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Production and Characterization of Monoclonal Antibody to Bovine Leukemia Virus gp51-SU

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Abstract: Enzootic bovine leukemia (EBL) is a disease of cattle with worldwide distribution. The bovine leukemia virus (BLV), is a typical type C retrovirus, has four genes: *gag*, *pol*, *pro* and *env*, flanked by long terminal repeats (LTR) and lacking oncogenes. Gene *env* encodes a precursor protein named $Pr72^{env}$ which undergoes glycosylation and lysis, giving rise to an envelope glycoprotein gp51-SU with a total of 12 epitopes. The objective of the present study was to produce a panel of monoclonal antibodies (mAbs) against BLV-gp51-SU following the standard immunization protocols. The antigen preparations included cellular lysates, a fraction of cellular lysates to study antibodies against BLV proteins precursors, whole virus particles and semipurified gp51-SU. Both route of injection (SC and IP) 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. Fusions produced five positive colonies in screening tests, two of which were against gp51-SU. The targeted hydrophobic domain of the gp51-SU molecule was detected by one of the colonies of mAbs. The two interested mAbs were used for immunohistochemistry on lymph node sections of a cow with lymphosarcoma which can detect immunoreactive materials in sections and also in H&E staining. We conclude the produced panel of mAbs is able to be used in detection of gp51-SU and a new epitope identified on this molecule could be used in IHC method for diagnosis of BLV infection.

Key words: Bovine Leukemia Virus · gp51-SU · Monoclonal antibody

INTRODUCTION

The causative agent of enzootic bovine leukemia (EBL), a disease of cattle with worldwide distribution, is the bovine leukemia virus (BLV), a lymphotropic retrovirus [1]. 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 leukemia virus (HTLV type I and II) and simian T-cell leukemia virus, STLV-I, show clear cut sequence homologies. BLV infection is characterized by the absence of chronic viremia even in the presence of circulating specific antibodies [2, 3].

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 gp51-SU (transmembrane and surface proteins, respectively) [4]. They both remain together by non-covalent binding and bisulphate bonds. The assumed molecular weights of SU and TM vary greatly, depending on the researchers and methods used. However, they have been named gp51-SU and gp30TM; gp51-SU is a 268-amino acids long polypeptide, has eight potential glycosylation sites and eight cysteine residues [5].

Glycosylation of gp51-SU is different depending on the cell lines or organ origins and the glycoprotein produced in bat or rat migrates electrophoretically as a

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double band, while bovine or ovine cells produce a single band using an anti-gp51-SU monoclonal antibody (mAb) [6]. A total of 12 epitopes has been identified in gp51-SU. 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 gp51-SU, which form the receptor-binding domain (RBD) [5, 7-9].

The transmembrane glycoprotein gp30TM is a highly glycosylated 214-amino acids long polypeptide. Of its six cysteine residues, four are conserved in all type C viruses, suggesting an invariant and crucial pattern of disulphide 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 gp51-SU and gp30TM serve as linkages between the two proteins after proteolytic cleavage of the precursor or whether they are artifacts [1].

Several methods to prepare antigen from BLV have been reported and some of them have been used in production of mAbs against BLV proteins [10, 11].

The objective of the present study was to produce a panel of mAbs against BLV-gp51-SU using various crude and semipurified 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 semipurified gp51-SU.

MATERIALS AND METHODS

Fetal lamb kidney cells (FLK-BLV), persistently infected by BLV was 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 Meraux 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 preparation was prepared to inoculate mice for mAb production and also for hybridoma screening:

Whole virus particles were harvested from FLK-BLV tissue culture medium through several steps of centrifugation as described before [11]. 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 and gp51-SU 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 the antigen preparation using either intraperitoneal (IP) or subcutaneous (SC) protocols [7, 8, 12]. The 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 the 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% hypoxanthine and thymidine (Sigma, USA).

For screening an indirect-ELISA was done according to the method described earlier, with a few modifications [12]. Microtiter plates (Nunc, Denmark) were coated with one µg (in 100 µl of coating buffer) of the 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 Ig G-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.

Indirect Peptide ELISA for Anti-N-Protein Abs: The peptide; H-PPQPDFPQ-OH; corresponding to the part of primary amino acid sequence of BLV envelope glycoprotein gp51-SU in one of the hydrophobic domains of the molecule were synthesized on solid phase, automatically, on an EPS 221 (ABIMED) automatic synthesizer using 9-fluorenylmethoxycarbonyl (Fmoc) chemical strategies and following standard protocols (Mimotops, Australia) [13, 14].

The ELISA was performed as described by standard protocols. In brief, wells of Nunc Maxisorp ELISA plates were coated with peptide (4 μg/well)in carbonate buffer pH 9.6 overnight and then incubated sequentially with blocking solution (PBS supplemented with 1% BSA and 0.5% Tween 20) at 37°C for 30 min and with dilutions of the indicated mAbs or pig sera in blocking solution at 37°C for 1 h. Then the wells were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma, 1:500) or alkaline phosphatase-conjugated rabbit anti pig IgG (Sigma, 1:500), respectively, at 37°C for 30 min and finally with alkaline phosphatase substrate. Between each step the wells were rinsed three to four times with PBS-Tween (PBS with 0.1%, v/v, Tween 20). At 15–30 min of incubation at room temperature the alkaline phosphatase product was quantified at $A405$ (A_{405}) with an automatic plate reader. The Ab titer was expressed as the reciprocal of the highest dilution that yielded an A_{405} value of about 1.0 after 15-20 min of incubation with substrate.

Purification and Isotypes Determination of the Antibodies: Hybridoma cells were injected into peritoneal cavity of BALB/c mice at 1×106 cell per mouse. Ascetic fluid was harvested 10 days later and antibodies were purified by protein A affinity column (PURE 1A, Sigma, US). Antibodies isotypes were determined using the mouse monoclonal antibodies isotyping kit according to manufacture's instructions (Iso-2, Sigma, US).

Immunoblotting: Whole virus particles 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-labeled 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.

Immunohistochemistry: The lymph nods of clinically ill animals were collected, fixed in 10% buffered formalin and processed by routine methods. Sections (4-5 μm) were cut and stained with hematoxylin and eosin (H&E). The diagnosis was based on the presence of pathogonomonic histologic changes typical of BLV infection such as effacement of architecture, loss of cortex and modularly differentiation, bridging of the peripheral sinus, fading cortical germinal centers with diffuse involvement of the nodes by neoplastic lymphocytes and involvement of perinodal tissues. The tumor cells had nuclei 1-2.5 red cells in diameter with irregular deposition of chromatin on nuclear membranes and large nucleoli.

Tissues presenting lesions suggestive of BLV infection were subjected to immunohistochemical examination. Sections (4-5 μm) were dewaxed in xylene and rehydrated through graded ethanol. Viral antigen was retrieved with trypsine (Sigma, USA) for 15 min at 37°C, followed by hit retrieval using citrate buffer (pH 6.1). Endogenous peroxidase was quenched by immersion in 3% H2O2. Slides were blocked by the application of casein solution (0.5%) for 20 min to decrease non-specific background staining. Sections were treated for 45 min at 37 °C with purified and diluted mAbs. A secondary horse raddish-peroxidase conjugated antibody (Komabiotech, S. Korea) was applied for 20 min at room temperature. Slides were then treated for 5 min with a diamiobenzidine solution (Sigma, USA) and counterstained with hematoxylin. Samples were washed between steps with Tris buffered saline solution. Sections were considered positive when structures consistent with the morphology of the parasite were immunolabeled by this method. A PCR was also performed on extracted DNA from tissues to confirm the presence of the provirus in the tissue sections (data not shown).

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.

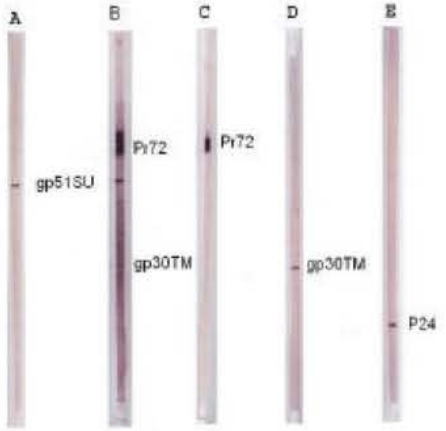

Fig. 1: The N-terminal extreme of peptide gp45 was characterized by the presence of high hydrophilicity amino acid residues (Hoop and Woods, 1981) and was predicted to be the most antigenic part of the whole sequence (Welling et al., 1985; Parker et al., 1986). The immunoreactivity of peptide gp45-I was significant only when it was used in its cyclic form. Even though synthetic peptides mainly mimic sequential epitopes, preferential conformations on the solid phase may play an important role in epitope functional activity

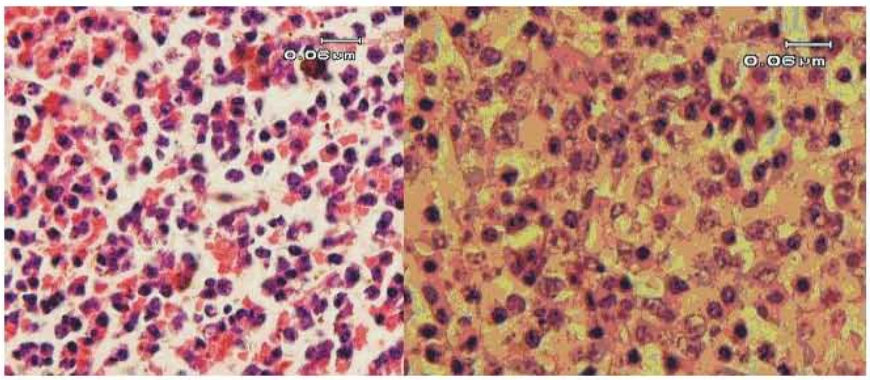

Fig. 2: Bovine leukemia, lymph node, the tumor cells have large nuclei with irregular deposition of chromatin on nuclear membranes, parachromatin clearing and prominent nucleoli (H&E, × 1000)

Fusions produced five positive colonies in screening tests; two of which were against gp51-SU and one was against its precursor as indicated by Western blotting (Fig. 1). The targeted hydrophobic domain of the gp51-SU molecule was detected by one of the colonies of mAbs.

The two interested mAbs were then used for immunohistochemistry on lymph node sections of a cow with lymphosarcoma which was confirmed by histopathological and PCR assays. As shown in Figure 2 mAbs can detect immunoreactive materials in sections and also in H&E staining.

Immunoglobulin were purified by protein A affinity chromatography generating high titer mAbs solutions at 0.5 to 2.5 mg/ml. A commercial typing kit was used to determine the isotype of mAbs (A to E), revealing that the mouse MAb contains the IgG1 heavy chain.

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 gp51-SU, 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 gp51-SU 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 gp51-SU 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 gp51-SU, 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.

It must be mentioned, 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 gp51-SU-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 gp51-SU 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 $Pr72^{env}$, gp51-SU 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 gp51-SU.[10] It has also been described that the molecular weight of gp51-SU varies between 51 and 77 kDa. In this study, the presence of gp51-SU in WVP and SP antigen preparations recorded as two close bands (60 and 70 kDa) in WB with polyclonal sera (Fig. 2). However, only one of these bands (60 kDa) was recognized by the mAbs against gp51-SU [6, 10, 12].

It is important to mentioned that two of the mAbs (SP I 6 and II 19) were able to detect the gp51-SU in WB followed by PAGE of viral proteins, while the same hybridoma colonies were not able to detect gp51-SU in WB followed by SDS-PAGE of viral proteins.

It seems that the molecule of gp51-SU 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 gp51-SU. There were also polyclonal serum antibodies that were able to detect gp51-SU which had undergone protein denaturation, so this is not in contrast with other findings [2, 5, 15].

Although it has been described that the purity of the antigen preparation for the production of mAbs against BLV is of little importance, other findings showed that more purification of the virus results in more production of positive hybridoma colonies [10, 16]. In this sense, semi pure antigen preparation (SP) had the highest ratio (8.5) of mAbs production against gp51-SU.

A total of 12 different epitopes were characterized on gp51SU. The synthetic peptide used in this study was localized in C-terminal half of gp51SU and flanked by N-terminal of epitope B/B; a sequential epitope from amino acid 175 to 190. The hybridoma colony A recognized the synthetic peptide in ELIZA. Based on this observation, this peptide might be a new sequential epitope on gp51SU although it needs more investigation to judge whether it is a sequential epitope or not.

Based on the results, we can conclude that the produced panel of mAbs is able to be used in diagnosis of gp51-SU in IHC method. Also, we identified a new epitope on this molecule that could be used in IHC diagnosis of BLV infection on tissue sections.

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