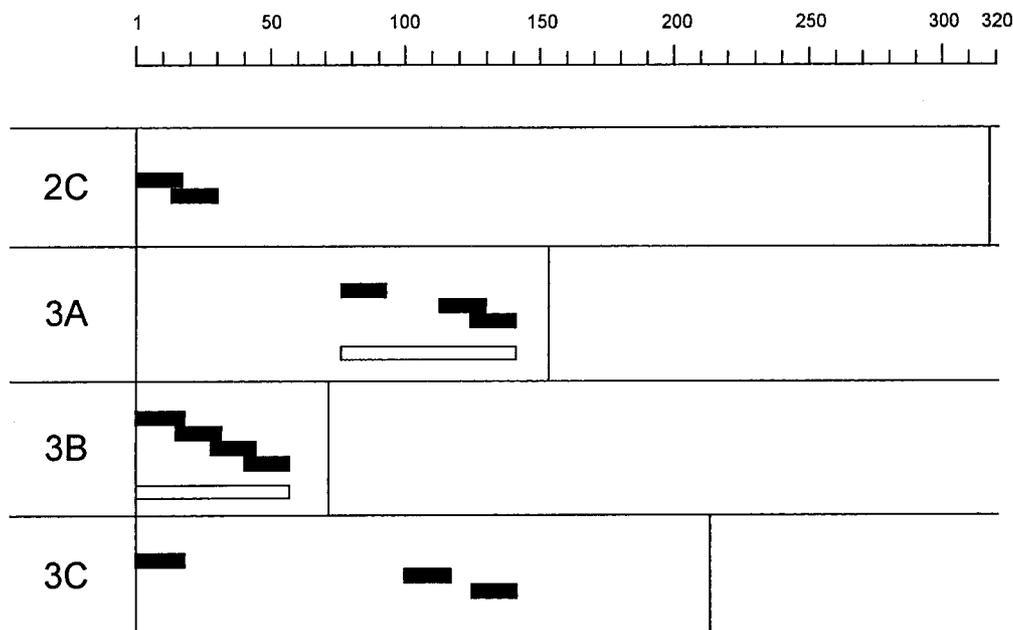
Fig. 1. Continuous epitopes on nonstructural proteins 2C, 3A, 3B, and 3C were mapped by antibody reactivities of 18mer and 19mer peptides. Peptides displaying absorbances on the screening ELISAs of ⩾0.290 with at least one of the asterisked sera of Panel 1 (Table 1), diluted 1:100, were scored as reactive. The reactive 18mer and 19mer peptides within the amino acid sequences of each nonstructural protein are shown by the solid bars. The open bars in the sequences for 3A and 3B represent the longer 3A and 3B peptide antigens used for 3A, 3B and 3A + 3B experimental ELISAs. The carboxy termini for the mature nonstructural proteins are indicated by the vertical lines.

swine sera, or HRP-conjugated ImmunoPure® Protein A/G (Pierce Chemical Co., Rockford, IL) for bovine, sheep, or guinea pig sera, were added to each well and incubated at 37°C for 30 min. (Bovine and guinea pig antibodies were detected in the initial mapping studies with polyclonal rabbit anti-bovine IgG-HRP and anti-guinea pig IgG-HRP conjugates.) The wells were again washed six times with UBI™ WASH BUFFER to remove unbound labelled conjugate and reacted with 100 μl of the substrate mixture containing 0.04% by weight *o*-phenylenediamine and 0.12% by volume hydrogen peroxide in sodium citrate buffer pH 5.0, for 15 min. Reactions were stopped by the addition of 100 μl of 1.0 M H_2SO_4 and the absorbance at 492 nm (A_{492}) was measured.

Peroxidase-labelled ImmunoPure® Protein A/G (Pierce) was selected for detection of ruminant and guinea pig IgG by the provisional ELISA because of its specificity for IgGl and IgG2 from multiple species. However, the background reactivity of the recombinant A/G conjugate was unacceptably high for swine IgG. Accordingly, the polyclonal anti-swine IgG HRP conjugate was selected for detection of captured swine immunoglobulins. The concentrations of the recombinant Protein A/G and polyclonal anti-swine conjugates were adjusted for optimum sensitivity and specificity on serum test panels (data not shown).

2. Results

2.1. Site-specific serology for mapping nonstructural protein epitopes

Sixty peptides comprising 18 or 19 amino acids were synthesised with overlaps of six residues across the entire amino acid sequence for the nonstructural proteins 2C, 3A, 3B, and 3C. Although specific secondary structures known to be associated with antigenic proteins were considered in the design of these overlapping peptides, emphasis was placed on an empirical selection of candidate immunodominant peptides by their immunoreactivities. Mixtures of two or three sequential peptides were used to coat ELISA microtitre plates at concentrations for each peptide of 2 μg/ml. For the initial screening, ELISA immunoreactivities were determined with Serum Panel 1 (Table 1) consisting of samples from nine guinea pigs experimentally infected with strains of types A, O, C, Asia 1, SAT-1, SAT-2, an unexposed guinea pig, a steer inoculated with multiple types, a weakly VIAA-positive bovine sample, and an unexposed bovine sample. The individual sera of the panel were added to the peptide-coated plates at a 1:20 dilution.

All the peptides from 3A, 3B, and 3C captured antibodies to the diverse types and subtypes of virus as
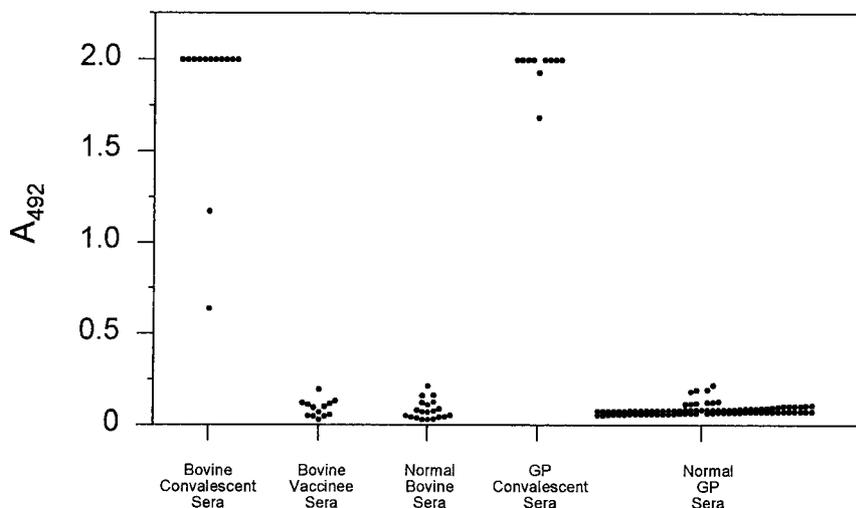
Fig. 2. Reactivities of the provisional differential ELISA on serum panels. Convalescent, vaccinee and unexposed bovine samples from Panel 2 (Table 2), convalescent guinea pig samples from Panel 1 (Table 1), and sera from 89 naive guinea pigs were tested for antibodies to nonstructural proteins by the peptide-based differential ELISA for ruminants. The results for the samples are grouped by species and exposure to virus.

shown by A_{492} values higher than 0.4 for the majority of the samples from infected animals (data not shown). Immunoreactivity to the 2C peptides was primarily to those from the amino terminal region of the protein, and was strongest with those sera that were also most strongly reactive with the 3ABC peptides (data not shown). Thus, the 2C peptides showed little potential for detection of additional infected samples over those also detected by the 3ABC peptides. No peptide provided an absorbance above 0.148 with either the uninfected guinea pig or bovine sera. Accordingly, all of the 3A, 3B, and 3C peptides remained as candidate antigens for sources of useful continuous epitopes while less benefit was ascribed to the 2C peptides.

The three sera marked with an asterisk in Table 1, from guinea pigs inoculated with strains of types A and O, and a steer multiply inoculated with strains of types A, O, and SAT-2, were used for a second round of peptide selection by serological validation in which the plates were coated with individual peptides at 2 µg/ml and the sera used at a dilution of 1:100. The results of this more discriminatory screen indicated the presence of potentially useful continuous determinants in the carboxy terminal half of 3A, throughout 3B except for the carboxy terminal region, and on three scattered areas in 3C contained within the amino terminal region and toward the carboxy terminal half (Fig. 1). Peptides from these regions were considered as candidate antigens for recognition of immunoreactivity associated with infection. An antigenic region from the amino terminus of 2C was also reactive with bovine serum BOV-RO1053, but the most antigenic 2C peptide gave lower absorbance with this serum than the

most reactive peptides from the 3A, 3B, 3C peptide collection and was not further investigated.

The immunodominant 18 and 19mer peptides from 3A, 3B, and 3C were selected for further investigation. These were re-synthesized as contiguous constructs of 29–60 amino acids to present longer processions of continuous epitopes and for greater ability to present conformational epitopes. These longer peptides were evaluated for reactivity to Serum Panel 1. Two of the longer peptides, corresponding to the antigenic regions of non-structural proteins 3A and 3B (shown in Fig. 1 by open bars), exhibited the highest seroreactivity (data not shown).

2.2. Serological validation of experimental ELISAs on mixed panel

Experimental differential ELISAs were formulated from the selected 3A and 3B peptide antigens and other test components in accordance with specifications that are preferred for a typical commercial ELISA. Thus, the antigen and other reagents were balanced so as to provide an absorbance value of about 2.0 at 492 nm on strongly reactive reference sera diluted 1:20, with non-reactive control values of less than 0.4. Three resulting ELISAs were assembled to have microtitre plates coated with (i) the 3A peptide at 2 µg/ml; (ii) the 3B peptide at 0.5 µg/ml, and (iii) a mixture of the coating peptides with the 3A antigen at 2 µg/ml and the 3B antigen at 0.5 µg/ml. These were tested under code on Serum Panel 2 (Table 2). The convalescent and vaccinee serum samples of this mixed panel represented each region, e.g. Iraq, India, Kenya, Turkey, UK, Campos (Brazil). These sera are charac-
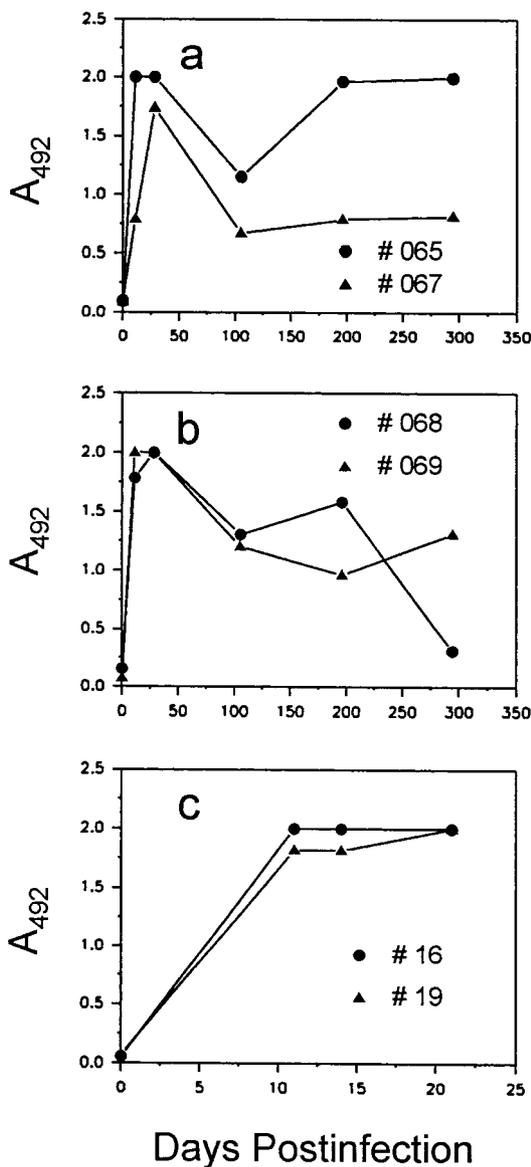
Fig. 3. Antibody reactivities of sera from sequential bleeds of swine experimentally infected with A24 [9] (a,b), and from swine infected by contact with O1 Taiwan (c). Animals were monitored by the provisional differential ELISA for swine, at the indicated intervals, for antibody to nonstructural proteins. Animal identification numbers shown for a and b are those used for the previous report [9].
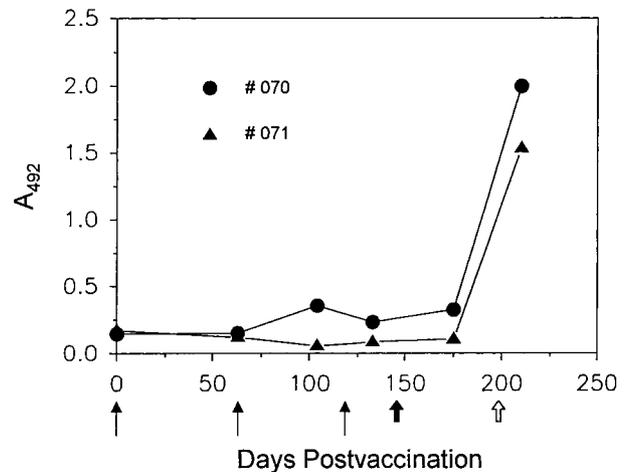

Fig. 4. Antibody reactivities of sera from sequential bleeds of swine receiving oil-adjuvanted A24 vaccine, homologous challenge, and heterologous challenge [9]. Animals were monitored by the provisional differential ELISA for swine at the indicated intervals. Animal identification numbers are those used for the previous report [9]. Animals were vaccinated on days 0, 63, and 119 (long arrows), challenged with homotypic virus A24 on day 147 (short solid arrow), and challenged with heterotypic O1 Tunisia on day 199 (short open arrow).

terized in Table 2 for FMDV status and for absorbances in the experimental ELISAs using sera diluted 1:20. All three peptide-based immunoassays identified the 13 convalescent samples for 100% sensitivity, and remained below the arbitrary cutoff value of 0.4 for nonreactive control sera. The serotype of the infecting viruses had no effect on sensitivity, but sensitivity for the convalescent samples was lower at serum dilutions of 1:50 and 1:100 (data not shown). However, absorbances of most of the nonreactive control sera were higher with peptide 3A than 3B. More significantly, the ELISA with the 3B antigen alone correctly distin-

guished all 13 vaccinee samples from the convalescent samples while the differential specificities of the 3A and the 3A + 3B tests were affected by crossreactive antibodies in the vaccinee sera. Three of 13 vaccinee sera of Panel 2 scored above the 0.4 A_{492} cutoff with the 3A peptide test (Sample ID Nos. 18, 25, and 27) with one sample (No. 36) being borderline. The 3 vaccinees with seroreactivity for 3A and the borderline sample were also reactive with the 3A + 3B combination (Table 2). Vaccinees 18, 25, 27, and 36 had been immunized with A, C, and SAT 2 viruses, respectively, so that crossreactivity to the non-structural protein A was not exceptional for any particular serotype. Consequently, the 3B peptide alone was selected for the solid-phase antigen of the test.

The data points from the 3B peptide-based ELISA shown in Table 2 were classified by the FMDV status of each animal and are summarized in Fig. 2. The satisfactory performance of the provisional assay is also shown by Fig. 2 for the infected guinea pig samples of Serum Panel 1 and for samples from 89 naive guinea pigs.

2.3. Sensitivity on seroconversion panels

Access to sera from experimentally infected and vaccinated swine and cattle enabled us to evaluate the synthetic peptide 3B-based differential assay for sensitivity and specificity with respect to early detection of seroconversion and duration of seroreactivity. Fig. 3 depicts the antibody responses of infected swine moni-
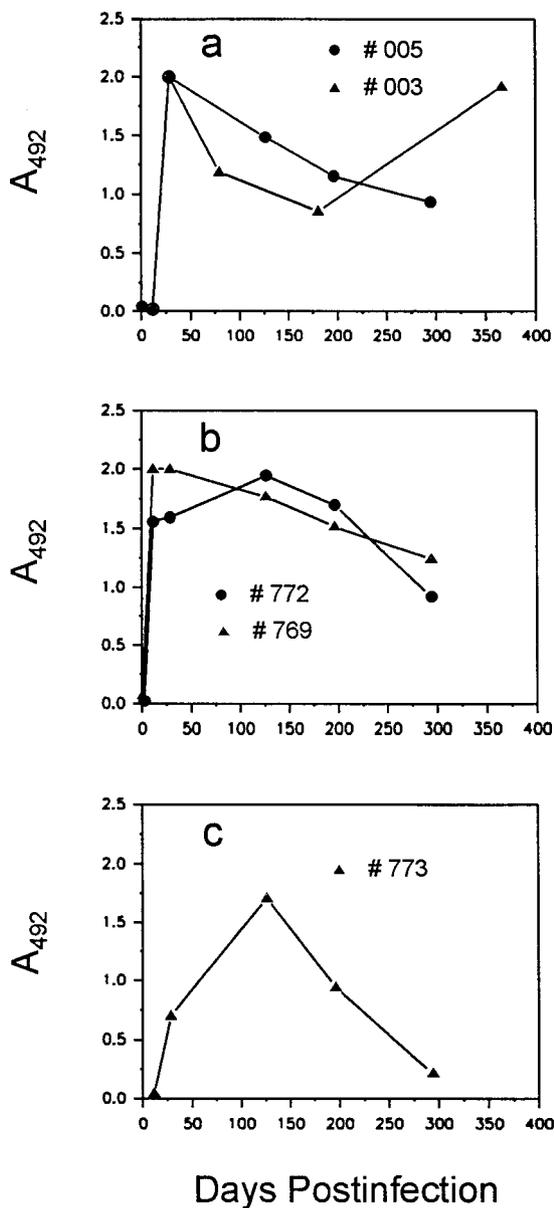
Fig. 5. Antibody reactivities of sera from sequential bleeds of steers experimentally infected with A24 at day 0. Animals were monitored by the provisional differential ELISA for ruminants, at the indicated intervals, for antibody to nonstructural proteins. Animal identification numbers are those used for the previous report [9].

Fig. 6. Antibody reactivities of sera from sequential bleeds of steer ♯774 [9]. This animal had been vaccinated with oil-adjuvanted A24 on days 0, 63, and 119 (long arrows), challenged with A24 on day 147 (short solid arrow), then challenged with 01 Tunisia on day 199 (short open arrow). Sequential serum samples were monitored by the provisional differential ELISA for ruminants at the indicated intervals.

bodies, reflecting the differential reactivity of the immunoassay based on the 3B nonstructural protein. On challenge with the homologous virus there was no increase in reactivity, but when challenged with heterologous virus of serotype 0, subtype 1 Tunisia, there was a considerable increase.

In four of the five seroconversion panels for experimentally infected cattle [Fig. 5(a–c)], reactivity was maintained through day 300. In the fifth animal, ♯773, seroreactivity persisted through day 200 [Fig. 5(c)]. Seroconversion was detected in infected cattle by day 21 [Fig. 5(a–c)], and as early as day 11 in animals ♯769 and 772 [Fig. 5(b)]. As was seen for the vaccinated swine of Fig. 4, steer ♯774 failed to develop seroreactivity in the assay after administration of three doses of an A24 vaccine, again despite the presence of high levels of neutralizing antibodies. On challenge with the homologous virus there was no increase in reactivity, but when challenged with heterologous 01 Tunisia virus, there was a considerable increase (Fig. 6).

2.4. Establishment of cut-off values and ELISA specificities

Adequate specificity for a validated ELISA requires that there is a wide separation between the reactivities of seropositive and normal sera. This had been incorporated as an original design element of our test by setting the sensitivity of the peptide antigen to exhibit absorbances of at least 2.0 on reactive reference samples and absorbances of less than 0.4 on samples from unexposed control animals. The distribution of reactivities of groups of infected animals was com-

tored for up to 300 days. Seroreactivity persisted through day 300 for three out of the four swine experimentally infected with A24, and through day 200 for the fourth [Fig. 3(a,b)]. Two additional swine, animals ♯16 and ♯19 [Fig. 3(c)] infected with O Taiwan demonstrated the ability of the assay to detect FMDV infection at 10 days post-exposure, the earliest sample that was drawn from each animal during the monitoring period.

Swine that had received an A24 vaccine on days 0, 63, and 119 [9] (Fig. 4) had no ELISA reactivity despite the presence of high levels of neutralizing anti-
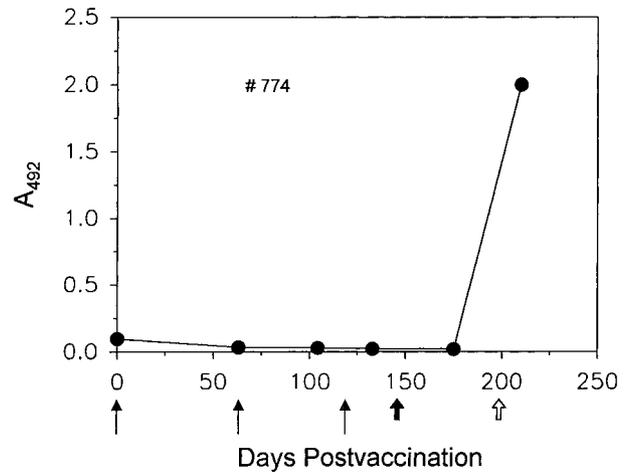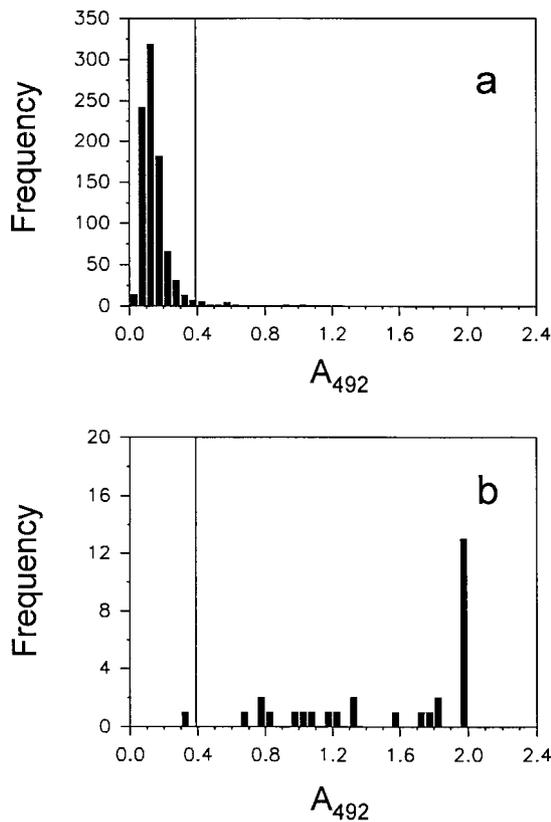
Fig. 7. Distribution of antibody reactivities to nonstructural proteins in sera from naive and infected swine. Samples were tested by the provisional differential ELISA for swine. (a) Samples from 887 naive animals; (b) sequential post-infection samples from swine experimentally infected at PIADC described in Figs. 3 and 4. The sole negative sample shown in (b) was from animal ♯068 bled on day 294 [Fig. 3(b)].
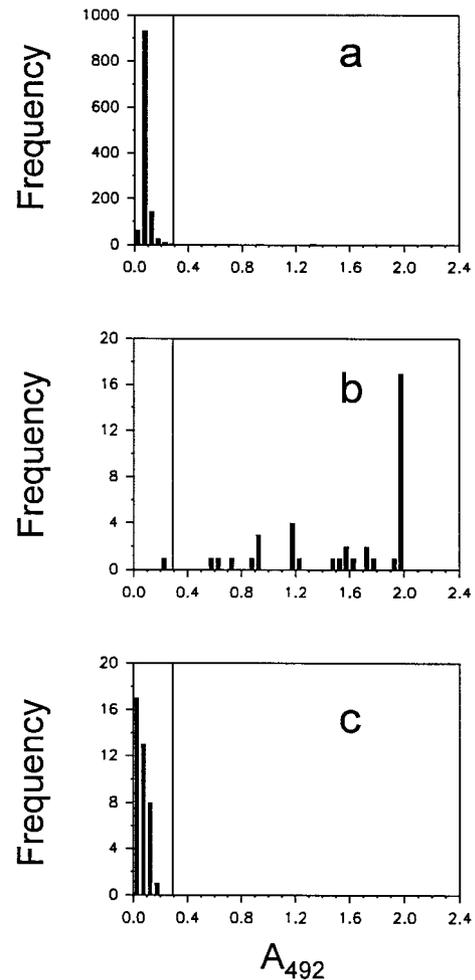

Fig. 8. Distribution of antibody reactivities to nonstructural proteins in sera from naive, infected, and vaccinated steers. Samples were tested by the provisional differential ELISA for ruminants. (a) Samples from 1202 naive animals; (b) sequential post-infection samples from steers experimentally infected at PIADC and from the 13 seropositive samples of panel 2 (Table 2) [the sole negative sample shown in (b) was from animal ♯773 bled on day 294, see Fig. 5(c); (c) distribution for the vaccinee samples from Panel 2 (Table 2) and other vaccinee samples described in the text.

pared to those of uninfected groups to demonstrate retention of this discrimination on actual populations, and to justify establishment of cut-off values for absorbances that clearly distinguished reactive from non-reactive samples.

The distribution of absorbances in the test are shown in Fig. 7(a,b) for 887 sera from naive swine and for 30 sequential samples taken following seroconversion in the series of swine experimentally infected at PIADC (Figs. 3 and 4). The sera from uninfected animals had a mean A_{492} of 0.148 ± 0.060. In comparison, the absorbances of the seroconversion series samples, including a minimum value of 0.311 taken beyond day 200 of convalescence, had a mean A_{492} of 1.538 ± 0.519. The separation validates the establishment of a clear cut-off value for reactivity to the test. A useful cut-off value for the swine test can be set at an A_{492} of 0.388 by adding 4 standard deviations to the mean for the normal swine sera of 0.148. This cut-off value, represented by the vertical lines in Fig. 7(a) and (b), defines a naive population [Fig 7(a)] that contains 17 outlier animals.

Similarly, the distribution of the absorbances on the ruminant test was plotted for 1202 naive cattle [Fig. 8(a)] and for 39 samples from infected cattle [Fig. 8(b)] that include the 13 convalescent serum samples of Panel 2 (Table 2) and 26 sequential bleeds taken following seroconversion in the series of cattle experimentally infected at PIADC (Figs. 5 and 6). The sera from the naive animals provided a mean absorbance of 0.086 ± 0.067. The absorbances of the infected samples, including one non-reactive sample collected on day 294 post-exposure [Fig. 5(c)], had a mean of 1.553 ± 0.518. Thus, a cut-off value for the provisional ruminant differential ELISA could be set at 0.288 by adding three standard deviations to the mean for the normal cattle sera of 0.086. This cut-off value is shown as a vertical line in the distributions of Fig. 8 and results in

a naive bovine population having 14 outliers. The characterization of reactivities to the test was expanded to include the distribution on sera from vaccinated cattle shown in Fig. 8(c). These sera include the 13 vaccinee samples listed in Table 2 and three week post-vaccination samples taken from: (i) five cattle immunized with Asia-1 Sau 32/92, (ii) five immunized with O1 BFS, (iii) four immunized with A10 Holland, (iv) four immunized with O1 Morocco, (v) five sequential bleeds taken over a course of 140 days from an animal vaccinated on days 0, 63, and 119, and (vi) four steers that had been administered three heterologous vaccines within the previous 3 years. The vaccinee serum samples showed a mean absorbance of 0.064 ± 0.045 [Fig. 8(c)], within the distribution of reactivities for naive cattle. None of the vaccinated cattle, including animals given multiple doses, was scored as seropositive according to the cut-off value determined from the 1202 naive samples.

The wide separation in the distributions for exposed and nonexposed populations suggest that in the field, a nonstructural peptide-based differential test will provide similarly clear cut-off values for distinguishing naive and vaccinated herds from exposed herds, and result in high specificity in the presence or absence of vaccination. For the naive samples shown in Fig. 7(a), including the 17 outlier sera from unexposed animals, and utilizing the cut-off value derived from the mean of unexposed swine of 0.388, the specificity of the swine test on a normal population was 98.0% ($887-17/887 \times 100 = 98.0\%$). From the cut-off value for cattle of 0.288 and 14 outlier samples, specificity was 98.8% ($1202-14/1202 \times 100 = 98.8\%$) on naive cattle [Fig. 8(a)] and 100% on a small population of vaccinated cattle [Fig. 8(c)].

In addition to cattle, swine, and guinea pig sera, we have also examined the sera from four sheep that had been immunized with an A10 virus vaccine and four that had been immunized with an O1 Morocco vaccine (data not shown). There was no rise in absorbance at 26 days post-vaccination. Moreover, the absorbance did not increase following challenge with the homologous viruses. However, there was an absorbance greater than 2.0 in two sheep infected with O1 Tunisia 15 days after infection (data not shown).

3. Discussion

The differential tests for swine and ruminants, based on a nonstructural peptide antigen from protein 3B, were examined for sensitivity and specificity on well-characterized repository samples from infected, vaccinated, and naive animals (Table 2 and Fig. 2), as well as on sequential bleeds of swine, cattle, and sheep which had been infected or vaccinated and then infected under strictly controlled experimental conditions (Figs. 3–6). Our results show that the test detected antibodies in the sera of convalescent cattle and swine up to 364 days and 301 days, respectively, after infection. The test identified the infected status of animals that were reminiscent of carrier animals in a vaccinated herd (Figs. 4 and 6), and also detected antibodies evoked by a full range of serotypes, thus providing a sensitivity that is sufficiently broad for heterogeneous epidemiological situations. Even at the later times, the immunoassays showed strong signals in selected animals suggestive of long-term persistence of seroreactivity. In contrast, sera from naive or vaccinated animals were rarely seropositive, even in animals that had been given multiple doses of vaccine.

The differential antigenicity seen for viral and recombinant proteins corresponding to the 2C and 3ABC nonstructural regions [7,9,10] was localized on epitope maps by site-directed serology using synthetic peptides and sera chosen for a wide range of serotype reactivities. The epitope maps were largely in agreement with the emergence of 3ABC as the consensus antigen for differentiating convalescent from vaccinated animals [13]. However, the observations with our site-specific antigens favoured a long 3B peptide alone as the optimum discriminatory antigen and led us to reject highly antigenic peptides taken from the 3A region. We observed that anti-3A antibodies were present in some of the sera from vaccinated animals, an observation in support of those by V. O'Donnell and by J. Lubroth (personal communications) who found antibodies against 3ABC in the sera of vaccinated swine and cattle. The interference with differential antigenicity by anti-3A antibodies had been previously suggested by Dekker and Gijsen [14] who predicted the presence of antibodies to 3A in the sera of vaccinated animals. They found that 3A was secreted into the medium of BHK cells infected with the virus and thus is present in the material used for vaccine production.

Synthetic peptides have been used in recent years for site-directed serology to define antibody recognition sites on proteins [15,16]. This process can now be used to identify both linear determinants and the conformational determinants that are more important for selective recognition [17]. When carefully performed with high quality peptides of longer lengths, site-directed serology has been used to identify useful serological markers for HIV, HCV, and HTLV I/II and has led to the development of validated immunoassays for serological detection [11,12,18–22]. These tests typically provide high sensitivity and a wide separation between the reactivities of infected and uninfected populations for good specificity, as shown here for the new peptide-based differential ELISA for FMDV. The excellent performance characteristics of the peptide-

based immunoassays are due to the high molar concentrations of immunodominant sites that had been selected for both antigenicity and specificity, and the absence of undesired crossreactivities, such as the reactivity of vaccinee sera with 3A protein. These peptide-based assays overcome the specificity problems associated with either complex virus-derived antigens or recombinant proteins produced by bacterial, viral, or host cell expression systems. A further advantage afforded by the synthetic peptide antigens is the ability of a chemically synthesized reagent to be adapted to the demands of a commercial product. The routine manufacture of a peptide-based ELISA can be readily accomplished at low cost, with the high reproducibility, rigorous quality control, and high degree of safety that is required for a commercial product. Concurrently, the peptide-based immunoassays, formatted as ELISAs, meet the needs of the user for rapid and simple tests, and for safety and long-term stability.

The peptide-based immunoassays are being developed to detect animals that have been infected with FMDV, whether or not they have also been vaccinated. By so doing, they will enable detection of residual viral activity in a vaccinated animal population. Further trials under field conditions will lead to the determination of cut-off values and performance characteristics such as diagnostic sensitivity and specificity. In a preliminary field trial in Taiwan, a country that had experienced a recent large outbreak, one out of 90 swine (1.1%) from a vaccinated but presumed to be unexposed herd was reactive in the ELISA for swine.

Based on the promising performance characteristics of the immunoassay, the ready, inexpensive and safe availability of the critical synthetic antigen, and the suitability of the simple indirect ELISA format, the synthetic peptide-based test has high potential as a method for: (i) the rapid detection of infectious animals in the presence or absence of vaccination, (ii) the detection of potential carriers among vaccinated herds, (iii) monitoring the progress of FMDV eradication programmes, (iv) epidemiological surveys in regions which practice vaccination, (v) encouraging more extensive vaccine coverage for control of FMD, and (vi) widespread serological surveys in all countries regardless of OIE status. Provided that these tests are proven by field testing to have the required levels of confidence, they will be applicable for import/export controls and for expedited evaluation of FMDV-free status in countries that retain immunized animals.

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