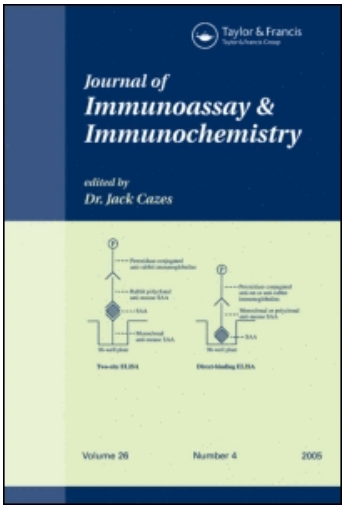


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# Temperature-Dependent Non-specific Adsorption of Recombinant Bovine Leukemia Virus Receptor BLVRcp1 in Immunoassay

Oto Orlika[a]; Cestmir Altaner[b]; Gary A. Splitter[a]
[a] Department of Animal Health and Biomedical Sciences, University of Wisconsin-Madison, Madison, WI, USA [b] Department of Molecular Virology, Cancer Research Institute, Bratislava, Slovak Republic

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# TEMPERATURE-DEPENDENT NON-SPECIFIC ADSORPTION OF RECOMBINANT BOVINE LEUKEMIA VIRUS RECEPTOR BLVRcp1 IN IMMUNOASSAY

Oto Orlik[a], Cestmir Altaner[b] and Gary A. Splitter[a]
[a]Department of Animal Health and Biomedical Sciences, University of Wisconsin-Madison, 1655 Linden Drive, Madison, WI 53706, USA
[b]Department of Molecular Virology, Cancer Research Institute, Slovak Academy of Sciences, 812 32 Bratislava, Slovak Republic



## ABSTRACT

Recombinant bovine leukemia virus receptor, BLVRcp1, possessed the unusual property of binding plastic plates after blocking nonspecific binding sites. Adhesiveness of BLVRcp1 to blocked plates hindered development of an antigen capture and receptor binding assay with this protein. Unexpectedly, non-specific adsorption of BLVRcp1 was dramatically influenced by temperature. Optimizing incubation temperature and antigen capture at 4 ° C instead of 37 ° C and the use of milk as blocking solution removed nonspecific binding of BLVRcp1 allowing development of a functional immunoassay. Thus, the temperature used for antigen capture can be a critical factor that influences performance of the immunoassay.

(KEY WORDS: immunoassay, antigen capture assay, ELISA, RIA, IRMA, monoclonal antibodies, bovine leukemia virus)

## INTRODUCTION

Immunoassays have become conventional in many areas of medical, veterinary and biological science, and solid-phase immunoassays are routinely used for qualitative and quantitative measurement of haptens, antigens and antibodies. (1, 2). A successful immunoassay requires a highly specific antibody-ligand interaction with minimal background interference. Non-specific binding is a major

factor limiting assay sensitivity (3), and the plastic surface must be blocked by unrelated proteins such as bovine serum albumin (BSA), ovalbumin and gelatin or non-ionic detergent Tween 20 (4). The amount and stability of the antigens bound to plastic can be affected by surface treatment (5), and specialized plasticware has been developed for immunoassays. For convenience, immunoassays can be performed at various temperatures, because the rate and extent of protein adsorption to plastic is only slightly influenced by temperature (6).

Recently, a cDNA encoding the putative bovine leukemia virus (BLV) receptor, BLVRcp1, has been isolated, and the chromosome localization in several animal species determined (7, 8, 9). Transfection of the non-permissive NIH 3T3 cell line with the BLVRcp1 cDNA conferred sensitivity to BLV infection; however, the phenotype of expressing cells and physiological function of the receptor has not been determined. Four MAbs to BLVRcp1 have been prepared and used for receptor characterization (our unpublished data). Here, we describe development of two-site immunoradiometric assay (IRMA) for detection of BLVRcp1 and the unusual property of recombinant BLVRcp1 protein to adhere to plates blocked with blocking solution. Surprisingly, non-specific adsorption was strongly dependent on the incubation temperature, as well as type of plates and blocking agent.



## MATERIAL AND METHODS

### Cells and monoclonal antibodies

MAbs to recombinant BLVRcp1 (2H1O, 3C7, 15C3, 5C9, our unpublished data) and MAb B22 to BLV gp51 (10) were used. BLV infected cell line FLK was used for virus production (11). Glycoprotein gp51 was purified by immunoaffinity chromatography from FLK supernatant as described (12). The MAbs were labelled

with $^{125}I$ by means of IODO-BEADS as recommended by the manufacturer (Pierce, Rockford, IL).

Preparation of bacterial lysates

To prepare BLVRcp1 protein, the *EcoRI-XbaI* cDNA fragment of BLVRcp1 (2.3-kbp) was inserted into *EcoRI-XbaI* digested plasmid pMAL-c2, transfected into *E. coli* DH5α and expressed as a fusion protein containing maltose binding protein (MBP). Bacterial lysates were prepared from clone BLVRcp1 containing inserted cDNA and mock transfected clone MBP (negative control) using 0.3 M isopropylthiogalactoside for 2 h, followed by centrifugation (4000 x g for 20 min) and lysis with lysozyme (5 mg/ml in PBS) and 1% sodium dodecyl sulfate for 10 min on ice. The lysate was clarified by centrifugation at 15,000 x g for 30 min, and the supernatant was used as a bacterial lysate. Recombinant protein was purified from bacterial lysate using amylose resin affinity chromatography according the manufacturer's recommendations (New England, BioLabs, Beverly, MA). Protein concentration in bacterial lysates was determined by BCA assay kit (Pierce, Rockford, IL). The content of expressed recombinant fusion BLVRcp1 in the lysates was estimated from 10 % SDS-PAGE gels stained with Coomassie blue.

Two-site Immunoradiometric assay (IRMA)

Ninety-six well plates were coated with primary MAb (1 μg/ml of immunoglobulin in PBS) overnight at 4 °C. After blocking non-specific binding sites, bacterial lysates (1 : 250 in PBS) were added and incubated at 4 °C overnight or at 37 °C for 4 h, respectively. Following three washings with PBS containing 0.1 % Tween 20, captured antigen was detected by [$^{125}I$]-labelled secondary

monoclonal antibody or a pool of antibodies. Then plates were extensively washed, and bound radioactivity was dissolved in 1 M potassium hydroxide and measured using a gamma counter.

Receptor binding assay

Ninety-six well plates were coated with affinity purified BLV gp51 dissolved in PBS overnight at 4 ° C. After blocking with 1 % BSA in PBS, the bacterial lysates diluted 1 : 250 in PBS were added and incubated for 4 h at 37 ° C. BLVRcp1 bound to gp51 was detected by anti-BLVRcp1 MAbs and visualized by peroxidase-labelled secondary antibody and ortho-phenylenediamine as a chromogenic substrate (1.0 mg/ml in 0.05 M citrate-phosphate buffer, pH 4.5) in the presence of 0.01 % hydrogen peroxide. The data represent the mean from duplicate samples, and are representative of at least two experiments.



## RESULTS

Non-specific binding of BLVRcp1 to microplates blocked with BSA

Monoclonal antibodies to BLVRcp1 (2H10, 3C7, 15C3 and 5C9) were used in an antigen capture assay for quantitative determination of recombinant BLVRcp1. Plates coated with primary antibody were blocked with 1% BSA in PBS followed by incubation with bacterial lysate BLVRcp1. Recombinant BLVRcp1 receptor captured by the primary MAb was detected using a pool of $^{125}$I-labelled anti-BLVRcp1 MAbs. Ninety-six well plates Costar TK (tissue culture plates, Cat. No. 3595) were used for assay development because the plates displayed high adsorption capacity for immunoglobulins and were successfully used for MAb-based ELISA for detection of anti-BLV antibodies (data not shown). However,

TABLE 1

Nonspecific binding of BLVRcp1 to BSA-blocked plates in IRMA[1]

| Coating MAb | Lysate | |
|---|---|---|
| | BLVRcp1 | MBP |
| none | 23002 | 300 |
| 15C3 | 2800 | 300 |
| B22 | 2700 | 300 |
| capture index[3] | 1.0 | |

[1] Plates were coated with primary antibody, blocked with 1 % BSA in PBS followed by incubation with lysates for 4 h at 37 ° C. 15C3 is a BLVRcp1 specific MAb; B22 is an anti-gp51 MAb, used as a negative control. BLVRcp1, lysate containing recombinant BLVRcp1; MBP, negative lysate containing maltose binding protein.

[2] Values represent the mean cpm of duplicate samples.

[3] Capture index distinguishes whether the antigen was captured by the capture antibody or whether it bound non-specifically to plastic. Capture index is the cpm ratio in wells coated with anti-BLVRcp1 MAb 15C3 and unrelated MAb B22.



binding of $^{125}$I-labelled antibodies in control samples (wells without primary antibody or with unrelated antibody B22) was comparable to wells with anti-BLVRcp1 antibodies indicating that recombinant BLVRcp1 adhered non-specifically to plastic surface and not captured by MAbs. All four anti-receptor MAbs displayed similar behavior. Representative data obtained with MAb 15C3 are shown in Table 1.

High $^{125}$I activity in control samples was not caused by non-specific binding of radio-labelled MAbs, because activity in plates incubated with MBP lysate instead of BLVRcp1 remained low. Similar binding of recombinant BLVRcp1 to a BSA-blocked plate was observed in the ELISA receptor binding assay. Plates coated with gp51 and blocked with 1 % BSA were incubated with BLVRcp1 lysate followed by

TABLE 2

Nonspecific binding of BLVRcp1 to BSA-blocked plates in receptor binding assay[1]

| Coating antigen | Lysate | |
|---|---|---|
| | BLVRcp1 | MBP |
| nothing | +++[2] | -[3] |
| gp51 | +++ | - |

[1] Plates coated with purified gp51 were blocked with 1 % BSA followed by incubation with bacterial lysates diluted 1 : 250 in PBS for 4 h at 37 °C. BLVRcp1 bound to gp51 was detected by BLVRcp1 specific MAbs and a horseradish-peroxidase labelled secondary; antibody. BLVRcp1, lysate containing recombinant BLVRcp1; MBP, negative lysate containing maltose binding protein.
[2] OD more than 0.6
[3] OD less than 0.1



detection of gp51-bound BLVRcp1 using a pool of anti-receptor MAbs and a secondary anti-mouse antibody. However, BLVRcp1 binding was detected in wells with or without g51 indicating that BLVRcp1 adhered directly to plastic and not gp51. Specificity of the reaction was verified by using MBP lysate, where low binding was detected (Table 2). To determine that non-specific adsorption was a feature of BLVRcp1 protein, and not caused by bacterial components in the lysate, BLVRcp1 lysate was incubated with anti-BLVRcp1 MAbs before adding to the plate. Lysate neutralization by receptor MAbs abolished BLVRcp1 non-specific binding to BSA blocked plates confirming the adhesive property of BLVRcp1 (Table 3).

Parameters influencing non-specific binding of BLVRcp1

Because BSA, the most commonly used blocking agent, did not prevent BLVRcp1 non-specific binding, several blocking solutions were tested.

TABLE 3

Neutralization of BLVRcp1 adhesiveness by anti-BLVRcp1 MAbs[1]

| Blocking agent | BLVRcp1 pretreated with | $^{125}$I- labelled MAb | |
|---|---|---|---|
| | | 2H10 | 3C7 |
| BSA | nothing | 4750[2] | 3300 |
| BSA | 15C3[3] | 900 | 800 |

[1] Costar TK plates blocked with 1% BSA were incubated for 4 h at 37 ° C with BLVRcp1 lysate pretreated or not with anti-BLVRcp1 MAbs. Bound BLVRcp1 was detected with $^{125}$I-labelled anti-BLVRcp1 MAbs 2H10 and 3C7, possessing different epitope specificities.
[2] Values represent cpm.
[3] All anti-BLVRcp1 MAbs displayed similar blocking effect as MAb 15C3.



TABLE 4

Influence of different blocking agents on BLVRcp1 non-specific binding[1]

| Blocking agent | Lysate | |
|---|---|---|
| | BLVRcp1 | MBP |
| none | 11500[2] | 850 |
| 1% BSA | 3900 | 300 |
| 1% ovalbumin | 8000 | 600 |
| 3% gelatin | 4800 | 400 |
| 3% Tween 20 | 11000 | 800 |
| 10% FS | 2400 | 200 |
| 5% milk | 1100 | 250 |

[1] Costar TK plates were coated with blocking solutions overnight at 4 ° C, and lysates were added and incubated for 4 h at 37 ° C. Detection of bound recombinant protein was done with a mixture of $^{125}$I-labelled anti-BLVRcp1 MAbs. BLVRcp1, lysate containing recombinant BLVRcp1; MBP, negative lysate containing maltose binding protein; FS, fetal bovine serum.
[2] Values represent the mean cpm from duplicate samples.

TABLE 5

Influence of the temperature and the plastic plates on BLVRcp1 non-specific binding[1]

| Coating MAb | Plate | | | | | |
|---|---|---|---|---|---|---|
| | Costar TK | | Costar ELISA | | Corning ELISA | |
| | 37°C | 4°C | 37°C | 4°C | 37°C | 4°C |
| 15C3 | 3500[2] | 2900 | 3350 | 3550 | 3500 | 4350 |
| B22 | 2700 | 1350 | 1200 | 3800 | 1030 | 1500 |
| capture[3] index | 1.3 | 2.1 | 2.8 | 0.9 | 2.8 | 2.9 |

[1] Plates coated with MAb immunoglobulins were blocked with 1 % BSA followed by incubation with BLVRcp1 lysate at indicated temperatures. Detection of captured BLVRcp1 was done with a mixture of $^{125}$I labelled anti-BLVRcp1 MAbs.
[2] Values represent the mean cpm of duplicate samples.
[3] Capture index is the cpm ratio in wells coated with anti-BLVRcp1 MAb 15C3 and unrelated MAb B22.



TABLE 6

Optimization of IRMA with BLVRcp1 receptor protein[1]

| Coating MAb | Blocking solution | | | |
|---|---|---|---|---|
| | 1% BSA | | 5% milk | |
| | 4°C | 37°C | 4°C | 37°C |
| 15C3 | 4350[2] | 3350 | 3550 | 3600 |
| B22 | 1500 | 1200 | 200 | 450 |
| capture[3] index | 2.9 | 2.8 | 18.0 | 8.0 |

[1] Corning ELISA plates coated with MAbs and blocked with indicated blocking solutions were incubated with lysates at indicated temperatures. Captured BLVRcp1 was detected with a pool of $^{125}$I-labelled anti-BLVRcp1 MAbs.
[2] Values represent the mean cpm of duplicate samples.
[3] Capture index is the cpm ratio in wells coated with anti-BLVRcp1 MAb 15C3 and unrelated MAb B22.

Surprisingly, plates treated with three other blocking agents (ovalbumin, gelatin and Tween 20) did not prevent BLVRcp1 nonspecific adsorption and adsorption was even higher than with BSA (Table 4). Nevertheless, non-fat milk markedly reduced BLVRcp1 binding, while blocking efficiency of fetal bovine serum (FS) was only slightly better than BSA.

Besides the influence of the blocking agent, several different plates and temperature effect on BLVRcp1 non-specific binding were tested. Plates coated with capturing MAbs and blocked with 1 % BSA solution were incubated with BLVRcp1 lysate at 4 $^{o}$ overnight or 37 $^{o}$ C for 4 hr. Unexpectedly, remarkable sensitivity of receptor non-specific binding on temperature was detected, and the pattern of temperature sensitivity depended on the plastic plate used. Non-specific BLVRcp1 binding to Costar TK plates was reduced when lysate incubation was performed at 4 $^{o}$ C instead of 37 $^{o}$ C, while nonspecific binding to specialized ELISA plates Costar (Cat. No. 3591) was low at 37 $^{o}$ C and increased by lowering the incubation temperature to 4 $^{o}$ C (Table 5). Corning ELISA plates (Cat. No. 25805-96) had the lowest background while non-specific binding in Linbro TK plates was increased at higher temperature (data not shown).

The importance of an incubation temperature on assay performance was clearly demonstrated even when the optimal combination of blocking solution and plastic plates was used. Corning ELISA plates combined with nonfat milk as a blocking solution yielded the best results; however, lysate-incubation at 4 $^{o}$ C but not 37 $^{o}$ C was required for maximal efficiency (Table 6). Final optimization of the assay using Corning plates blocked with milk and incubated with lysate at 4 $^{o}$ C resulted in development of a reproducible and specific assay for BLVRcp1 detection. Detection limit was determined as a final dilution when counts in the wells coated

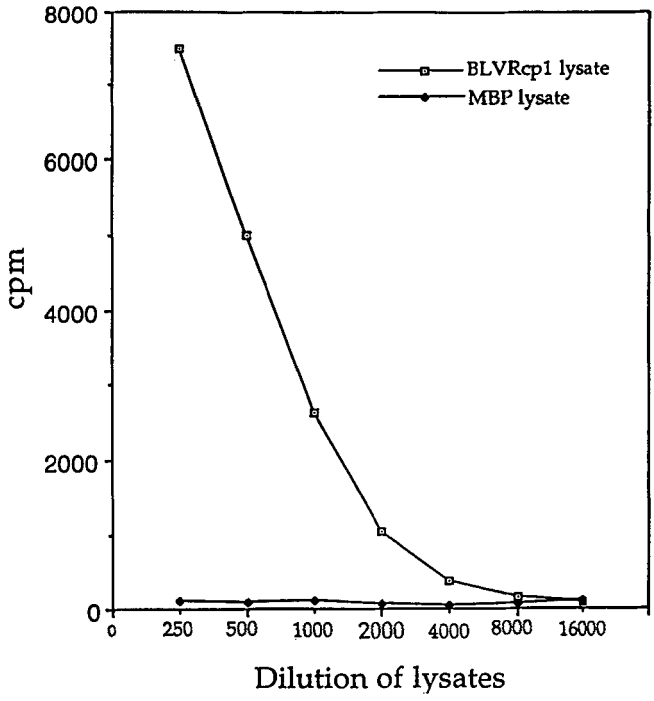


**Figure 1.** Titration of bacterial lysates in IRMA. Corning ELISA plates coated with anti-BLVRcp1 MAb 15C3 and blocked with 5 % non-fat milk were incubated with lysates at 4 ° C overnight. Captured BLVRcp1 was detected with $^{125}$I-labelled anti-BLVRcp1 MAb 2H10.

with BLVRcp1 were at least twice the counts in the wells coated with MBP lysate. Bacterial lysates with recombinant receptor diluted up to 8,000 times yielded specific signal (Fig. 1). Assay sensitivity was calculated from the lysate protein concentration (15 mg/ml) and estimated content of the recombinant BLVRcp1 in the lysate (1-5 %). IRMA allowed us to detect specifically approximately 19-95 ng/ml of recombinant BLVRcp1 protein in the diluted bacterial lysates. Considering the molecular mass of the fusion BLVRcp1 protein (120 kDa), the assay sensitivity was in the range of 0.16 - 0.80 nM.

## DISCUSSION

Here we describe an unusual property of recombinant bovine leukemia virus receptor, BLVRcp1, to bind non-specifically blocked plastic plates in a temperature-dependent manner. Protein adsorption to plastic is only marginal influenced by temperature (6), and plate coating, blocking and incubation performed at 4 $^{\circ}$ C or 37 $^{\circ}$C do not qualitatively influence immunoassay results. However, temperature revealed a dramatic effect on BLVRcp1 nonspecific adsorption. The mechanisms by which proteins adhere to plastic are not completely understood although charge and hydrophobicity are believed to be important. Different plastics have variable affinity for different proteins, and it is impossible to predict the adsorption behavior of a particular protein (13, 14). Therefore, in our case a change of temperature likely introduced modification of parameters that influenced plastic-BLVRcp1 affinity and non-specific binding. Nonspecific adsorption of BLVRcp1 was abrogated by preincubation with anti-BLVRcp1 MAbs providing evidence that adhesiveness was an intrinsic feature of BLVRcp1. We speculate that antibody binding and immune complex formation modified BLVRcp1 charge and conformation similar to temperature-induced changes resulting in loss of nonspecific adsorption.

Most of the blocking reagents tested did not sufficiently prevent BLVRcp1 adsorption to blocked plates. Most likely only blocking proteins possessing similar charge and hydrophobicity as BLVRcp1 would bind analogous sites on the plates and block BLVRcp1 nonspecific adsorption. Milk, containing a broad mixture of different proteins fulfilled this condition and was the best blocking reagent for BLVRcp1. The level of nonspecific binding differed substantially depending on plastic plates used. Plate-surface treatment was probably the reason for great differences, and tissue culture plates ensuring optimal cell attachment revealed the

highest level of nonspecific adsorption. However, use of specialized ELISA plates did not guarantee low background and revealed substantial nonspecific adsorption of BLVRcp1 comparable with tissue culture plates. Strong nonspecific adsorption was described by others when polycationic proteins were coated on plates as the antigen for measuring specific antibodies (15). In this case, nonspecific binding of antibodies to polycationic proteins was prevented by adding the sulphatated polyanions heparin and dextran sulphate.

Physiologic function of BLVRcp1 has not been determined; however, the adhesiveness of BLVRcp1 may be a reflection of its physiologic function. If BLVRcp1 was a cell adhesion molecule, temperature sensitivity would have a very important physiologic consequence. The concomitant increase of BLVRcp1 adhesiveness with temperature would predispose BLVRcp1 engagement at the site of inflammation, where temperature is usually elevated. The antigen capture assay for BLVRcp1 will be useful for receptor detection in cell-lysates from different cell populations. Correlating BLVRcp1 expression with cell susceptibility to BLV infection will provide information regarding BLV-tropism requirements and the possible BLVRcp1 physiologic function.



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**Corresponding author**:
Gary A. Splitter
Department of Animal Health and Biomedical Sciences
University of Wisconsin-Madison
1655 Linden Drive
Madison, WI 53706
Phone: (608) 262-1837; FAX: (608) 262-7420

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