Using various antigen preparations to produce monoclonal antibodies against bovine leukaemia virus (BLV) gp51SU

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Summary

The objective of this study was to compare different antigen preparations to produce monoclonal antibodies against bovine leukaemia virus gp51SU. The four antigen preparations for immunization of BALB/c mice were: CL: BLV-FLK cell lysate, UF: a fraction of CL (between 30 and 100 kDa), WVP: whole virus particles and SP: with ion exchange chromatography, gp51SU was semipurified. A total of nine successful fusions were performed which resulted in production of 23 monoclonal antibodies (mAbs) specific against gp51SU. The highest ratio of specific hybridoma colonies in each fusion was with SP preparation. Based on the reactivity of the mAbs in Western blotting, mAbs were classified into four groups: anti-gp51SU (23 mAbs), anti-gp30TM (8 mAbs), anti-Pr72 (5 mAbs) and antibodies against other viral proteins (7 mAbs). Some of the anti-gp51SU mAbs reacted with more than one band in Western blotting, suggesting that these colonies recognized not only gp51SU but also its precursors.

Key words: Monoclonal antibody, Bovine leukaemia virus (BLV), gp51SU

Introduction

The causative agent of enzootic bovine leukaemia (EBL), a disease of cattle with worldwide distribution, is the bovine leukaemia virus (BLV), a lymphotropic retrovirus (Kettmann *et al*., 1994). BLV is a member of Retroviridae and its predominant targets are B cells, but other cell types such as T cells or even macrophages, might be infected. BLV, human T-cell leukaemia virus (HTLV type I and II) and simian T-cell leukaemia virus, STLV-I, show clear cut sequence homologies. BLV infection is characterized by the absence of chronic viraemia even in the presence of circulating specific antibodies (Gatei *et al*., 1993; Domenech *et al*., 2000).

The virus is a typical type C retrovirus lacking oncogenes. It has four genes: *gag*, *pol*, *pro* and *env*, flanked by long terminal repeats (LTR). Gene *env* encodes a precursor protein named $Pr72^{env}$ which undergoes glycosylation and lysis, giving rise to the envelope glycoproteins gp30TM and gp51SU (transmembrane and surface proteins, respectively). They both remain together by non-covalent binding and di-sulphate bonds. The assumed molecular weights of SU and TM vary greatly, depending on the researchers and methods used. However, they have been named gp51SU and gp30TM; gp51SU is a 268-amino acid long polypeptide, has eight potential glycosylation sites and eight cysteine residues (Mamoun *et al*., 1990). Glycosylation of gp51SU is different depending on the cell lines or organ origins, and the glycoprotein produced in bat or rat migrates electrophoretically as a double band, while bovine or ovine cells produce a single band using an anti-gp51SU monoclonal antibody (mAb) (Altaner *et al*., 1993). A total of 12 epitopes has been identified in gp51SU. Of these, those named F (amino acids 64-73), G (amino acids 38-

57) and H (amino acids 98-117) are of conformational type, the most accessible and are associated with infectivity and neutralization. These conformational epitopes, F, G and H are located in the N-terminal half of gp51SU, which form the receptor-binding domain (RBD) (Bruck *et al*., 1982a; Bruck *et al*., 1982b; Mamoun *et al*., 1990; Johnstone *et al*., 2002).

The transmembrane glycoprotein gp30TM is a highly glycosylated 214-amino acid long polypeptide. Of its six cysteine residues, four are conserved in all type C viruses, suggesting an invariant and crucial pattern of di-sulphide bonding. gp30TM anchors the envelope proteins in the membrane of the infected cell and virus particles. It is not known whether S-S bridges between gp51SU and gp30TM serve as linkages between the two proteins after proteolytic cleavage of the precursor or whether they are artifacts (Kettmann *et al*., 1994).

Several methods to prepare antigen from BLV have been reported and some of them have been used in production of mAbs against BLV proteins (Buck *et al*., 1988; Llames *et al*., 2000).

The objective of the present work was to produce a panel of mAbs against BLV-gp51SU using various crude and semi-purified BLV antigen preparations as immunogenes following the standard immunization protocols. The antigen preparations included cellular lysates; a fraction of cellular lysates (between 30 and 100 kDa) (to study antibodies against BLV proteins precursors); whole virus particles and semi-purified gp51SU.

Materials and Methods

Fetal lamb kidney cells (FLK-BLV), persistently infected by BLV, were used as the source of the virus. SP2/0 myeloma cell line (Pasteur Institute of Iran) was used for the production of mAbs. Cell culture was grown in media containing DMEM (Sigma, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma, USA) and maintained with 5% CO_2 at 37°C.

Five female BALB/c mice (4–5-week-old) (Pasteur Institute of Iran) were inoculated three times subcutaneously (days 0, 14 and 28), each time with 50 mg of standard antigen from a commercial agar gel immunodiffusion (AGID) kit (Leukoassay B, Rhone Merieux Inc, USA) and diagnosed as positive by immunoperoxidase assay in Razi Institute, Karadj, Iran. The sera from these animals, before and after inoculation, were used as negative and positive control sera, respectively in ELISA, dot-ELISA and Western blot (WB). Commercial BLV-positive sera from the AGID kit was used as BLV-positive control sera.

The following BLV antigen preparations were prepared to inoculate mice for mAb production and also for hybridoma screening:

(CL): FLK-BLVcell lysate, FLK-BLV cells (4×10^6) were lysed in 250 µl of lysis buffer for 30 min on ice (0.15 M NaCl, 0.05 M Tris-HCl pH = 7.2, 0.1% sodium dodecyl sulphate, 1% Triton X-100, 1% sodium deoxycholate, 10^{-4} M PMSF (phenylmethyl-sulfonylfluoride)). After centrifugation (10,000 × g for 15 min at 4°C) the supernatant was collected, aliquoted and stored at -20°C.

(UF): A fraction of the said antigen preparation between 30 and 100 kDa was prepared using ultrafilters (Eppendorf, Germany) with respective cut offs.

(WVP): whole virus particles were harvested from FLK-BLV tissue culture medium through several steps of centrifugation as described before (Buck *et al*., 1988). Purified virions were lysed in Tris-HCl buffer, 0.01 M, pH = 7.2 containing 1% (w/v) Triton X-100 and 10^{-4} M PMSF.

(SP): WVP was lysed as described above, and the gp51SU was then semipurified by chromatography on a diethylaminoethyl (DEAE) anion-exchange column.

Female BALB/c mice (4–6-week-old) (Pasteur Institute of Iran) were immunized with different BLV preparations using either intraperitoneal (IP) or subcutaneous (SC) protocols (Protetelle *et al*., 1980; Bruck *et al*., 1982a). Each antigen was emulsified in complete (for the first inoculation) or incomplete (for subsequent inoculations) Freund's adjuvant. Fusion between SP2/0 myeloma cells line (Pasteur Institute of Iran) and spleen cells from the hyperimmunized mice were performed two days after a final intravenous booster inoculation of respective

antigen using PEG 1450 (Sigma, USA). The resulting hybridomas were resuspended in enriched fusion medium (DMEM) (Sigma, USA), 20% FBS (Sigma, USA), hypoxanthine-aminopterin-thymidine supplement, (HAT) (Sigma, USA)) containing peritoneal macrophage cells (5×10^4 cells/well) and plated in 96-well plates (Nunc, Denmark).

Three to five weeks after the fusion, selection of antibody-secreting hybridomas was performed employing various screening methods (as described below). Hybridomas of interest were expanded and recloned twice by limiting dilution method in media containing DMEM, 20% FBS, peritoneal feeder cells and 1% hypoxantine and thymidine (Sigma, USA).

For screening an indirect-ELISA was done according to the method described earlier (Protetelle *et al.*, 1980), with a few modifications. Microtiter plates (Nunc, Denmark) were coated with one µg (in 100 µl of coating buffer) of different antigen preparations for two hrs at 37°C. After coating, wells were incubated sequentially with 1% bovine serum albumin (BSA), colonies supernatant and rabbit anti-mouse IgG-labeled with horse radish peroxidase (HRP) (DAKO, Denmark) (one hr for each step, followed by thorough washing). Substrate solution containing tetramethyl benzidine (TMB) was added and the absorbance was read at 450 nm.

Dot-ELISA

Nitrocellulose membrane (Bio-Rad, USA) was prepared in accordance to the number of hybridoma colonies to be tested, and washed for 15 min in PBS containing 1% Tween 20 (PBS-T) and for 30 min in transfer buffer (25 mM Tris-HCl and 192 mM glycine). One µl of each antigen preparation (approximately containing 100–400 ng of protein) was placed in the centre of each grid and incubated for two hrs at 37°C. Hybridoma colonies supernatant (one µl of each) was applied and incubated for one hr at 37°C. After washing three times with PBS-T, HRP-labeled second antibody (rabbit anti-mouse polyclonal IgG and IgM (1:5000) (DAKO, Denmark) was added and incubated for one hr at 37°C. Reactions were revealed by using diamino benzidine (DAB) and 4-chloro-1-naphthol substrates.

Immunoblotting

WVP and CL proteins were separated using 10% acrylamide and were then electroblotted on nitrocellulose membrane (Bio-Rad, USA) (0.400 mA for four hrs). The membranes were blocked with 3% BSA for one hr and incubated sequentially with ascetic fluid (1:100 in PBS-T) or concentrated antibodies with the aid of freeze dryer (1:100 in PBS-T) or undiluted colonies supernatant and HRP-labelled polyclonal rabbit anti-mouse IgG and IgM (1:3000) (DAKO, Denmark), with washing in between. The reaction was revealed using the substrate described for dot-ELISA. Molecular weights of BLV proteins identified by mAbs were determined by standard molecular weight markers (Sigma, USA).

Specific antibody-secreting hybridoma colonies were screened when they occupied 40–70% of the well; it took generally three weeks after fusion and five days after the last change of the medium. Hybridoma colonies with three sequentially negative results in screening tests were discarded.

Results

Both route of injection resulted in hyperimmunized mice as indicated by indirect-ELISA and also yielded high fusion efficiency. The hybridoma colonies were stable and had a tendency to save their ability to secrete antibodies.

From the different antigen preparations, CL produced the highest number of hybridoma colonies after two consecutive successful fusions—I and II (83 positive colonies in screening tests). However, lots of those colonies (n = 76) were against different proteins rather than gp51SU and/or its precursors in Western blotting. SP antigen preparation produced eight positive colonies in screening tests; five of which were against gp51SU, but none was against its precursor. Even though this antigen preparation produced the lowest number of hybridoma colonies—after two successful fusions (I and II)—the proportion of gp51SU-specific mAbs was highest. UF antigen preparation produced 19 positive hybridoma colonies but only 10 of which

were against gp51SU and/or its precursor. WVP antigen preparation produced 21 positive colonies after two successful fusions (I and II); six colonies of which were against gp51SU (Table 1).

CL and WVP were antigen preparations applied for Western blot. Using BLV-positive serum taken from animals which were found positive by immunoperoxidase assay in Razi Institute, Karadj, Iran, we compared the positive hybridoma colonies with the polyclonal serum in Western blot (Fig. 1).

Based on different antigen preparations

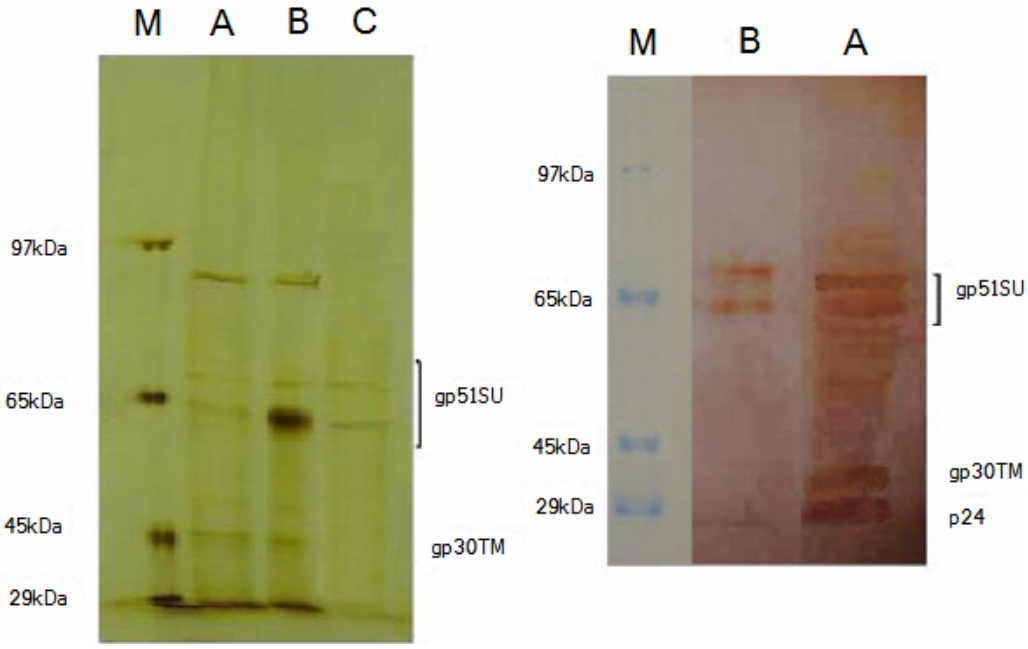

Fig. 1: Left: SDS-PAGE profiles of whole virus particles (WVP) proteins, after first step of high-speed centrifugation (Lane B); after third step of high-speed centrifugation (Lane A) and semipurified gp51SU (Lane C). Right: Western blot (WB) using cow's BLV-positive serum and WVP antigen preparation (Lane A) and semipurified antigen preparation (SP) (Lane B); molecular weight marker (Lane M)

Table 1: The specificity of the mAbs; different antigen preparations were: CL (BLV-FLK cell lysate), UF (a fraction of CL between 30 and 100 kDa), WVP (whole virus particles) and SP (semipurified gp51SU)

Specificity	Bands (kDa)	CL	UF	WVP	SP	Number
gp51SU	70	I,9	I,7	I,3	I,6	
		I,15	I,13	I,7	I,8	
		I,23	I,24	II,10	II,11	
		II,29	I,38	II,14	II,15	
		II,33	II,45	II,15	II,19	
			III,56	II,19		
			III,59			23
gp30TM	35	I,13	I,13	I,5		
		I,15	III,49	I,9		
		II,25		II,13		8
$Pr72^{env}$	90	I,15	I,13			
		II,37	I,38			
			III,50			5
Other viral		I,6	I,11	I,4		
proteins		II,31	I,30	I,11		
				II,25		7
Total		12	14	12	5	43

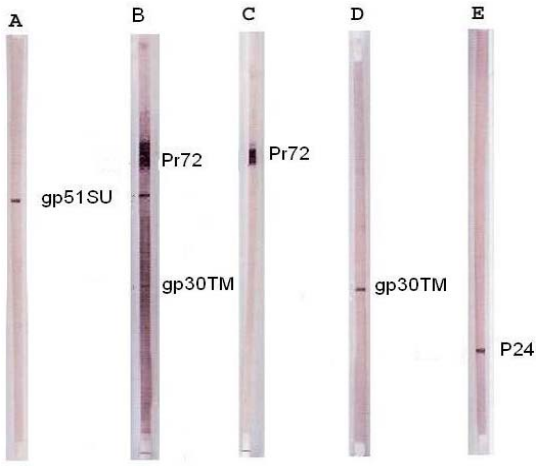

Fig. 2: Some of the mAbs produced in this study. Lane A–E: concentrated supernatant and FLK-BLV cell lysate antigen preparation (CL) were used for Western blotting (WB). Lane A: (SP II 15); B: (UF III 56); B: (UF I 13); C: (CL I 23); D: (CL I 13); E: (WVP I 4)

(CL, UF, WVP, SP) and the number of fusions (I, II or III), we classified the 43 mAbs against different viral proteins into four groups (Table 1). A selected mAbs prototype (which gave the clearest reaction) of each group is shown in Fig. 2.

Discussion

According to the antigen preparation, the BLV proteins would be different and have different conformations. Thus, in immunization using CL or UF, we basically expected to obtain mAbs against intracellular viral proteins and also against BLV precursor proteins. In immunization with WVP or SP antigen preparations, fundamentally, the mAbs would identify the virus proteins or gp51SU, respectively.

The purity of immunogen for the production of mAbs is a really important criterion. The semipure antigen (SP) preparation is the best preparation for production of mAbs against gp51SU as 62.6% of the positive hybridoma colonies in screening tests were against the target molecule. However, the majority of the mAbs produced in this study reacted against gp51SU or its precursors, suggesting that this molecule is the most immunogenic BLV protein regardless of the type of antigen preparation.

Indirect ELISA is the most frequently used screening test for the detection of hybridoma colonies due to its simplicity, reproducibility and its ability to test several colonies simultaneously. For the initial analysis, only FLK-BLV cell lysate (CL) was used. Once expanded, hybridoma colonies were tested also with other antigen preparations (UF, WVP and SP). Even though WVP and SP were good antigen preparations for the detection of mAbs against gp51SU, their preparation was tedious and costly. Western blot was used to detect the specificity of the antibody-secreting hybridoma colonies. Supernatant from hybridoma colonies was generally obtained after expansion in 24-well plates.

The dot-ELISA technique which was used in this study for the detection of mAbs only required one µl of each reagent (antigen, antibody and conjugate), used 100–200 times less than what is necessary for indirect-ELISA. Its sensitivity permitted the detection of antibodies that would have otherwise been undetected by indirect ELISA and Western blot. In addition, not all mAbs recognized BLV proteins in all immunological tests. Some of the colonies—e.g., UF I 38 and CL I 9, both against gp51SU—had false-negative results in indirect-ELISA independent of antigen preparation used. On the contrary, these mAbs reacted positive in Western blot and dot-ELISA. In this respect, dot-ELISA proved far more sensitive for the screening of mAbs in this study. This technique allowed us to detect 14 positive colonies against gp51SU compared to only nine positive results using indirect-ELISA.

The fact that some of the mAbs produced by CL or UF, reacted with more than one band in Western blot (even after two steps of recloning), may be explained by the fact that aside from reacting with mature proteins, these mAbs also recognized viral precursors. For example UF I 13, always react in three different bands (in Western blot) corresponding to $Pr72^{env}$, gp51SU and gp30TM (Fig. 2). So we can interpret it, in this sense that, this colony has detected a shared part among these three proteins.

As earlier researcher mentioned, sometimes several close bands were recognized by a mAb, reacting against gp51SU (Llames *et al*., 2000). It has also been described that

the molecular weight of gp51SU varies between 51 and 77 kDa. In this study, the presence of gp51SU in WVP and SP antigen preparations recorded as two close bands (60 and 70 kDa) in WB with polyclonal sera (Fig. 1). However, only one of these bands (60 kDa) recognized by the mAbs against gp51SU (Protetelle *et al*., 1980; Altaner *et al*., 1993; Llames *et al*., 2000).

It is important to mentioned that two of the mAbs (SP I 6 and II 19) were able to detect the gp51SU in WB followed by PAGE of viral proteins, while the same hybridoma colonies were not able to detect gp51SU in WB followed by SDS-PAGE of viral proteins.

It seems that the molecule of gp51SU may be altered during SDS-PAGE and many of its epitopes are chemically denatured and conformationally altered so that the epitopes are no longer recognized by the antibodies. Nevertheless, there were colonies that reacted with denatured form of the gp51SU. There were also polyclonal serum antibodies that were able to detect gp51SU which had undergone protein denaturation, so this is not in contrast with other findings (Mamoun *et al*., 1990; Gatei *et al*., 1993; Kittelberger *et al*., 1996).

Although it has been described that the purity of the antigen preparation for the production of mAbs against BLV is of little importance (Llames *et al*., 2000), other findings showed that more purification of the virus results in more production of positive hybridoma colonies (Platzer *et al*., 1990). In this sense, semipure antigen preparation (SP) had the highest ratio (8.5) of mAbs production against gp51SU.

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