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Antigenic variation in the bovine ephemeral fever glycoprotein. Bovine ephemeral fever and related rhabdoviruses. (Eds. T.D. St.George, M.F. Uren, P.L. Young and D. Hoffman)

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Cybinski, D.H. and St.George, T.D.

Antigenic Variation in The Glycoprotein of Bovine Ephemeral Fever Virus. (Eds M.F. Uren and B.H. Kay)

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Cybinski, D.H., Davis, S.S. and Zakrzewski, H.

Mapping of antigenic sites on the bovine ephemeral fever virus glycoprotein using monoclonal antibodies.

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Cybinski, D.H., Walker, P.J. and Byrne, K.A.

The task to produce a library of monoclonal antibodies as a basis for future research into the BEF virion began in 1984. I was set the general task by Dr St.George and developed the monoclonal antibodies to the G, N and M_2 proteins as set out in the abstract to my PhD thesis. The four papers above on antigenic variation derived directly from this. Ms Zakrzewski assisted with the IFA section of the work and provided the ELISA test which she developed. Mr S.S. Davis provided informed technical assistance at every stage. Dr St.George provided the bank of ephemeral fever viruses for assay and the sets of field and lab oratory sera with known histories essential for testing the project. He has permission to abstract data from the papers where he is not an author. The same bank of monoclonal antibodies was used by Wang Yonghong in her PhD Thesis on Adelaide River virus. Jasjit used the library of monoclonal antibodies and the supporting data in her PhD Thesis

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Antigenic Variation in the Bovine Ephemeral Fever Virus Glycoprotein

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Abstract

Antigenic variation in the bovine ephemeral fever virus glycoprotein was demonstrated using monoclonal antibodies and comparing a particular passage level of the BB7721 strain with (1) other Australian isolates of BEF virus, (2) the Beijing 1 strain of BEF virus, isolated in China and (3) batches of the BB7721 strain with different passage histories. Escape mutants of BEF virus were selected from cell culture and suckling mice, by growing virus in the presence of neutralising monoclonal antibodies. Escape frequencies were calculated for 14 monoclonal antibodies and an epitope map constructed of antigenic sites on the BEF viral glycoprotein which induce the production of neutralising antibodies.

RABIES viruses isolated from different animal species in various parts of the world were formerly considered to be closely related (Wiktor and Clarke 1973). However, more recent studies using monoclonal antibodies against the nucleocapsid protein and the glycoprotein of the virus have provided evidence of antigenic differences among several strains of rabies virus (Wiktor and Koprowski 1980). It has been suggested that these differences might have been responsible for occasional failures in postexposure vaccination of rabies patients. Furthermore, when mice were vaccinated with standard rabies vaccine and then challenged with street viruses isolated from fatal cases of human rabies, some instances of vaccine failure were observed (Wiktor and Koprowski 1980). Considerable genetic diversity has also been demonstrated for strains of vesicular stomatitis virus, using T1 ribonuclease fingerprinting (Nichol 1988). Using monoclonal antibodies (MAbs), it has now been shown that variation also exists in the bovine ephemeral fever (BEF) virus glycoprotein (Cybinski et al. 1990, 1992).

Materials, Methods and Results

Viruses

The Australian virus isolates which were compared to the BB7721 strain of BEF virus included the insect isolates CSIRO 42 and CSIRO 53 viruses (Standfast et al. 1984) and the closely related Berrimah virus (Gard et al. 1983). The Beijing 1 strain of BEF virus from China (Zhai et al. 1980) was also compared. CSIRO 42 virus was isolated from *Anopheles bancrofti* mosquitoes collected at Beatrice Hill in the Northern Territory. CSIRO 53 virus was isolated from a mixed mosquito pool of Culicine mosquitoes collected at Etna Creek near Rockhampton in Queensland. Berrimah virus was isolated from the blood of a healthy steer located in the north of the Northern Territory. Neutralisation testing carried out on this animal for two years revealed high levels of neutralising antibody to both Berrimah and BEF viruses prior to the isolation of Berrimah virus (Cybinski 1987). This indicated a previous infection with at least one and probably several BEF-related viruses (Figure 1). The Beijing 1 strain of BEF virus was isolated in China from bovine blood.

Cross-reactivity of BEF virus isolates using polyclonal antibodies

Cross neutralisation tests on the Australian isolates, using polyclonal antibodies indicated some minor differences among the BB7721, CSIRO 42 and CSIRO 53 viruses, while Berrimah virus was considered to be a different virus, although closely related (Table 1). Testing has not been completed on the Beijing 1 virus, although the results so far, using polyclonal antibodies, indicate that there is little difference between the Chinese and Australian viruses.

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Table 1. Cross-neutralisation titres of Australian BEF virus strains and Berrimah virus, using polyclonal antibodies

Ascitic fluid	BEF Virus (BB7721)[1]	(CSIRO42)[1]	(CSIRO53)[2]	(DPP63)[1]
BEF BB7721	1024[3]	512	128	4
BEF CSIRO 42	64	384	8	<2
BEF CSIRO 53	512	1024	1024	32
Berrimah 1	192	32	32	4096
Berrimah 2	<2	6	4	384

[1] Bovine isolate
[2] Mosquito isolate
[3] Reciprocal of antibody dilution which neutralised 100 $TCID_{50}$ of virus in 50% of the wells

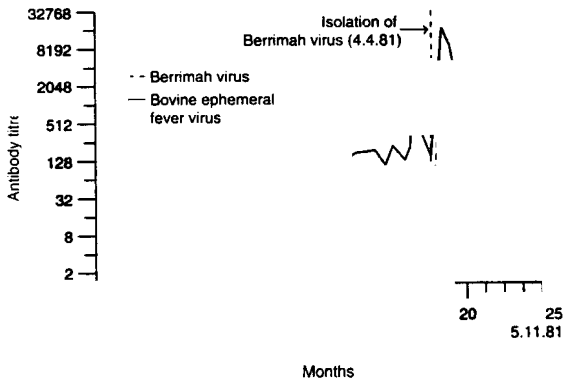

Fig. 1. Antibody titres to BEF and Berrimah viruses found in serial serum samples from a steer from which Berrimah virus was isolated.

It is thought that those antibodies which bind without neutralisation may act as blocking antibodies by binding to the virus and preventing or at least inhibiting the binding of neutralising antibodies. This may explain the mechanism by which Berrimah virus was able to escape neutralisation and cause an infection in the presence of high levels of neutralising antibody. Non-neutralising MAbs to the G, M2 and N proteins were tested by indirect immunofluorescence and the results are shown in Table 3. Of these, two MAbs did not bind to CSIRO 42 virus while five failed to bind to the CSIRO 53 isolate of BEF virus. Six non-neutralising G protein Mabs failed to bind to Berrimah virus.

The Beijing 1 strain of BEF virus was obtained in China, and processed by Peter Walker and Helen Zakrzewski. The virus was purified, the proteins separated by PAGE then transferred to nitrocellulose. In Australia, the virus protein preparations were tested by immunoblotting, for reactivity with 22 MAbs to the G, M2 and N proteins. Results are shown in Table 4. Four of the G protein-specific MAbs that bound to the BB7721 strain failed to bind to the Chinese strain.

Strain variation using monoclonal antibodies

Monoclonal antibodies were prepared against the BB7721 strain of BEF virus and these reacted with the G, M2 and N proteins according to results obtained by polyacrylamide gel electrophoresis (PAGE) and immunoblotting (Tables 2 and 3). Fourteen of the MAbs against the BEF virus G protein were neutralising. Indirect immunofluorescence and neutralisation tests on ascitic fluid from these MAbs were used to determine their ability to bind and neutralise the two other Australian strains and Berrimah virus. The results are listed in Table 2. Only one MAb, 8D3, failed to neutralise CSIRO 42 virus while two MAbs, 8B6 and 3D6, failed to neutralise or bind CSIRO 53. Berrimah virus was neutralised by only eight of the neutralising MAbs. However, three additional MAbs were capable of binding to the virus without neutralisation.

Batch variation

To determine what effect, if any, passage level may have on antigenic variation, three batches of the BB7721 strain of BEF virus with different passage histories were tested against 14 neutralising MAbs. Batch 1 was passaged six times in mouse brain, 34 times in BHK 21 cells, twice in Vero cells then plaque cloned three times in Vero cells. Batch 2 received the same number of passages as batch 1 but was plaque

Table 2. Viral specificity of neutralising monoclonal antibodies by neutralisation and immunofluorescence to isolates of BEF virus.

G protein monoclonal antibody	Neutralisation titres[1] of ascitic fluids (immunofluorescence reaction)			
	BEF (BB7721)	BEF (CSIRO42)	BEF (CSIRO53)	BER (DPP63)
DB5	512(+)	256(+)	256(+)	<2(−)
13C6	4096(+)	2048(+)	2048(+)	<2(+)
13A3	1024(+)	3072(+)	1024(+)	<2(−)
17B1	1024(+)	2048(+)	1024(+)	<2(+)
9C5	128(+)	384(+)	128(+)	96(+)
1C6	256(+)	256(+)	384(+)	128(+)
12A5	256(+)	512(+)	512(+)	24(+)
15B5	512(+)	256(+)	512(+)	128(+)
11D1	4(+)	16(+)	8(+)	8(+)
3D6	512(+)	256(+)	<2(−)	<2(+)
8B6	16(+)	6(−)	<2(−)	<2(−)
16A6	8(+)	16(+)	8(+)	8(+)
8D3	8(+)	<2(+)	8(+)	8(+)
5A5	512(+)	32(+)	128(+)	16(+)

[1] Reciprocal of antibody dilution which neutralised 100 $TCID_{50}$ of virus in 50% of the wells
+ = immunofluorescence − = no immunofluorescence

Table 3. Viral specificity (indirect immunofluorescence) of non-neutralising monoclonal antibodies to isolates of BEF virus.

Monoclonal antibody	BEF protein specificity	Immunofluorescence reactions of hybridoma supernatants			
		BEF (BB7721)	BEF (CSIRO42)	BEF (CSIRO53)	BER (DPP63)
18D2	G	+	−	−	−
3A2	G	+	+	+	+
11B5	G	+	+	−	−
10B1	G	+	+	+	+
13B5	G	+	+	+	−
2C4	G	+	−	−	−
18C2	G	+	+	−	−
EB4	G	+	+	+	−
2C6	M2	+	+	+	+
FD2	M2	+	+	+	+
4A4	M2	+	+	+	+
17A3	M2	+	+	+	+
20A6	M2	+	+	+	+
11A3	N	+	+	+	+
6C1	N	+	+	+	+
11B5	N	+	NT	NT	+
2D5	N	+	NT	NT	+
18A3	N	+	NT	NT	+
12DI	N	+	NT	NT	+
9D1	N	+	NT	NT	+
14B5	N	+	NT	NT	+
14A4	N	+	NT	NT	+
4B4	N	+	NT	NT	+
14A6	N	+	NT	NT	+
12B3	N	+	NT	NT	+

+ = binding; − = no binding NT = not tested

cloned separately. Batch 3 was passaged six times in mouse brain and 25 times in BHK 21 cells then cloned three times by limiting dilution in Vero cells. Batch 3 was not plaque cloned.

Neutralisation titres for the 14 MAbs against the three batches of BEF virus are listed in Table 5. Most MAbs gave the same titres with each batch of virus. However, batch 1 contained a proportion of virus which was partially resistant to the MAb 13C6, shown by virus breakthrough at low dilutions. Batches 2 and 3 were partially resistant to the MAb 5A5 while batch 2 was also partially resistant to the MAb 3D6.

Having demonstrated that various batches and isolates of BEF virus are not homogeneous, variants were isolated by incubating the BB7721 BEF virus with 13 of the neutralising MAbs and selecting plaques which escaped neutralisation. Escape frequencies for these variants, calculated as plaque forming units/ml in the presence of MAb divided by plaque forming units in the absence of MAb, are shown in Table 6. The average escape frequency was between 10^{-4} and 10^{-5}, although high escape frequencies were observed for 13C6, 3D6 and 5A5, while low escape frequencies were obtained for 16A6 and 8B6.

From the BB7721 parent strain of BEF virus, a total of 43 variants were selected which escaped neutralisation by the MAb used for selection and in most cases by other MAbs as well, resulting in 23 patterns of resistance as shown in Figure 2. To determine whether a variant was resistant or partially resistant, titres were compared with the titre of the parent virus. A virus was considered to be resistant to neutralisation only if the neutralising titre was less than two. In some cases, there was a greater than 10-fold reduction in the titre, which was considered to be partial resistance, although viruses showing only partial resistance to the selecting MAb were excluded from the study. It was assumed that when a mutant virus escaped neutralisation by a MAb, then the epitopes defined by these MAbs were functionally linked. In this way, patterns of neutralisation and resistance to neutralisation allowed the variants to be grouped into at least six clusters representing viruses with mutations affecting the same antigenic site.

Monoclonal antibody resistance in mice

MAb-resistant mutants were also detected in mice. Suckling mice inoculated intraperitoneally with neutralising or non-neutralising MAbs, then challenged intracerebrally with BEF virus, were generally protected from paralysis and death by the neutralising MAbs. Protected mice often survived longer than 14 days compared to 4 or 5 days for the controls. However, some mice survived no longer than the controls despite high levels of passive protecting antibody, indicating the presence of MAb-resistant virus (Table 7).

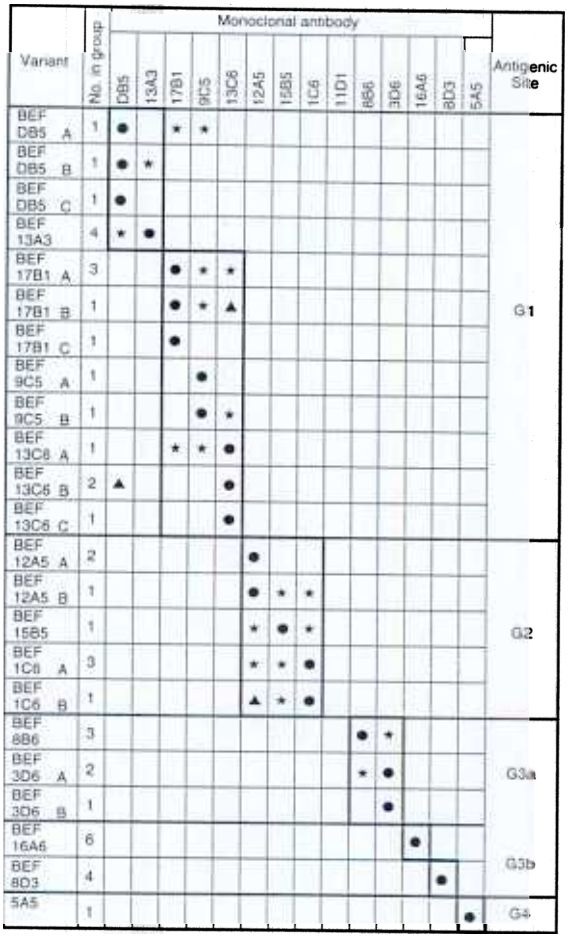

Fig. 2. Antigenic map of the BB7721 strain of bovine ephemeral fever virus. Neutralisation resistant variants were selected using monoclonal antibodies (MAbs), then tested for susceptibility (□) or resistance to neutralisation by the MAb used in selection of the variant (●) and by another MAb in the panel (★). (▲) denotes partial resistance. Labelling of antigenic sites as G1, G2, G3a, G3b is taken from Cybinski et al. (1990). Antigenic site G4 is a new site detected by competitive binding assay and by MAb resistance.

Table 4. A comparison of the Beijing strain 1 and the BB7721 strain of BEF virus by immunoblot analysis using monoclonal antibodies specific for the BB7721 strain.

Monoclonal antibody	Protein specificity	BB7721	Beijing 1
DB5	G	+	+
13C6	G	+	+
13A3	G	+	+
17B1	G	+	+
9C5	G	+	+
1C6	G	+	+
12A5	G	+	+
15B5	G	+	+
11D1	G	+	+
3D6	G	+	−
16A6	G	+	+
8D3	G	+	+
5A5	G	+	+
3A2	G	+	+
11B5	G	+	−
2C4	G	+	−
18C2	G	±	±
EB4	G	+	−
2C6	M2	+	+
FD2	M2	+	+
20A6	M2	+	+
11A3	N	+	+

+ = binding; − = no binding

Table 5. Neutralisation of BEF virus batches with monoclonal antibodies.

Monoclonal antibody	Neutralising titre[1]		
	BEF virus batch number		
		2	
DB5	256	256	256
13A3	1024	1024	1024
13C6	16	4096	4096
17B1	>8192	>8192	>8192
9C5	256	256	256
1C6	256	256	256
15B5	256	256	256
12A5	1024	1024	1024
11D1	8	8	8
16A6	128	128	128
8D3	256	256	256
3D6	512	16	512
8B6	64	64	64
5A5	256	16	16

[1] Titres expressed as the reciprocal of the highest dilution required to neutralise 100 $TCID_{50}$ of virus in 50% of the wells

Discussion

Previous serological studies using polyclonal antibodies have failed to show any significant differences between strains of BEF virus from within Australia or in strains from different countries (Snowdon 1970; Inaba 1973; Tian et al. 1987). However, MAb studies on rabies and vesicular stomatitis virus have shown evidence of extensive antigenic diversity in these viruses (Wiktor and Koprowski 1980; Luo et al. 1988) and this diversity is thought to be one cause of vaccine failure for rabies virus.

The data presented here clearly indicate that considerable antigenic diversity also exists in BEF virus strains from different sources and that variants can be selected experimentally from cloned BEF virus either by passaging or by growing in the presence of MAb. The vaccine currently available in Australia is based on the BB7721 strain of BEF virus (Vanselow 1985) and this is known to be different from viruses now circulating (Cybinski et al. 1992). However, the viruses investigated shared the majority of epitopes as indicated by MAb reactions, therefore

Table 6. Frequency with which BEF virus (BB7721) escaped neutralisation by monoclonal antibodies.

Selecting monoclonal antibody	Escape frequency
DB5	10^{-4}
13A3	$5x10^{-5}$
13C6	$6x10^{-3}$
17B1	$2x10^{-5}$
9C5	$4x10^{-5}$
1C6	$3x10^{-5}$
15B5	10^{-4}
12A5	$5x10^{-5}$
16A6	$5x10^{-7}$
8D3	10^{-5}
3D6	$5x10^{-3}/10^{-5}$*
8B6	$5x10^{-8}$
5A5	10^{-3}

* small plaques/large plaques

Table 7. Passive protection of mice inoculated with monoclonal antibodies and challenged with BEF virus.

Monoclonal antibody	Neut. titre	No.	Number of deaths recorded each day											
			4	5	6	7	8	9	10	11	12	13	14	>14
6.13C6	4096	7	—	—	1	1	—	2	—	—	—	—	—	3
6.17B1	2048	6	—	1	—	—	—	—	—	—	—	—	—	5
1.DB5	1024	7	—	—	—	—	—	—	—	—	—	—	—	7
4.13A3	1024	6	—	1	1	—	1	1	—	—	1	1	—	—
6.3D6	1024	5	2	—	—	1	—	1	—	—	—	—	1	—
4.1C6	256	9	—	1	—	—	1	1	—	2	—	—	3	1
4.12A5	256	6	—	—	1	—	1	—	—	—	1	—	—	3
4.15B5	192	6	—	—	2	1	—	1	1	1	—	—		
6.9C5	128	7	—	—	2	4	1	—	—	—	—	—		
6.8B6	16	6	—	1	1	—	2	2	—	—	—	—		
5.8D3	8	7	—	—	4	1	1	1	—	—	—	—		
1.16A6	8	7	—	1	4	2	—	—	—	—	—	—		
6.11D1	4	6	—	5	—	1	—	—	—	—	—	—		
6.18C2	<2	6	—	5	1	—	—	—	—	—	—	—		
6.3A2	<2	6	—	6	—	—	—	—	—	—				
6.18D2	<2	4	—	2	2	—	—	—	—	—				
6.13B5	<2	6	1	2	3	—	—	—	—	—				
5.11B5	<2	6	—	4	2	—	—	—	—	—				
6.10B1	<2	7	4	2	1	—	—	—	—	—				
5.2C4	<2	7	—	—	—									
4.17D6	<2	6	2	3	1									
Control	—	42	16	23	3									

— No deaths recorded

it is not surprising that the vaccine is protective. For the same reason, it is likely that vaccines against the BB7721 and the Beijing 1 strains of BEF virus would cross protect. On the other hand, there is at least one virus, Berrimah virus, which has a high degree of homology with BEF virus strains, but can escape neutralisation by high levels of BEF antibody. Berrimah virus is not known to be pathogenic but other pathogenic viruses could occur which avoid neutralisation by the same mechanism. These factors need to be considered when producing an ephemeral fever vaccine or a diagnostic test based on MAbs. Monitoring of current field strains of BEF virus is therefore essential.

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