Application of Colorimetric Assay for Detection of Tumor-associated Antigens on Bovine Leukemic Cells

Rieko YONEDA, Misao ONUMA*, Rikio KIRISAWA, and Yoshimi KAWAKAMI

Department of Veterinary Microbiology, College of Dairy Agriculture, Ebetsu 069, Japan

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ABSTRACT. A rapid colorimetric assay was used to quantitate the cytotoxic activity of monoclonal antibody against tumor-associated antigen (TAA) of bovine leukemia cells. The viability of specific antibody-treated target cells was assessed by using a tetrazolium dye that is reduced to a purple formazan by living but not dead cells. The results of cytotoxicity determined by colorimetric assay were similar to that of trypan blue dye exclusion method. Using this colorimetric assay, we detected TAA in peripheral blood lymphocytes (PBL) from leukemic cattle as well as in PBL from most cattle with lymphocytosis but no evidence of tumor.—KEY WORDS: bovine leukosis, colorimetry, cytotoxicity, monoclonal antibody, tumor associated antigen.

Enzootic bovine leukosis (EBL) is a lymphoproliferative disease caused by bovine leukemia virus (BLV). Leukemia cells from cattle with EBL have tumor-associated antigen (TAA) on their surface [8]. Previously, we obtained monoclonal antibodies against TAA expressed on EBL tumor cells [2]. One of the monoclonal antibodies, c143 which recognizes a polypeptide with a molecular weight of 74,000 reacted with all EBL tumor cells tested but not with BLV antigen nor normal bovine lymphoid cells [1, 2]. The c143 was found to be a useful diagnostic tool for detecting leukemic cells by complement-dependent antibody cytotoxicity (CDAC) test [6]. Viable cells were measured by using trypan-blue dye exclusion method in the CDAC test.

Recently, Mosmann [5] developed a rapid colorimetric assay, based on a tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), that is reduced by mitochondrial dehydrogenases to a purple formazan. The assay detects living but not dead cells, and therefore it can be used for measuring cytotoxicity. Furthermore, the results can be read on a multiwell scanning spectrophotometer (ELISA reader) with a high degree of precision.

This paper describes the usefulness of MTT assay as a quantitative marker for TAA positive cells which show lysis by the specific antibody in the presence of rabbit complement.

MATERIALS AND METHODS

Cells: Blood samples were obtained from 33 healthy cattle negative for BLV antibodies, 42 BLV-infected but clinically healthy cattle, 15 BLV-infected cattle with lymphocytosis or persistent lymphocytosis and 13 cattle with EBL. Animals, over one year old with BLV antibodies were considered positive for BLV infection. Peripheral blood lymphocytes (PBL) were separated from blood by Ficoll-Conray method as described previously [3]. A bovine lymphoid B cell line, BLSC-KU1 established from tumor of affected cow with EBL, was also

* CORRESPONDENCE TO: M. Onuma, Dept. of Veterinary Microbiology, College of Dairy Agriculture, Ebetsu 069, Japan
used. This cell line has TAA on the cell surface and bovine leukemia provirus in the genome [7].

**Monoclonal antibody against TAA:** Monoclonal antibody c143, specific for the common TAA expressed on EBL tumor cells was used to detect EBL tumor cells. This antibody was previously characterized and it reacted with all EBL tumor cells but not with normal bovine lymphocytes nor with BLV antigens [2, 6].

**CDAC test:** The CDAC assays were performed by the trypan-blue dye exclusion method as described previously [9]. Briefly, a mixture of 5 μl of test cells (1×10^6/ml), 5 μl of c143 (1:300 dilution of ascites) and 5 μl of rabbit complement (1:2 dilution) were incubated at 37°C for 45 min and stained with trypan blue. Cytotoxic index (Cl) was calculated as follows:

\[
\text{Cl} = \frac{\% \text{ viable cells in control wells} - \% \text{ viable cells in test sample}}{\% \text{ viable cells in control wells}} \times 100
\]

A Cl value greater than 31.7 was considered positive in this test system [2].

**MTT-CDAC assay:** Viability of c143-treated PBL was determined by the MTT assays as described previously [4, 5] with modifications. We used DMSO instead of acid-isopropanol to solubilize the MTT formazan in the present MTT-CDAC assay [10]. The dye, a tetrazolium salt, MTT (Sigma, USA) was dissolved in PBS at 5 mg/ml. Twenty μl of test cells (1×10^7/ml), 10 μl of c143 (1:300 dilution) and 20 μl of rabbit complement (1:2 dilution) were mixed together in the wells of 96-well flat-bottomed microplate and incubated at 37°C for 45 min. After incubation, an aliquot of 5 μl of MTT stock solution was added to each well and incubated for 16 hr. When MTT incubation was completed, the medium was removed completely by aspiration after the centrifugation of the plate at 800×g for 5 min and dried in incubator. To each well, 50 μl of DMSO was added to solubilize the MTT formazan. The optical density (OD) reading in sample solubilized with DMSO was higher than that of the same sample solubilized with isopropyl alcohol (0.04 N HCl in isopropanol) (Fig. 1). Therefore, we used DMSO for solubilization of MTT formazan. Complete solubilization of the dye was achieved by repeated pipetting of the solution. The plate was read on Immuno Reader (NJ2000, Inter Med.) using a test wavelength of 540 nm, and a reference wavelength of 620 nm. As control, the antibody was replaced by medium for each test. The mean and standard deviation (SD) was determined from triplicate samples. The cytotoxic index (CI) was calculated by the following equation:

\[
\text{CI} = \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \times 100
\]

To know the maximum nonspecific cytotoxicity of c143 by MTT assay, PBL from 33 cattle negative for BLV antibodies by immunodiffusion test and negative for TAA by CDAC test were examined by the MTT-CDAC test. The mean Cl was 11.8±9.4 (SD). A Cl value greater than 30.6 (two fold of SD plus mean value of control) was considered positive in the MTT-CDAC assay.

**RESULTS**

**Standardization of MTT assay for bovine PBL:** MTT as a quantitative marker for viable cells was tested by determining the relationship between target cell number and the amount of MTT formazan solubilized by isopropanol or DMSO. For bovine PBL, a linear relationship was observed between the absorbance of the dye and cell number of 1×10^3 to 8×10^3 cells/well. At low cell number (5×10^4 cells/well), the relationship
COLORIMETRIC ASSAY FOR CYTOTOXIC ACTIVITY

Fig. 1. MTT dye reduction as a function of cell number
Microplate wells were seeded with various number of bovine PBL in 50 μl of medium and 5 μl of MTT dye (5 mg/ml), and the plate was incubated at 37°C for 16 hr. The medium was discarded and 50 μl of DMSO (●) or acidified isopropanol (○) were added to each well and the OD_{490-620nm} was determined.

Fig. 2. MTT dye reduction as a function of cytolytic antibody concentration
TAA positive target cells were incubated with various dilutions of c143 ascites and rabbit complement at 37°C for 45 min. Following incubation, cytotoxicity of the antibody was determined by trypan blue dye exclusion method (CDAC ●) or MTT assay (MTT-CDAC ○) as described in materials and methods. The data represents mean CI of triplicate plates.

became nonlinear. Absorbance of the dye solubilized with DMSO always showed higher OD values than that of dye solubilized with isopropanol (Fig. 1). The rate of MTT dye reduction, determined after various lengths of incubation time (4 to 16 hr), was found to have the highest OD value at 16 hr incubation period (data not shown). Therefore, in the following MTT assay, we used 2×10^5 cells/well of bovine PBL, 16 hr incubation period of MTT dye and DMSO for solubilization of MTT formazan.

Serial two-fold dilutions of c143 ascites were tested to determine the antibody titers by cytotoxic assay using TAA positive, BLSC-KU1 cells as target cells (Fig. 2). Cytotoxicity as determined by trypan blue dye exclusion test and MTT assay showed a good correlation, and it decreased gradually when c143 was serially diluted. Since cytotoxicity of c143 was still observed at a dilution of 1:640 in both assays, a 1:300 dilution was used for CDAC and MTT-CDAC tests in later experiments.

Correlation of CDAC and MTT-CDAC tests: To know the relationship between CDAC and MTT-CDAC tests for the detection of TAA on bovine PBL, PBL from 29 BLV-negative cattle and from 17 cattle with EBL were tested. As shown in Fig. 3, all PBL from BLV-negative cattle were within the negative range, whereas all PBL from EBL cattle were within the positive range of the CI values by both tests, indicating a good correlation between both tests. The relationship of CI values by both tests was
examined using 49 samples which were positive by CDAC test and/or MTT-CDAC test. CI value of the CDAC assay was slightly higher than that of the MTT-CDAC assay and the correlation coefficient of CI values was 0.53 (Fig. 4).

A total of 108 samples, randomly collected from cattle was tested for the detection of TAA positive animals by CDAC and MTT-CDAC tests, and the results were compared (Table 1). Of the 108 samples, 44 were positive and 57 negative by both tests. A good agreement by both tests was observed in 101 (93.5%) out of 108 samples tested. A discrepancy was only seen in 7 samples, indicating a good correlation between the two tests.

We examined the presence of TAA in PBL from cattle with different stages of BLV infections (Table 2). TAA was positive in PBL from cattle with EBL by CDAC and MTT-CDAC tests and their CIs were 75.3±18.7 and 57.3±18.6, respectively. The c143 did not react with PBL from BLV-negative cattle. Most of the cattle (14 out of 15 tested) that showed lymphocytosis at the time of the test had TAA in their lymphocytes and the CIs of CDAC and MTT-CDAC were 54.7±13.9 and 43.8±8.7, respectively. Thirteen and 16 of 42 clinically normal cattle but positive for BLV infection showed positive reaction by MTT-CDAC and CDAC tests, respectively.
Table 2. Detection of the TAA in peripheral blood lymphocytes (PBL) from various stages of BLV infection by CDAC and MTT-CDAC tests

<table>
<thead>
<tr>
<th>PBL tested</th>
<th>CDAC test</th>
<th>MTT-CDAC test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. pos/tested</td>
<td>Range of CIa)</td>
</tr>
<tr>
<td>BLV-negative</td>
<td>0/33</td>
<td>Neg: 12.5± 8.3b)</td>
</tr>
<tr>
<td>Healthy cattle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BLV-positive</td>
<td>16/42</td>
<td>Pos: 49.0± 9.0</td>
</tr>
<tr>
<td>Healthy cattle</td>
<td></td>
<td>Neg: 17.8± 7.6</td>
</tr>
<tr>
<td>Lymphocytosis</td>
<td>14/15</td>
<td>Pos: 54.7±13.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Neg: 21.0</td>
</tr>
<tr>
<td>Leukemic</td>
<td>13/13</td>
<td>Pos: 75.3±18.7</td>
</tr>
</tbody>
</table>

a) Cytotoxic index.
b) Average CI±SD.

DISCUSSION

MTT is reduced by all living, metabolically active cells but not by dead cells or erythrocytes [5]. The amount of formazan generated was directly proportional to the number of bovine peripheral blood lymphocytes tested (Fig. 1). BLSC-KU1 cells, which is a TAA positive lymphoid cell line, when treated with serially diluted c143, showed an inverse relationship with c143 concentration. The amount of reduced MTT and cytotoxicity as expressed by CI gradually decreased with the dilutions of c143 (Fig. 2). Since MTT assay has a potential value for quantitative and rapid measurement of cell death, this assay can replace trypsin blue dye exclusion test for cytotoxicity. The main advantage of the colorimetric assay over the trypsin blue dye exclusion method is the rapidity of the test. This is especially so during the final stages of the assay, that is, reading of the plate takes much less time than counting viable cells microscopically in the trypsin blue dye exclusion method. Also, the data of MTT assay is more objective than the dye exclusion method. Comparative analysis of MTT-CDAC with CDAC indicated that both were equally sensitive as shown in Table 1. It is possible that MTT-CDAC might have failed in detecting the animals with CI value around the cut-off point which were shown to be positive by CDAC. This may account for the CI value in MTT-CDAC being a bit lower than that of CDAC (Table 2).

Monoclonal antibody used in the present study proved to be useful diagnostic tool for diagnosing EBL as suggested in the previous paper [6]. The TAA was detected by MTT-CDAC assay in PBL from 14 out of 15 cattle with persistent lymphocytosis or lymphocytosis at the time of the test, and 13 out of 42 clinically normal cattle but positive for BLV infection (Table 2). Lymphocytosis is characterized by an increase in the number of PBL, mainly B cells with or without unusual morphological features but absence of clinical signs. Most cattle with lymphocytosis showed TAA on their lymphocytes as reported in the previous [6] and present papers. In our preliminary experiment, we obtained an evidence that atypical lymphocytes in cattle with lymphocytosis as determined by Giemsa staining had TAA in their cytoplasm by immunofluorescence test. Therefore, cattle with persistent lymphocytosis or lymphocytosis which were TAA positive and had atypical lymphocytes in their PBL may be at the early stage of EBL and they have a high potential to develop tumors in future as compared to other
BLV-infected but TAA negative cattle. Until the present, we had obtained 4 cases which were TAA positive but with no clinical signs at the time of the test. However, all the four animals later developed tumors. We are following up TAA positive but clinically normal animals to see whether they will develop clinical signs of EBL. MTT-CDAC assay presented in this paper may be a useful technique for mass screening of BLV-infected herds in order to determine the cattle with potential to develop tumor in the future.

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REFERENCES