Topographical Analysis of Tumor-associated Antigens on Bovine Leukemia Virus-induced Bovine Lymphosarcoma

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ABSTRACT

Tumor-associated antigens (TAs) expressed on tumor cells from cattle with enzootic bovine leukemia were divided into three groups by using 13 monoclonal antibodies: common TAA; partially common TAA; and individually distinct TAA.

TAA was extracted from tumor cells and purified by ion exchange chromatography on diethylaminoethyl-cellulose and isoelectric focusing. The common TAA, which was detected on all tumors tested, was eluted with 0.6 M KCl by ion exchange chromatography on diethylaminoethyl-cellulose, and the isoelectric point of the antigen was 6.8. The partially common TAA, which was detected on some (but not all) of the tumors tested, was eluted with 0.4 to 0.8 M KCl, and the isoelectric points of the antigen were 5.3, 5.8, and 6.4. The individually distinct TAA was present in the fractions eluted with 0.4 to 0.8 M KCl, and the isoelectric point of the antigen was 5.5.

Results of competitive binding assay and Western blot analysis showed that the common TAA was a polypeptide with a molecular weight of 74,000; that it has at least two independent antigenic regions; that the partially common and individually distinct TAs were a polypeptide with a molecular weight of 64,000; and that the antigenic determinants on the common TAA, partially common TAA, and individually distinct TAA existed independently from each other.

INTRODUCTION

Antigenic analyses of compounds expressing various undefined distinct antigens have been difficult to achieve by using conventional immune serum. This difficulty is primarily due to the fact that conventional immune serum is a mixture of antibody populations directed against the various determinants of an antigenic molecule. Thus, conventional polyspecific antiserum delineate the overall antigenic molecule, rather than the individual epitopes. Therefore, polyspecific antiserum, in general, are unable to discriminate among small antigenic differences. Monoclonal antibodies circumvent these problems, because they present a single population of antibody-combining sites. For example, analyses of antigenic determinants of structural proteins of viruses and the relationship between antigenic determinants and biological activities have been studied in detail with monoclonal antibodies (3, 5, 8, 11, 16, 19). Moreover, specific monoclonal antibodies have been used to identify and characterize the molecular properties of various types of membrane antigens of human tumor cells (2, 4, 6, 9, 18, 20, 23, 24).

In the previous paper, we have obtained 13 monoclonal antibodies that identified TAs on tumor cells from cattle with EBL (1). From the reactivities of the monoclonal antibodies with individual EBL tumor cells, TAs may consist of common, partially common, and individually distinct TAs (1). In the present studies, we attempted to purify the TAs, analyze the topography of the antigenic determinants reacting with the monoclonal antibodies by using competitive binding assay, and identify the antigenic molecules by Western blotting.

MATERIALS AND METHODS

Purification of TAs from Tumors. The extraction of TAs by 0.2% DOC and partial purification by IEC were described previously (1). Tumor cells from cattle with EBL were solubilized and centrifuged at 105,000 g for 60 min, and the lower one-third of the supernatant was collected (Step 1). Ten ml of the concentrated supernatant were applied to a column of DEAE-cellulose, and the column was eluted stepwise with 0.1 to 1.0 M KCl. Fractions were dialyzed against PBS and examined for antigenic activity by the ELISA inhibition test with 11 monoclonal antibodies (c453, c444, c432, c164, c153, c143, 885, 903, 4134, 4366, and 311). Fractions which were eluted with 0.4 to 0.8 M KCl were found to be antigenic (Chart 1), pooled, and then concentrated to approximately 13 ml (Step 2). For competitive binding assay, further purification of TAA was performed by IF in a pH range of 3 to 10. Focusing was performed for 60 hr at 900 V using 1% ampholyte in a 110-ml column (LKB productor) (22). Fractions of 3 ml were collected, the pH was measured at room temperature, and the fractions were examined at A280. Each fraction was dialyzed against PBS before testing for antigenic activity. Antigenic activity was detected by the ELISA inhibition test with 11 monoclonal antibodies as described below (Chart 2, Step 3).

Standard ELISA. The titration of TAs against monoclonal antibodies was carried out by standard ELISA utilizing peroxidase-conjugated anti-mouse IgG. Serial 2-fold dilutions of purified TAs were adsorbed to individual wells at 37° for 3 hr. Excess antigen was removed, and the wells were postcoated with 50 µl of 1% BSA containing 0.75% glycine in PBS for 60 min at 37° to block nonspecific protein adsorption sites. Ascorbic fluid of predetermined concentrations was added to duplicate wells (50 µl/well) and incubated for 60 min at 37°. After 3 washes with 0.05% Tween 20 in PBS, 50 µl of a predetermined concentration (1:800) of peroxidase-conjugated IgG fraction of sheep anti-mouse IgG (Cappel Laboratories, West Chester, PA) were added to each well and incubated for 60 min at 37°. The wells were washed as described above before 0.2 ml 2.2-azino-di(3-ethylbenzthiazoline sulfonic acid) and 0.004% H2O2 in 0.05 M citrate buffer, pH 4.0, were added as substrates and were then incubated at 37° for 60 min. A492 was scored by Microelisa automatic reader (Flow Laboratories, Inc., McLean, VA).

ELISA Inhibition Test. For detection of antigenic activities against 11 monoclonal antibodies in fractions of IECD and IF, the ELISA inhibition test was performed for 60 min at 37°. The wells were washed as described above before 0.2 ml 2.2-azino-di(3-ethylbenzthiazoline sulfonic acid) and 0.004% H2O2 in 0.05 M citrate buffer, pH 4.0, were added as substrates and were then incubated at 37° for 60 min. A492 was scored by Microelisa automatic reader (Flow Laboratories, Inc., McLean, VA).
test was performed. Twenty-five \(\mu\)l of each fraction and 25 \(\mu\)l of each diluted ascitic fluid were mixed and incubated at 37° for 45 min. After incubation, residual antibody activity was detected by standard ELISA using the following antigens: Step 1 antigen (lower one-third of supernatant) for each fraction of IEC and Step 2 antigen (pooled 0.4 to 0.8 M KCl eluates) for each fraction of IF (Table 3). Parallel tests were done with antibodies using PBS in place of the fractions. Antibiotic activity was determined from the ability of the fractions to reduce the binding of mononuclear antibodies to Step 1 or Step 2 antigen in ELISA, when these antibodies were preincubated with each fraction before performance of the test.

**Isolation and Biotinylation of IgG Fraction of Monoclonal Antibody.** Mouse IgG monoclonal antibody from ascitic fluid was fractionated by precipitation with 50% ammonium sulfate, followed by IEC (14). Biotinylation of the IgG fraction was performed as described previously (1).

**Determination of Binding Activity of Monoclonal Antibodies.** Previous experiments showed that the binding activity of antibodies of Groups 2 and 3 against the 0.6 M KCl eluate of IEC was lower than that of Group 1 antibodies (1). Therefore, the following antigens were used for the determination of binding activity of the antibodies: the 0.6 M KCl eluate or Fraction 25 of IF for Group 1 antibodies and different fractions of IF that showed the highest antigenic activities against individual antibodies for antibodies of Groups 2 and 3. For example, Fractions 18 for clone 885, 19 for clone 311, 20 for clones 4134 and 4366, and 23 for clone 903 were used as antigens. Serial 2- or 3-fold dilutions of IgGs were tested by standard ELISA with a predetermined concentration of the antigens.

**Competitive Binding Assay.** The competitive binding assay was performed by Lab-ELISA as described previously (1). The antigen used for each antibody was the same antigen used for the determination of binding activity of the particular antibody. Fifty \(\mu\)l of purified TAA diluted with 0.05 M phosphate buffer (pH 8.0) containing 0.5 M NaCl were adsorbed to individual wells at 37° for 3 hr. The plate was washed 3 times with 0.05% Tween 20 in 0.05 M phosphate buffer containing 0.5 M NaCl. Serial 10-fold dilutions for each competing cold IgG, which was about 10-fold in excess of biotin-labeled IgG, were added to the TAA-adsorbed wells. Growth medium of P3X63Ag8.653 was used as control. After incubation at 37° for 2 hr, the wells were washed as described above, and biotin-labeled IgG diluted to 1:200 was added. After incubation, peroxidase-labeled avidin (1:800 dilution) and substrate were added sequentially as described before. The amount of competitive binding was estimated from \(A_0\) in the presence of antigen or absence of unlabeled competing antibodies.

\[
\% \text{ of competition} = \frac{100 (A - N)}{A - B}
\]

where \(A\) is \(A_0\) in the absence of homologous antibody, \(B\) is \(A_0\) in the presence of homologous antibody, and \(N\) is \(A_0\) in the presence of competitor as described previously (8).

**Western Blotting.** The concentrated lower one-third of the supernatant fluid (Step 1 antigen) and molecular weight standards were electrophoresed on a 10% polyacrylamide gel in the presence of sodium dodecyl sulfate and 2-mercaptoethanol (10, 13). The resulting protein bands were electroblotted onto nitrocellulose as described (21). Transferred proteins were detected either by staining the nitrocellulose sheet with Coomassie blue or by immunological reaction. For immunological detection, a portion of the sheet was blocked with 3% BSA containing 0.75% glycine and reacted with mouse ascitic fluid containing either monoclonal antibody or control IgG. Specific binding of monoclonal antibody was detected using peroxidase-conjugated goat anti-mouse IgG (Fab')2 fragment (Zymed Laboratories, Inc., South San Francisco, CA) by ELISA.

**Protein Estimation.** The amount of protein was estimated by the method of Lowry et al. (12).

**RESULTS**

**Purification of TAA from EBL Tumor.** The TAAs were extracted from EBL tumor cells and purified by IEC and IF. When the Step 1 antigen was subjected to IEC and eluted with KCl (Chart 1A), antigenic activity against c453 (Chart 1B) and other monoclonal antibodies from Group 1 were found in the shoulder of the 0.6 M KCl elution. On the other hand, antigenic activities against antibodies of Groups 2 and 3 were found in the 0.4 to 0.8 M KCl eluates (Chart 1, C to G). The specific activity of the pooled fractions (0.4 to 0.8 M KCl eluates by IEC, Step 2) against c453 increased by 56.1-fold relative to the starting material (Step 1) (Table 1). When the pooled fractions were subjected to IF (Chart 2A), antigenic activity against Group 1 antibodies was found in fractions (Nos. 18 to 29) in which the isoelectric points ranged from 5.3 to 7.9 (Chart 2B). Antigenic activity against antibodies of Groups 2 and 3 was found in

**Table 1**

<table>
<thead>
<tr>
<th>Steps</th>
<th>Total volume (ml)</th>
<th>Protein (mg/ml)</th>
<th>Total protein (mg)</th>
<th>Titer (ELISA)</th>
<th>Purification (titre/protein)</th>
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</thead>
<tbody>
<tr>
<td>1. DOC-extracted tumor ultracentrifugation (lower 1/3)</td>
<td>10.0</td>
<td>12.1</td>
<td>121.0</td>
<td>32</td>
<td>1</td>
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<tr>
<td>2. DEAE-cellulose column chromatography (KCl eluates)</td>
<td>13.5</td>
<td>3.2</td>
<td>43.2</td>
<td>640</td>
<td>56.1</td>
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<td>3. IF (pH 3-10)</td>
<td>3.0</td>
<td>2.2</td>
<td>6.6</td>
<td>10,240</td>
<td>5,877.0</td>
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</table>

* Purification = \(\frac{\text{titer}}{\text{total protein}} \times \frac{32}{121}\)
fractions (Nos. 15 to 24) in which the isoelectric points ranged from 4.7 to 6.6 (Chart 2, C to G). The isoelectric points of antigens that were reactive with individual antibodies were 6.8 (clone c453 of Group 1), 5.3 (clone 885), 6.4 (clone 903), 5.5 (clone 311), and 5.9 (clones 4134 and 4366) (Table 3; Chart 2). The specific activity of the fraction (No. 25) against c453 increased by 5800-fold relative to the Step 1 antigen (Table 1). With purification, the specific activities of clone 903 against antigens of Steps 2 and 3 increased by 900-fold and 3000-fold, respectively, relative to the Step 1 antigen (data not shown).

Binding Activity of Monoclonal Antibodies against the Common TAA. The binding activities of Group 1 antibodies recognizing the common TAA (1) were compared in antibody-binding assay using the 0.6 M KCl eluate as antigen (Chart 3). Serial 2-fold dilutions of IgGs from the 6 antibodies were tested by ELISA using a 1-ng/ml concentration of the antigen. The results showed that, although these antibodies reacted with the antigen, the binding activities differ slightly. The relative activities of these antibodies were as follows: high-avidity (c153); middle-avidity (c432 < c164 < c444 < c453), and low-avidity (c143) antibodies.

Topographical Analysis of the Common TAA Determinants. Competitive binding assay was performed to analyze the topography of the common TAA epitope to which the monoclonal antibodies reacted. Competition of antibody binding was based on the premise that, if 2 antigenic sites are very close or overlapping, then binding of an antibody to one of the epitopes would hinder the binding of another antibody to the neighboring epitope. Also, if the competitor is directed against the same epitope, then the competitor will completely block the binding of the conjugated antibody. To minimize any effects due to differences in avidity, competitors were prepared by serial 10-fold dilutions and incubated in the wells of microplates before adding the biotin-labeled antibody. All combinations of the 6 monoclonal antibodies that recognized the common TAA were tested with the 0.6 M KCl eluate (Chart 4, Table 2). Biotin-labeled c444 was blocked completely by the homologous antibody and c164 but not by the others. Biotin-labeled c164 was completely blocked by the homologous antibody and partially blocked by c444 (69% inhibition), c432 (66% inhibition), and c143 (78% inhibition). Biotin-labeled c143 was completely blocked by the homologous antibody as well as c444 and c432. Biotin-labeled c432 was completely blocked only by the homologous antibody and partially by c444 (54% inhibition) and c143 (74% inhibition). Biotin-labeled c153 was blocked completely by the homologous antibody and c143 and partially by c444 (88% inhibition). Biotin-labeled c453 was blocked only by the homologous antibody. The results of competitive assay showed the existence of at least 2 distinct antigenic regions on the common TAA molecule: one which is recognized only by c453 and another which is recognized by the rest of the Group 1 antibodies.

Table 2

<table>
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<th>Competitor</th>
<th>c453</th>
<th>c432</th>
<th>c164</th>
<th>c153</th>
<th>c444</th>
<th>c143</th>
<th>885</th>
<th>903</th>
<th>4134</th>
<th>4366</th>
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*a*: +, positive inhibition (>50%); -, negative inhibition (<50%).

CANCER RESEARCH VOL. 45 MARCH 1985
Binding Activity of Monoclonal Antibodies against Partially Common and Individually Distinct TAA. Binding activities of the 5 monoclonal antibodies (885, 903, 4134, 4366, and 311) that reacted with the partially common or individually distinct TAA were compared in antibody-binding assay using the different fractions of IF that showed the highest antigenic activity against the individual antibodies. The fractions used were Fractions 18 (pH 5.3), 19 (pH 5.5), 20 (pH 5.9), 23 (pH 6.4), and 25 (pH 6.8) (Chart 2). The 6 monoclonal antibodies that recognized the common TAA (Group 1 antibodies) were also tested for binding with the antigens. Serial 3-fold dilutions of IgGs were tested by ELISA with a single antigen preparation (Chart 5). The results showed that, although all of the IgGs tested reacted with each fraction, IgGs from 885, 4134, 4366, and 311 had low avidity for each fraction, since plateau values could not be reached, even when a 40:1 ratio of antibodies to antigen was used. IgGs from Group 1 antibodies and 903 showed relatively high avidities for the 5 different fractions.

Topographical Analysis of Common, Partially Common, and Individually Distinct TAA. Since antibodies of Groups 2 and 3 showed different binding activities against purified TAA in different fractions of IF (Chart 5), different fractions were used as antigens for each of the biotin-labeled antibodies in competition assay. Epitope specificities of the 5 antibodies from Groups 2 and 3 were compared. Biotin-labeled 885, 903, 4134, 4366, and 311 were blocked only by homologous antibody and not by the other competitors from Groups 2 and 3. These results indicate that the epitope recognized by each of these monoclonal antibodies exists independently (Table 2). Epitope specificities of all 11 antibodies were compared to elucidate the topography of the common, partially common, and individually distinct TAAs. As shown in Table 2, 6 biotin-labeled monoclonal antibodies from Group 1 were blocked by some competitors from Group 1 (see Chart 4), but not by the competitors from Groups 2 and 3. Five biotin-labeled antibodies from Groups 2 and 3 were blocked completely by homologous antibodies but not by antibodies from Group 1. This result indicates that the antigenic regions of the partially common and the individually distinct TAA exist independently from the antigenic region of the common TAA.

Detection of TAA by Western Blotting. From the results of purification procedures (Charts 1 and 2) and competitive binding assay (Table 2), the common, partially common, and individually distinct TAAs were thought to exist on different molecules. Western blotting was performed to identify the molecule that reacted with each monoclonal antibody (Table 3). All of the
antibodies from Group 1 reacted with an M, 74,000 polypeptide. However, all of the antibodies from Groups 2 and 3 reacted with an M, 64,000 polypeptide (Fig. 1).

DISCUSSION

Previously, we described 13 monoclonal antibodies against tumor cells from cattle with EBL that identified common, partially common, and individually distinct TAAs (1). The TAAs were solubilized from tumor cells with DOC and partially purified by IEC. Although the monoclonal antibodies from Group 1 reacted strongly with the partially purified TAA (0.8 m KCl eluate of IEC), the antibodies from Groups 2 and 3 showed a lesser reaction. We thought that this might have been due to the lack of purity of the antigen. Thus, we attempted further purification of TAAs by IF. During the purification procedure, the ELISA titer and specific activities of antigens of Steps 2 and 3 against c453 increased remarkably as compared with those of the starting material (Step 1 antigen) (Table 1). The ELISA titer and specific activities of antigens of Steps 2 and 3 against 903 also increased remarkably. The increase of ELISA titer during the purification procedures may be due to elimination of extraneous substances that interfere with the ELISA reaction of TAA.

Antigenic activities against antibodies of Groups 1 to 3 were found in different fractions of IF (Chart 2). Antibodies from Groups 2 and 3 except 903 showed low avidity, even when an adequate amount of antigen and antibody was used (Chart 5; Table 3). The exact reason for this is not known yet. However, different interpretations are possible: (a) some antigenic sites could be indeed poorly antigenic; (b) the TAA sites recognized by antibodies of Groups 2 and 3 could be partially hidden on the plastic-adsorbed TAA; or (c) the affinity of the respective hybridoma antibodies for the antigenic determinant could be low. In a preliminary experiment, we found that even antibodies of Groups 2 and 3 which showed low avidity to the solubilized TAA had the same binding activity to intact tumor cells by cell ELISA binding assay as that of Group 1 antibodies. Therefore, the low avidity of antibodies of Groups 2 and 3 may be due to the second possibility and not to the first or the third possibility.

When the solubilized TAA was subjected to IF, the highest antigenic activity against Group 1 antibodies was found in Fraction 25 with an isoelectric point of 6.8, whereas the highest antigenic activities against antibodies of Groups 2 and 3 were found in fractions other than Fraction 25. The isoelectric points of antigens reactive with individual antibodies from Groups 2 and 3 were 5.3 for clone 885, 5.5 for clone 311, 5.9 for clones 4134 and 4366, and 6.4 for clone 903 (Table 3; Chart 2). The differences of isoelectric points of antigens reactive with the individual antibodies may indicate that the common, partially common, and individually distinct TAAs are located in different molecules.

In order to identify the antigenic molecule, we performed Western blot analysis. The common TAA with an isoelectric point of 6.8 showed a M, 74,000 polypeptide, whereas the partially common and individually distinct TAA whose isoelectric points ranged from 5.3 to 6.4 showed a M, 64,000 polypeptide. These results indicate that the common TAA may exist as an independent molecule distinct from the partially common and individually distinct TAAs. In the case of the competitive binding assay using Group 1 antibodies, partial blocking was observed by heterologous antibodies. This partial competition may be due to a structural overlapping of determinants or a conformational change induced by binding of the competing antibody. On the basis of the results, determinants of clones c444, c432, c164, c153, and c143 are considered to be very close or overlapping, and these determinants exist on the same antigenic region. In contrast, antigenic determinants of clone c453 exist independently, because no competition was observed by heterologous competitors. When epitope specificities of the 5 antibodies from Groups 2 and 3 were compared, each antigenic determinant was found to exist independently. Furthermore, antigenic determinants of the partially common and individually distinct TAAs exist independently from the antigenic determinants of the common TAA. The results of competitive binding assay and Western blot analysis suggest that the common TAA is an M, 74,000 polypeptide and exists in at least 2 independent antigenic regions. Although the partially common and individually distinct TAAs were an M, 64,000 polypeptide, whether the epitopes are expressed on the same or different M, 64,000 molecule is not known. Even if the partially common and individually distinct TAAs existed on the same molecule with a molecular weight of 64,000, the antigenic determinants of the individual antibodies might exist at different regions. Further topographical analysis by using intact cell ELISA competitive binding assay and characterization of the chemical nature of TAAs are in progress.

By using monoclonal antibodies, many TAAs were identified in cells of human cancers (2, 4, 6, 9, 18, 20, 23, 24). Human melanomas expressed cell surface-localized tumor antigens, some of limited distribution and some shared by many different melanomas identified by sera from melanoma patients (9, 17, 23). Dippod et al. (4) reported that 6 distinguishable systems of melanoma surface antigens were identified by using 18 mono-

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* Y. Aida, M. Onuma, and H. Izawa, unpublished data.
clonal antibodies. Two of these antigenic systems are glycoproteins, and others have properties of a glycolipid. A monoclonal antibody can show a high degree of restriction to melanoma, while others can react to epithelial cancers and normal cells as well as melanomas. In the present experiment, we did not obtain monoclonal antibodies which reacted with normal bovine cells as well as EBL cells, because of the elimination of these antibodies in the initial screening (1). The M, 74,000 antigen which exists in EBL cells should be a good surface marker of EBL. Since this antigen is expressed in EBL cells in vivo, while the expression of BLV genomes is suppressed in vivo (7), the TAA may be coded by cellular genes but not by the BLV genome. However, since BLV has a pX region in the genome (15), the possibility still exists that TAA is a pX product of BLV. In consideration of leukemogenesis or maintenance of tumor phenotype, the expression of the TAA should play an important role in the maintenance of tumor phenotypes.

REFERENCES


Table 3

<table>
<thead>
<tr>
<th>Clones</th>
<th>Classification of TAAs</th>
<th>DEAE-cellulose column chromatography</th>
<th>Molecular wt (× 103)</th>
<th>Binding activity on purified TAAa</th>
<th>Site</th>
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<tr>
<td>c453</td>
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<td>0.6 M KCl 6.8</td>
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<td>0.4–0.8 M KCl 5.5</td>
<td>65</td>
<td>Low</td>
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</table>

a Binding activity was expressed as high or low when the specific absorbance at the plateau level was > or < 1.0 in ELISA, respectively.
Topographical Analysis of Tumor-associated Antigens on Bovine Leukemia Virus-induced Bovine Lymphosarcoma

Yoko Aida, Misao Onuma, Takeshi Mikami, et al.


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