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IDENTIFICATION OF AN IMMUNODOMINANT REGION ON THE ISOLATED BOVINE LEUKAEMIA VIRUS (BLV) MAJOR ENVELOPE PROTEIN gp51 BY MONOCLONAL ANTIBODIES PRESUMABLY NOT EXPOSED DURING NATURAL BLV INFECTION

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Summary. — A panel of newly isolated monoclonal antibodies (MoAbs) is described which are specific for bovine leukaemia virus (BLV) envelope protein gp51. Epitope mapping using competition antibody binding assays and binding studies with gp51-related fusion proteins and synthetic peptides show that they are directed against seven independent antigenic determinants. Four of them are unrelated to the epitopes described earlier (Bruck *et al.*, 1982*a*). We define three binding regions for the MoAbs on the gp51 molecule. The region between amino acids 170 and 217 is highly immunogenic when the isolated protein is used for immunization, and seems to be inaccessible for immune recognition when gp51 is associated with the virus envelope as it occurs during natural BLV infection.

Key words: *bovine leukaemia virus*; *envelope protein gp51*; *monoclonal antibodies*; *epitope mapping*

Introduction

The bovine leukaemia virus (BLV), an exogenous retrovirus, is the causative agent of a lymphoproliferative disease of cattle, the enzootic bovine leukosis (Miller *et al.*, 1969). Gp51, the major glycoprotein of BLV envelope, was partially characterized (Devare *et al.*, 1977) and was shown to be responsible for several biological activities of the virus (for review see Burny *et al.*, 1980).

By means of monoclonal antibodies (MoAbs) eight independent epitopes (named A—H) on the gp51 molecule were defined (Bruck *et al.*, 1982*a*). Four of them (A, B, D, E) represent sequential antigenic determinants found in both the natural viral protein and in recombinant gp51-related polypeptides comprising the C-terminal half of the gp51 molecule (Siakkou *et al.*, 1990).

Epitopes F, G and H were shown to be involved in virus infectivity and fusion activity. One of them (epitope G) is exposed on the surface of BLV-producing cells and can be a target of complement-mediated cytotoxicity (Bruck *et al.*, 1982). The epitopes F, G and H are depending on a three-dimensional structure involving glycosylation (Bruck *et al.*, 1984*b*) and are most likely localized within the N-terminal half of the gp51 molecule (Bruck *et al.*, 1982*b*). After natural infection cattle produce antibodies (Abs) against the epitopes F, G and H, but not against the epitopes A, B, C, D, and E (Bruck *et al.*, 1984*a*).

In the present paper we describe a panel of new MoAbs directed against 7 independent antigenic determinants on the gp 51 molecule, which were used for further characterization of the antigenic structure of gp51. The MoAbs recognize 4 hitherto unknown epitopes in addition to epitopes A—H, described above. We describe an immunodominant region on the isolated envelope protein gp51 representing an antigenic domain presumably not accessible during natural BLV infection.

Materials and Methods

Preparation of BLV and gp51. Virus was produced by BLV- infected foetal lamb kidney (FLK) cells (Van Der Maaten and Miller, 1976). We used the subline FLK 44/2 (kindly provided by D. Scholz, Institute of Virology, Humboldt University, Berlin, G.D.R.). Culture medium was Eagle MEM (Staatliches Institut für Immunpräparate und Nährmedien, Berlin, G.D.R.) supplemented with 10% foetal calf serum (Kombinat Veterinärimpfstoffe Dessau, Dessau, G.D.R.). BLV was purified from culture supernatant by ultracentrifugation in continuous 5—40% metrizamide density gradients. Gp51 was prepared from disrupted virus by chromatography on DEAE 52-cellulose (Whatman) (Portetelle *et al.*, 1980).

Production of monoclonal antibodies. The MoAbs were derived from two fusion experiments. Immunization of female Balb/c/Han mice was performed with enriched gp51 (50% enrichment as estimated by liquid phase radioimmunoassay) according to the following schedule: (i) 50 μg gp51 in complete Freund's adjuvant (1+1) intraperitoneally (i.p.); (ii) 4 weeks later 50 μg gp51 in incomplete Freund's adjuvant (1+1) i.p.; (iii) 4 weeks later 50 μg gp51 in PBS i.p.; (iv) final boost with 20 μg gp51 in PBS was given intravenously after another 4 weeks. Four days after the final injection spleen cells were fused with cells of the myeloma cell line P3/X63-Ag8653. Hybridoma clones were prepared as described (Köhler and Milstein, 1975; Karsten *et al.*, 1985). Culture supernatants of hybrid cells were screened for Ab production in an ELISA with the enriched gp51. Ascitic fluids containing MoAbs were produced by intraperitoneal inoculation of 2×10^6 hybrid cells into syngeneic pristane-primed mice.

Immunoglobulins were precipitated from ascitic fluids by addition of ammonium sulphate (Merck) to a final concentration of 30% or PEG 6000 (Sigma) to a final concentration of 10%. After resuspending in phosphate-buffered saline (PBS) and dialysis the immunoglobulin concentration was read by spectrophotometry at 280 nm (E = 1.35 for 1 mg/ml).

Subclass estimation. The subclass of MoAbs was estimated by a haemagglutination inhibition assay using antisera to murine isotypes from Miles Laboratories.

Affinity constant measurement. Affinity constants were determined with horse-radish peroxidase (HRP)-labelled MoAbs (labelling was performed according to Tijssen *et al.*, 1984) as described (Friguet *et al.*, 1985; Stevens, 1987).

Electrophoresis and Western blotting. Purified disrupted BLV and crude extracts from transformed bacterial and yeast cell cultures expressing different gp51-related polypeptides (Table 1) were separated by electrophoresis on a 10% SDS polyacrylamide gel (Laemmli, 1970) and transferred to nitrocellulose paper (Schleicher and Schüll, Ba85) as described (Kyhse-Andersen, 1984). The blots were incubated for 2 hr with the MoAb diluted to a final concentration of 0.01 mg/ml in 10% normal horse serum (Kombinat Veterinärimpfstoffe Dessau, Dessau, G.D.R.) in pho-

sphate-buffered saline containing 0.1% Tween (PBST). After washing with PBST the blots were incubated with anti-mouse IgG antibody, conjugated to HRP (Staatliches Institut für Immunpräparate und Nährmedien, Berlin, G.D.R.) in PBST containing 10% normal horse serum for another 2 hr. After washing, the bound HRP was visualized by 0.2 mg/ml 2-bromo-1-naphthol (Noll *et al.*, 1984) in PBS with 0.015% H_2O_2.

Competition binding assays. a) Microtitre plates (Czechoslovak Academy of Sciences) were coated with enriched gp51 (25 ng gp51/well) in PBS. Unlabelled MoAb or a bovine standard serum (serial dilution from 1.0 mg/ml to 100 ng/ml and from 1 : 10 to 1 : 10,000, respectively) were added and allowed to react with the antigen (Ag) overnight at 4 °C. After removing unbound antibodies the plates were incubated with an optimal concentration of HRP-labelled MoAb (E_{492} around 1.5 without competitor antibody) for 2 hr. Antibodies were considered to be competing, if the binding of HRP-labelled MoAb was inhibited up to at least 40%. b) The wells of the microtitre plates, coated with purified disrupted BLV (1 μg/well) were incubated simultaneously with MoAb and a serially diluted synthetic peptide (Portetelle *et al.*, 1989) or purified BLV (denaturated or native). Binding of the MoAb to the adsorbed Ag was estimated by HRP-labelled anti-mouse IgG antibody. A decreased binding of the MoAb to the adsorbed antigen was considered to be due to a specific reaction of the MoAb with the peptide or with BLV.

All dilutions were made in PBST with 10% normal horse serum, washing buffer was PBST.

Results

Production of anti-gp51 MoAbs and their general properties

From two fusion experiments 18 anti-gp51 antibody-producing hybridoma clones were selected. Their specificity was confirmed by Western blotting using purified BLV (Fig. 1). Fourteen of them could be characterised in more detail. All MoAbs belong to the IgG subtype. Affinity of most MoAbs to the soluble antigen was estimated (Table 2).

Determination of epitope specificity of the MoAbs

Almost all possible combinations between the gp51 specific MoAbs (including the MoAbs against epitopes A—H; Bruck *et al.*, 1982*a*) were tested in competition binding assays. Two MoAbs are considered to detect the same or spatialy closely related antigenic determinants if the binding of one MoAb was inhibited by the other MoAb completely or partially (at least 40%). By this way six independent epitopes could be discriminated. The results are summarized in Table 2.

Table 1. Fusion proteins synthesized by E. coli and S. cerevisiae

Host	Plasmid	Expressed fusion proteins (amino acids of BLV-proteins)	Reference
E. coli	pENV1	βgal/gp51 (55—103)	Ulrich *et al.*, in press
E. coli	pENV4	βgal/gp51 (135—268)/gp30 (1—45)	Ulrich *et al.*, in press
E. coli	pBDB4	pre (1—33)/gp51 (1—56)/NPT	Merkel (1989)
E. coli	pB3/4	gp51 (105—217)/NPT	Merkel (1989)
S. cerev.	YEpDIT9/2	gp51 (170—217)/NPT	Merkel (1989)

NPT — Neomycinephosphotransferase II
βgal — β-Galactosidase

The MoAbs 1 and 2; MoAbs 9, 10, 13, and 22; MoAbs 5 and 11 are related to the epitopes A; B/B′; D/D′, respectively, as described earlier (Bruck *et al.*, 1982*a*). The MoAbs 14 and 30 did not compete with any of the other gp51-specific MoAbs. We referred to the new epitopes recognized by them as I and K, respectively. MoAbs 3, 6, 7, and 8 compete with each other but not with the MoAbs mentioned above and, therefore, define a new epitope named L.

Reactivity of MoAbs with BLV gp51 recombinant fusion proteins and synthetic peptides

In order to map the epitopes recognized by the MoAbs, fusion proteins containing different and in three cases partially overlapping regions of gp51 (Table 1) were used in Western blot experiments. Results of epitope mapping are summarized in Table 2 and Fig. 3.

MoAb 14 (epitope I) recognized the pENV1-expressed polypeptide which spans a part of the N-terminal half of gp51 (result not shown).

The MoAbs 9, 10, 11, 13, 22, and 30 (epitopes B, B′, D/D′ and K) and also the MoAbs against the epitopes B/B′, D/D′, and E (Bruck *et al.*, 1982*a*) reacted with the fusion proteins covering amino acids 170-217 (region II in Fig. 3). Fig. 2-2 shows the binding of MoAb 13 as a representative example for this group of antibodies.

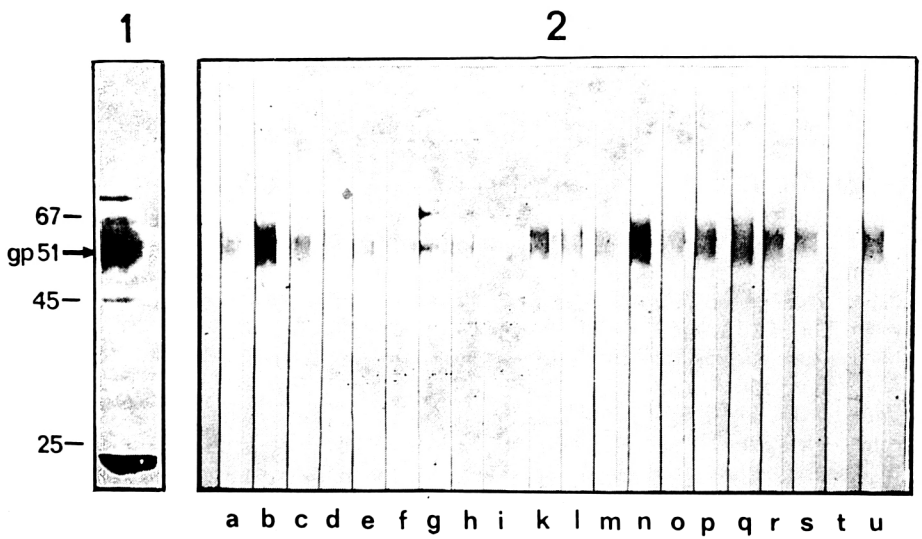

Fig. 1

Reactivity of MoAbs with purified disrupted BLV. (1) Coomassie blue-stained proteins; (2) Western blots, incubated with MoAbs 1 (a), 2 (b), 3 (c), 4 (d), 5 (e), 6 (f), 7 (g), 8 (h), 9 (i), 10 (k), 11 (l), 12 (m), 13 (n), 14 (o), 20 (p), 22 (q), 23 (r), 30 (s), normal mouse serum (t), and MoAb against epitope B (u).

Table 2. Characterization of MoAbs against BLV-gp51

MoAb No.	IgG-subclass	Affinity (1/mol)	Competing MoAb	Epitope location (amino acid of gp51)	Epitope name	Competition with BS D12
14	1	1.0×10^{7}	—	57—67	I	—
13	1	5.9×10^{8}	9, 22,*B	170—217	B	—
22	1	1.0×10^{10}	9, 13,*B	170—217	B	—
9	1	1.5×10^{8}	13, 22	170—217	B	—
10	1	n.d.	*B	170—217	B′	+
30	2a	2.5×10^{7}	—	170—217	K	—
11	2a	2.4×10^{7}	5, *D, *D′	170—217	D/D′	—
5	1	5.8×10^{8}	11, *D, *D′	217—268	M	+
1	2b	1.4×10^{8}	2, *A	217—268	A	+
2	2a	1.4×10^{8}	1, *A	217—268	A	+
3	1	3.2×10^{8}	6, 7, 8	—	L	+++
6	2a	7.3×10^{8}	3, 7, 8	—	L	+++
7	1	1.7×10^{8}	3, 6, 8	—	L	+++
8	2a	1.5×10^{9}	3, 6, 7	—	L	+++

BS — bovine serum
* — MoAb of Bruck *et al.* (1982) against the epitopes A, B, B′, D, D′
+++ — positive reaction
\+ — weakly positive reaction
— — no reaction
n.d. — not determined

The MoAbs 1, 2, 5, and also the MoAbs against the epitope A (Bruck *et al.*, 1982*a*), bound to the pENV4-expressed polypeptide but not to the pB3/4- and YEpDIT9/2-expressed polypeptides. Fig. 2-3 shows as an example the binding of MoAb 5. Based on these results we can conclude that the binding sites of MoAbs 1, 2, and 5 are located at the C-terminus of gp51, between the amino acid positions 217 and 268 (region III, Fig. 3).

On the one hand, MoAb 5 and 11 inhibit each other in competition assays, on the other hand, they recognize different gp51 fragments. We, therefore, assume that they are directed against different epitopes. Thus, we can discriminate a seventh epitope named M. However, the epitopes D/D′ and M should be in adjacent regions causing a steric or allosteric hindrance for the binding of both MoAbs in competition binding assays. Although the MoAbs 3, 6, 7, and 8 recognized denatured viral gp51 in Western blotting, they showed no reaction with any of the recombinant polypeptides.

We then tried to map the binding sites of MoAbs using synthetic peptides (Portetelle *et al.*, 1989). Location of these peptides within the gp51 molecule is shown in Fig. 3. Only the peptide 57—67 (region I, Fig. 3) competed with plastic-adsorbed BLV for binding of MoAb 14 as did denatured BLV,

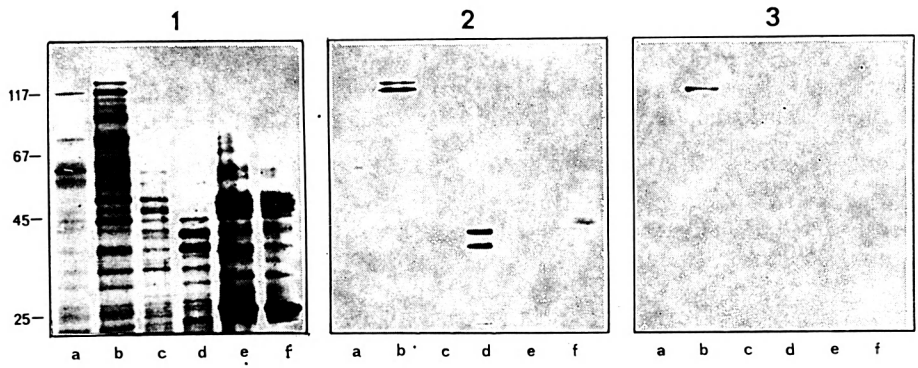

Fig. 2

Reactivity of MoAbs with gp51-related fusion proteins, synthesized in *E. coli* and *S. cerevisiae* (1) Coomassie blue-stained proteins; Western blots incubated with MoAb 13 (2) and MoAb 5 (3). Total lysates of *E. coli* transformed with pEX (a), pENV4 (b), pBD4 (c), pB3/4 (d), and of *S. cerevisiae* transformed with YEpDIT24/3 (e) and YEpDIT9/2 (f) were loaded. The lysates pEX, pBD4, and YEpDIT24/3 were negative controls.

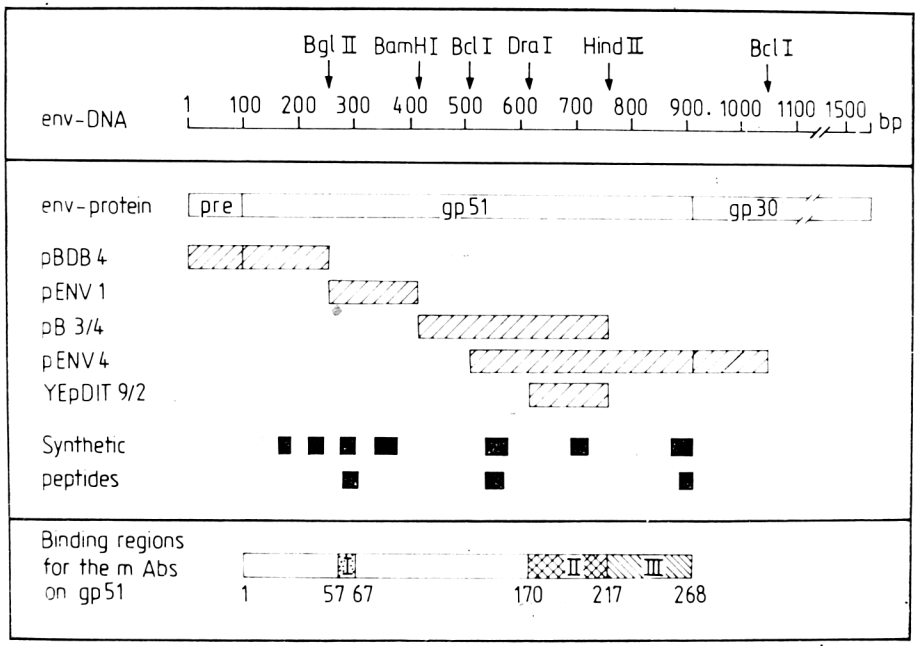

Fig. 3

Binding regions for the MoAbs found by epitope mapping with gp51-related fusion proteins from *E. coli* and *S. cerevisiae* and synthetic peptides

(I) Region (peptide 57—67) recognized by MoAb 14; (II) region (peptide 170—217) recognized by MoAb 9, 10, 11, 13, 22, 30, and also by MoAbs against epitopes B, B′, D, D′, and E; (III) region (peptide 217—268) recognized by MoAbs 1, 2, 5, and also by MoAbs against epitope A.

whereas the peptide 59—69 and native BLV did not compete with plastic-adsorbed BLV for Ab binding (Figs. 4-I and 4-II). Thus, we were able to determine the exact position of the epitope I between the 57th and 67th amino acids. None of the other MoAbs reacted with any of the peptides.

Competition of MoAbs with a serum of BLV-infected cattle

In order to determine the ability of the MoAbs to compete with Abs from a naturally BLV-infected cow for binding to gp51 we used the bovine standard serum D12 from a tumour case (gp51-end point titre 1 : 40,000, determined in a capture ELISA with MoAb 22). If any, the MoAbs related to the epitopes A, B/B′, D/D′, I, K, and M (1, 2, 5, 9, 10, 11, 13, 14, 22, 30) competed only weakly with the bovine Abs. In contrast to these findings, binding of the MoAbs against epitope L (3, 6, 7, 8) to gp51 was strongly inhibited by the bovine Abs (Figs. 5 -I-II and 5-III).

Discussion

We report on the characterization of 14 new MoAbs against the major envelope protein gp51 of BLV. We defined seven independent, non overlapping epitopes and three binding regions for our MoAbs (region I, II, and

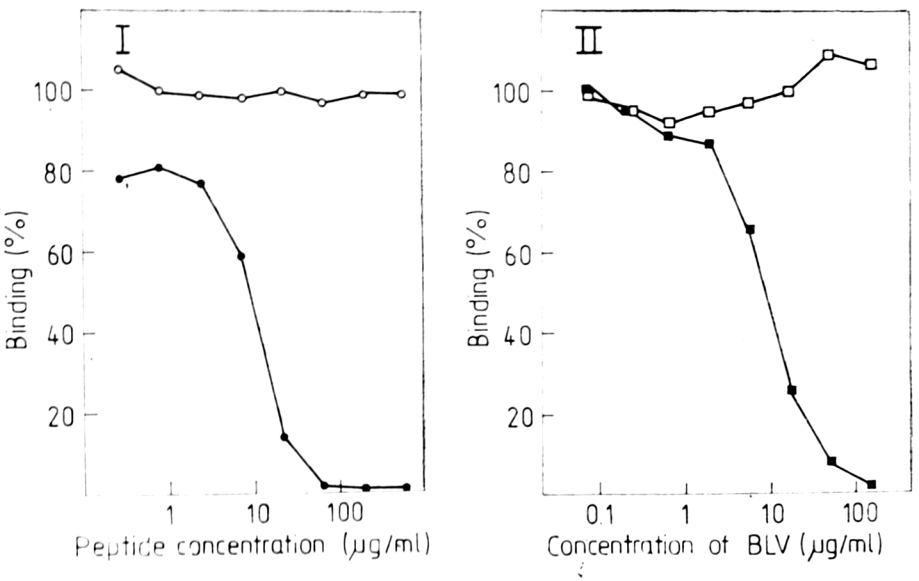

Fig. 4

Reactivity of MoAb14 with the synthetic peptide 57—67, peptide 59—69 and purifiede denaturated and native BLV

(I) Binding of MoAb 14 to plastic-adsorbed BLV in the presence of the peptide 57—67 (●) and of the peptide 59—69 (○) and (II) after incubation with denaturated BLV (■) and with native BLV (□). Ascites containing the MoAb 14 was diluted 1 : 1,000. Binding of MoAb was visualized by HRP-labelled anti-mouse-IgG antibody.

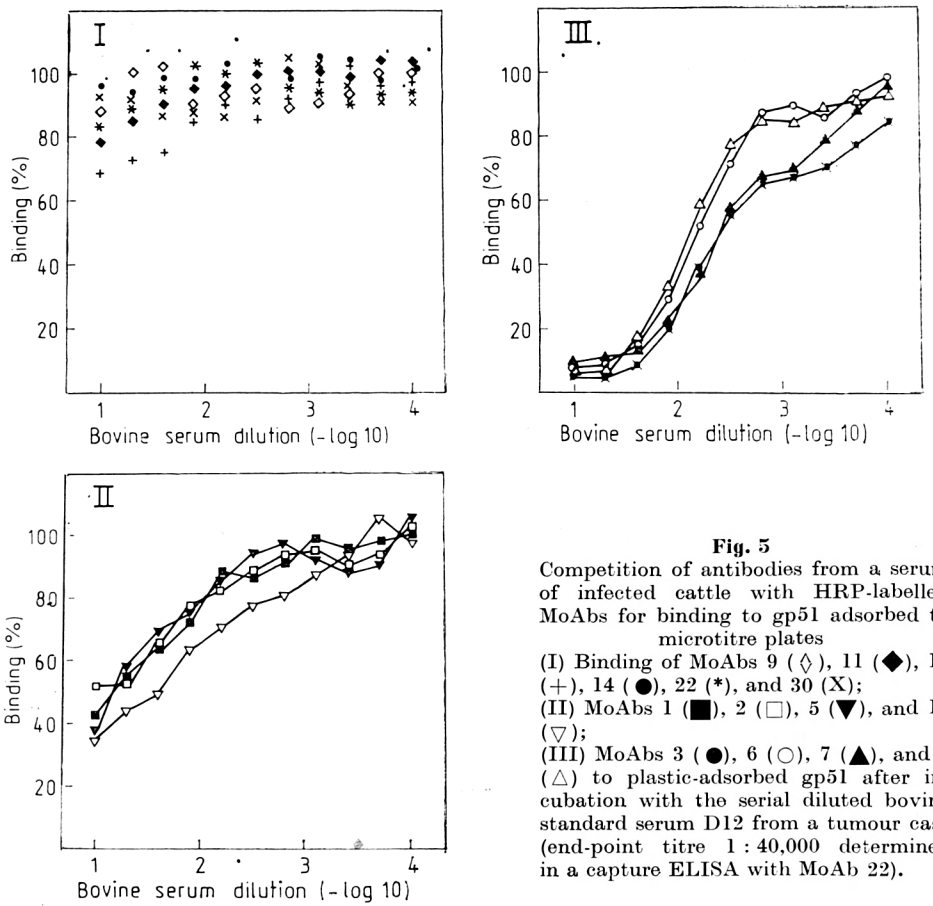

Fig. 5
Competition of antibodies from a serum of infected cattle with HRP-labelled MoAbs for binding to gp51 adsorbed to microtitre plates
(I) Binding of MoAbs 9 (◊), 11 (◆), 13 (+), 14 (●), 22 (*), and 30 (X);
(II) MoAbs 1 (■), 2 (□), 5 (▼), and 10 (▽);
(III) MoAbs 3 (●), 6 (○), 7 (▲), and 8 (△) to plastic-adsorbed gp51 after incubation with the serial diluted bovine standard serum D12 from a tumour case (end-point titre 1 : 40,000 determined in a capture ELISA with MoAb 22).

III) on the gp51 molecule. Four epitopes referred to as I, K, L, and M are unrelated to epitopes A—H identified earlier, whereas three are identical or related to the epitopes A, B/B′, and D/D′ (Bruck *et al.*, 1982*a*).

In summary we found that 50% of the MoAbs raised against enriched gp51 (16 out of 32 MoAbs from 4 mouse spleens, including 14 MoAbs kindly provided by C. Bruck), are directed against 4 epitopes (B/B′, D/D′, E, and K) localized in region II (Fig. 3). The other 16 MoAbs recognize 8 epitopes on the gp51 molecule: epitope I (region I); epitopes A, M (region III), and epitopes C, F, G, H, L (not yet localized). Therefore, the peptide covering amino acids 170 to 217 seems to be an immunodominant region exposed on the isolated gp51 molecule. Prediction of antigenic determinants by different methods confirm this assumption (Grau, personal communication).

Evaluating competition of our MoAbs with Abs of a serum of naturally infected cattle, we found that MoAbs against the epitopes localized in region

II (B/B′, D/D′, and K) did not display significant competition (Figs. 5-I and 5-II). This is in agreement with the observation that in sera of infected cattle there are no Abs with specificities against epitopes B/B′, D/D′, and E (Bruck *et al.*, 1984*a*), while sera from cattle, immunized with enriched gp51 contained Abs against all the epitopes characterized so far (Burkhardt *et al.*, in press; Siakkou, unpublished). These results suggest that the immunodominant region between amino acid position 170 and 217 is not exposed when gp51 is associated with the virus envelope, as it occurs during BLV infection.

MoAbs 3, 6, 7, and 8 (epitope L) showed significant competition with a serum of infected cattle (Fig. 5-III). Because they did neither bind to the fusion polypeptides synthesized in *E. coli* and *S. cerevisiae*, nor to the synthetic peptides it is assumed that this epitope depends on authentic glycosylation of gp51. It was also found that the epitopes F, G, and H recognized by bovine Abs are destroyed by deglycosylation and denaturation (Bruck *et al.*, 1984*b*).

These results suggest that the induction of an effective humoral immune response depends on antigenic complexity of the immunogen. It seems to be obvious that several gp51-related products from *E. coli* and *S. cerevisiae* and also synthetic peptides are weak immunogens and are unsuitable for protection against BLV infection. They are also poor antigens for diagnostic purposes. Our results confirm the findings made earlier (Portetelle *et al.*, 1989; Siakkou *et al.*, 1990). Whether this is generally the case with microbially synthesized gp51 fragments and synthetic peptides remains to be investigated.

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