Human Antibodies Binding to the Mouse Mammary Tumor Virus: A Nonspecific Reaction?

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Introduction

MTV\(^3\) shares a number of biophysical, biochemical, and morphological characteristics with virus-like particles found in human milk and human breast cancer (8, 12–16). Recently, a number of reports have appeared that suggest that an immunological relationship between MTV and the putative human particle also exists (5, 6, 9–11). Charney and Moore (5) observed that sera from breast cancer patients apparently neutralize MTV. Müller and Grossmann (11) have observed antigens in human sera that react with antisera against MTV. Hoshino and Dmochowski (6), using human immunoglobulin tagged with horseradish peroxidase, demonstrated that absorbed human sera reacted with the envelope of B-particles. Müller et al. (10), using immunoferritin, demonstrated a reaction between absorbed human sera and intracytoplasmic A particles. One can infer from these reports that an immunological relationship between MTV and the putative human breast cancer agent exists. It is important, therefore, to verify previous observations and to examine the nature of the reactions involved.

Recently, we developed a rapid, accurate method of studying antibody-antigen reactions in the MTV system using radioimmune precipitation (3). In this technique, antibodies are caused to react with the surface antigens of intact iodinated virions, and the resulting antibody-antigen complexes are then precipitated with antoglobulin. The specificity of the precipitation reaction can be analyzed using competition by unlabeled inhibitors.

Preliminary experiments indicated that not only sera from rabbits immunized against MTV but also sera from rabbits immunized with MTV-free mouse tissues could precipitate MTV (3). We now report that human antisera also precipitate MTV-1-MTV. The specificity of each of these 3 reactions was examined with competitive inhibitors, and each was found to differ from the other 2.

Materials and Methods

Antigens. MTV purified from supernatants of primary BALB/cfC3H mouse mammary tumor cell cultures was iodinated using the lactoperoxidase technique (3, 4). Following dialysis, the iodinated MTV was subjected to isopyknic centrifugation, and \(^{125}\)-MTV banding at the 1.17-g/ml region was collected and used for radioimmune precipitation. Ninety \% of the \(^{125}\)I from the 1.17-g/ml density region migrated as a single peak in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (3, 17). This peak corresponds to the 52,000-dalton polypeptide which is the major envelope glycoprotein (3, 4, 17). No murine leukemia virus gs antigens were detectable by immunodiffusion assay (4, 17), and anti-AKR sera did not cause a radioimmune precipitation of MTV (3). MTV purified from the same tissue culture source was used as an unlabeled competitive inhibitor. The unlabeled MTV was also devoid of detectable murine leukemia virus gs antigen (4, 17).

BALB/c/Crgl LMG, liver, spleen, and kidney were extracted by homogenization and differential centrifugation as previously described (1, 3). Tissues from BALB/c/Crg1 mice from Cancer Research Laboratory, University of California, Berkeley, Calif., have been reported to be free from detectable B-particles (1, 2). Our assay systems have confirmed these observations using electron microscopy, immunodiffusion, radioimmune precipitation inhibition, and reverse transcriptase.

Dog milk was kindly donated by Dr. L. J. Faulkin, University of California at Davis, Calif., from his beagle colony. We have been unable to detect C-type gs antigen or MTV antigens in this milk by immunodiffusion assay. Electron mi-
croscopy and reverse transcriptase assays have also been negative (L. J. Faulkin, unpublished observations).

**Sera.** Sera were obtained from 3 sources. Control antisera were obtained from a rabbit immunized with sucrose gradient-purified MTV (anti-MTV) obtained from BALB/cfC3H milk (1) and from a rabbit immunized with an extract of BALB/c/Crgl LMG (anti-LMG) (1, 2). The specificity of these antisera has been previously characterized (3). Experimental sera tested by radioimmune precipitation were obtained from 162 humans in 4 categories: breast biopsy patients, breast carcinoma patients, normal females, and normal males. Only 111 of these sera were available for inhibition studies. The patients' sera were obtained through Sutter Memorial Hospital, Sacramento, Calif., from sera discarded after type- and cross-matching for surgery. The normal sera were obtained fresh from healthy human volunteers. Antiglobulins were obtained from a commercial source (Antibodies, Inc., Davis, Calif.); they were tested by immunoelectrophoresis and found to react against IgG and IgM.

**Radioimmune Precipitation.** The control or experimental sera were caused to react with surface antigens of intact 125I-MTV, and the resulting complexes were precipitated with antiglobulin (3). The specificity of the reactions was examined using competitive inhibitors (3, 4).

In competitive inhibition studies, rabbit anti-MTV was used at a dilution of 1:200,000, rabbit anti-LMG at 1:700, and human serum at 1:100. In each test 50 \( \mu l \) of serial dilutions of each inhibitor were added to duplicate tubes of 50 \( \mu l \) of the primary antiserum, and the mixture was incubated at 37°C. After 2 hr 45 ng of 125I-MTV in 50 \( \mu l \) of radioimmune precipitation buffer (0.14 M NaCl-0.1 M Tris-HCl, pH 7.4) and 1 mg bovine serum albumin per ml were added, and the mixture was incubated at 37°C. After 2 hr, 50 \( \mu l \) of antiglobulin were added and the mixture was incubated at 37°C. After 1 hr, the tubes were placed at 4°C for 12 to 18 hr. The tubes were then centrifuged for 1 min at 15,000 \( \times g \); 100 \( \mu l \) were collected and counted in a y counter as the supernatant fraction, and the remaining 100 \( \mu l \) were counted as the pellet fraction (3). The inhibited sample was determined as a percentage of the uninhibited control sample. Other controls included: (a) buffer without antiserum; (b) antiglobulin without test sera; (c) test sera without antiglobulin; (d) precipitation control; and (e) physical precipitate control. The precipitation control consisted of test sera and anti-test immunoglobulin to judge completeness of the precipitation by observation of the flocculent precipitate and supernatant. The physical precipitate control consisted of the addition of test sera and anti-test immunoglobulin before the addition of MTV; this prevented the test serum from precipitating MTV and was used to indicate the degree of precipitation caused by physical entrapment of MTV. Precipitation by physical entrapment was generally below 10%. Repeated tests with the anti-MTV revealed a standard deviation of ±3.5% for a single dilution point.

**Results**

**Anti-MTV.** The rabbit anti-MTV antiserum precipitated 95% of the 125I-MTV in the presence of goat anti-rabbit immunoglobulin (3). A 50% end point of 1:200,000 was obtained when the antiserum was diluted in normal rabbit globulin (3). The specificity of this reaction was examined by competitive inhibition. The radioimmune precipitation of 125I-MTV by anti-MTV was inhibited by the addition of unlabeled MTV but not by the addition of mouse LMG, mouse spleen, skimmed dog milk (Chart 1A), or other unrelated antigens (3).

**Anti-LMG.** The 2nd antisera examined was a rabbit antiserum against BALB/c/Crgl LMG extract (anti-LMG) (3). The BALB/c/Crgl is a strain of mouse that does not ordinarily express MTV and was used as a “virus-free” control (1–3, 18). Serial dilutions of the anti-LMG gave a maximum radioimmune precipitation of 49% at a 1:100 dilution and a 50% end point at 1:700 (3). In contrast to the anti-MTV, this reaction at 1:700 dilution was inhibited by extracts of BALB/c/Crgl LMG and spleen as well as MTV (Chart 1B). Dog milk did not inhibit this reaction (Chart 1B).

**Human Sera.** The 3rd source of antisera was human sera. A direct radioimmune precipitation system for human IgG was also developed. The technique was similar to that used to precipitate the rabbit antibodies. Human sera were diluted in buffer and precipitated with goat anti-human globulin.

**Chart 1.** The effects of the addition of unlabeled MTV (●), “virus-free” BALB/c/Crgl LMG extract (○), “virus-free” BALB/c/Crgl spleen extract (△), and dog milk (△) to the radioimmune precipitation of 125I-MTV in the presence of (A) rabbit anti-MTV at 1:200,000, (B) rabbit anti-LMG at 1:700, and (C) human serum Case 20 at 1:100. The percentage of radioimmune precipitation was normalized to express the percentage of the control sample.
Antibody titration by serial dilution, however, could not be done because: (a) dilutions of human sera below 1:100 resulted in nonspecific physical entrapment of \(^{125}\text{I}-\text{MTV}\), as judged by Control 5; (b) dilutions of human sera beyond 1:100 in buffer were in antiglobulin excess and did not form precipitates; and (c) the presence of anti-MTV reactivity in most, if not all, human sera prevented the use of human sera as a nonreactive diluent (3). The human sera were, therefore, used at a 1:100 dilution. This dilution resulted in radioimmune precipitation, but the resulting precipitate did not physically trap the virion.

Using the 1:100 dilution, 160 of 162 human sera precipitated MTV with a range from 10 to 80% (Chart 2). All 4 groups had similar means and ranges (Chart 2). The radioimmune precipitation of \(^{125}\text{I}-\text{MTV}\) indicates that human immunoglobulins can bind to the virion surface. No correlation, however, could be found between MTV reactivity, age, sex, blood group, or health status.

The specificity of human MTV reactions was examined by competitive inhibition. In contrast to the rabbit anti-MTV and anti-LMG, the radioimmune precipitation of MTV by human sera could be inhibited by dog milk (Chart 1C). Mouse mammary gland extracts, spleen extracts, and MTV also inhibited the reaction.

One hundred eleven of the human sera were available for competitive inhibition studies using a single concentration of BALB/c/Crgl LMG extract (22 \(\mu\text{g}\)) or dog milk (450 \(\mu\text{g}\)). The radioimmune precipitation of MTV by each human serum tested was inhibited by at least 30% with either mouse LMG extract or dog milk (Chart 3). The majority of sera was inhibited by both. No differences in the patterns of inhibition were observed among the 4 groups of human sera.

A more comprehensive search for MTV-specific human antibodies has been carried out with competition curves using serial dilutions of dog milk looking for terminal plateaus that would indicate more specific antibodies. Thus far, 30 human sera have been examined. End points have not yet been reached with all of the human sera. There has, however, been no evidence of plateaus at high inhibitor concentrations (Chart 4).

**Discussion**

Three types of antisera, rabbit anti-MTV, rabbit anti-LMG, and human sera, were shown to precipitate radiolabeled MTV. The specificity of each differed from the other 2, thus establishing 3 classes of reactions: (a) radioimmune precipitation reactions inhibited only by the addition of MTV (anti-
MTV); (b) radioimmune precipitation reactions inhibited by the
addition of either MTV or extracts of BALB/c/Crg1 tissue
but not dog milk (anti-LMG); (c) radioimmune precipitation
reactions inhibited by dog milk (most human sera).

We have observed the Class 1 reaction only with antibod-
ies from animals immunized with MTV or MTV polypeptides
(3, 4). Class 1 has been the only reaction that could be
considered to be directed against antigens coded by the
MTV genome.

The Class 2 reaction has been observed with antibodies
against LMG from "virus-free" strains of mice (3). Antibod-
ies against I and C57BL LMG also show a Class 2 reaction
(R. D. Cardiff and P. B. Blair, unpublished observations). The Class 2 reaction was inhibited by a variety of mouse cell
extracts and MTV. It was not, however, inhibited by dog
milk. Class 2 reactions appeared to identify antigenic deter-
minants that the virion surface shares with the mouse cell.
Host cell determinants on virions have been described in
other oncornavirus systems (7).

The Class 3 reaction was inhibited by dog milk as well as
MTV and extracts of mouse spleen or LMG. The Class 3
reaction was found exclusively in the human sera. All of the
human sera thoroughly studied thus far fall into Class 3.
Because the reaction was inhibited by nonvirion, nonspe-

cies antigens, it was the least specific of the 3 classes and
could not be considered to involve MTV-specific determi-
nants.

The Class 3 reaction is critically important because it does
involve human sera. Failure to recognize such a class of
antibodies could lead to misinterpretation of human serum
reactivity with MTV. The specificity of such reactions can
obviously be determined only by exhaustive absorption or
competitive inhibition such as done here.

Our attempts to detect Class 1 specificities in human sera
have thus far been negative. These results do not eliminate
the possibility that some Class 1 antibodies against MTV
envelope or nonenvelope antigens do exist in human sera.
Nevertheless, our results demonstrate the caution that must
be used in interpreting any antibody-viral interaction in the
absence of stringent criteria for specificity.

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