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Brief Communication

Rosetting of Antibody-sensitized Tumor Cells with Anti-Immunoglobulin Antibody-coated Erythrocytes by a New Method for Detecting Antigens on Cells[1]

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SUMMARY

Tumor cells were treated with rabbit antibody to tumor-associated cell surface antigens and tested with erythrocytes coated with antibody specific for the sensitizing rabbit immunoglobulin. The sensitized tumor cells formed rosettes with the indicator cells. By this method, we confirmed that line 1 and line 10 hepatoma cells (from two tumors independently induced by diethylnitrosamine in strain 2 guinea pigs) bear antigens not present on normal liver cells. We also confirmed that line 1 and line 10 cells bear antigenically different tumor-associated cell surface antigens. This method appears simpler than other serological methods for detecting tumor-associated cell surface antigens on tumor cells. Also, this method may be a general one for detecting and enumerating cells bearing surface antigens.

INTRODUCTION

Experimental tumors induced by oncogenic viruses or chemical carcinogens may have tumor-specific antigens. These antigens have been demonstrated by *in vivo* methods such as specific rejection of a tumor or cutaneous delayed hypersensitivity to a tumor by tumor-sensitized animals. The *in vivo* methods, however, are time consuming and are not quantitative. Tumor-specific antigens have also been demonstrated by *in vitro* serological methods, namely, cytotoxicity, complement fixation, immunodiffusion, immunofluorescence, immuno-electron microscopy, and mixed antiglobulin reaction (10). These methods have detected some tumor-specific antigens that are not detectable by the transplantation rejection method, such as the T-antigen of DNA virus-induced tumors or the TL-antigens of the mouse leukemias. *In vitro* methods for detecting TSA[5] have obvious advantages over *in vivo* methods. Here we describe a new serological method, exploiting the avid reactivity of Ab-E (6, 7). TSA-bearing cells were sensitized with rabbit antibody to TSA and rosetted with Ab-E specific for the sensitizing rabbit Ig.

MATERIALS AND METHODS

Tumor Cell Lines. Line 1 and line 10 ascitic variants of diethylnitrosamine-induced hepatomas in strain 2 Sewell-Wright inbred guinea pigs were originally obtained from Dr. Berton Zbar at the National Cancer Institute. These tumors were maintained in our laboratory by serial i.p. transplantation into strain 2 inbred guinea pigs. The tumor cells used were obtained from frozen stocks after thawing or from fresh ascites fluids of tumor-bearing animals. Line 1 cells were from transplantation passages 43 to 55 or 99, and line 10 cells were from passages 13 or 99 to 110.

Tumor Cell Suspensions. The ascitic fluids were aspirated from guinea pigs with palpable ascites. A 1:3 dilution of the ascites was made in HBSS. The cell suspension was centrifuged at 250 × *g* at 4° for 10 min. Contaminating RBC in the cell suspension were lysed by hemolytic shock with distilled water. The cells were then washed twice with HBSS. The final pellet was resuspended in about 1 ml of HBSS. A single-cell suspension was obtained by passing the cells through a syringe fitted with needles of decreasing diameters. The cells were then filtered through glass wool.

Liver and Spleen Cell Suspensions. The liver cell suspensions were prepared as described in another paper (G. A. Molinaro, E. Maron, W. C. Eby, and S. Dray. A General Method for Enumerating Single Cells Secreting Antigen: Albumin-secreting Hepatocytes Detected as Plaque-forming Cells, submitted for publication) and the spleen cell suspensions were prepared as described previously (8), except that the cells were washed with HBSS.

[1] A preliminary report of this work was published (9).

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[5] The abbreviations used are: TSA, tumor-associated cell surface antigen(s); Ab-E, erythrocytes coated with antibody; line 1 and line 10 cells, transplantable ascites variants of hepatomas separately induced by diethylnitrosamine in strain 2 guinea pigs; HBSS, Hanks' balanced salt solution, containing NaN_3 (0.2 mg/ml) and bovine serum albumin (1 mg/ml); (anti-Ig)Ab-E, erythrocytes artificially coated with purified antibody specific for the allotypic specificities of κ-light chain (*b* locus) of rabbit immunoglobulin.

Antisera. Adult rabbits (homozygous at the *b* locus of the κ light chain of Ig) were injected with line 1 or line 10 tumor cells (3×10^6 cells in 1.0 ml 0.15 M NaCl) i.v. every 3 weeks. The animals were bled 1 week after the 3rd injection. Aliquots of antisera were adsorbed first with spleen cells and then with liver cells from normal guinea pigs. The anti-line 10 antiserum was also adsorbed with line 1 cells. In each adsorption, the antitumor antiserum was mixed with an equal volume of packed adsorbing cells and incubated with gentle shaking at 4° for at least 60 min. The antiserum was recovered by centrifugation.

Coating of Erythrocytes with Purified (Anti-b4 or Anti-b5) Anti-Ig Antibodies. The anti-b4 or anti-b5 antibodies were purified by acidic elution from immunosorbents as described previously (8). Sheep erythrocytes were coated with purified anti-b4 or anti-b5 antibodies by the chromium chloride method as described in another paper (G. A. Molinaro, E. Maron, W. C. Eby, and S. Dray. A General Method for Enumerating Single Cells Secreting Antigen: Albumin-secreting Hepatocytes Detected as Plaque-forming Cells, submitted for publication).

Rosetting of Antibody-sensitized Cells with (Anti-Ig)-Ab-E. The washed tumor cell suspensions were adjusted to a concentration of 3×10^6 cells/ml. One-tenth ml of the cell suspensions (3×10^5 cells) was incubated with 0.1 ml of serial dilutions of antitumor antiserum in 12- x 75-mm disposable culture tubes on a shaker at room temperature for 60 min. Following incubation, the sensitized cells were washed 4 times with HBSS and resuspended in approximately 0.1 ml of HBSS. Next, 0.1 ml of the (anti-Ig)Ab-E suspension (3×10^7 cells) was added to the sensitized cell suspension. The tubes were shaken and then spun in a centrifuge (serofuge) at room temperature for 2 min. The pellet was resuspended, and 0.1 ml of toluidine blue solution (0.1 g/100 ml of HBSS) was added. One drop of the suspension on a slide was examined under a light microscope at ×800 for rosetted and unrosetted cells. A cell was considered rosetted when firmly surrounded by at least 5 Ab-E's. Three hundred consecutive cells were scored.

Soluble Tumor Surface Antigens. Soluble tumor surface antigen was extracted from line 10 cells according to the method of Reisfeld *et al.* (11) as modified by Meltzer *et al.* (4). The preparation was then dialyzed against HBSS.

RESULTS AND DISCUSSION

Cell Surface Antigens. Line 10 cells, treated with serial dilutions of unadsorbed rabbit antiserum to line 10 cells, were tested with erythrocytes coated with antibody against rabbit Ig [(anti-Ig)Ab-E] for rosette formation. Antiserum-treated cells rosetted whereas normal serum-treated cells did not (Table 1, Lines 1 and 2). Microscopically, the rosetted cells appeared as single cells surrounded by a complete row of (anti-Ig)Ab-E (Fig. 1*A*). This ring of indicator cells was resistant to mechanical disruption. Mixing of the cell suspension by pipet or vortex, tapping of the coverslip on the microscope slide, or centrifugation of the suspensions on a microscope slide by cytocentrifuge did not break the rosettes. Overnight storage of the preparations did not lower the percentage of rosetted cells. The percentage of rosetted cells decreased with increasing dilutions of the antiserum. At the end point of the sensitizer, the cells bound fewer Ab-E, thereby appearing as single cells partially surrounded by the indicator cells (incomplete rosettes) (Fig. 1*B*). Even these "incomplete" rosettes were quite firm. Thus, the different antigens on cell surfaces were detected indirectly by sensitizing the cells with unadsorbed antiserum and rosetting them with Ab-E specific for the sensitizing Ig.

TSA. The anti-line 1 and anti-line 10 antisera were adsorbed with spleen and liver cells. After 2 adsorptions with spleen cells, about 5% of normal splenocytes, sensitized with a 1:10 dilution of the adsorbed antisera, formed rosettes (incomplete) with the (anti-Ig)Ab-E. After 2 more adsorptions with liver cells, 1% of splenocytes and less than 10% of hepatocytes, sensitized with a 1:10 dilution of the adsorbed antisera, formed rosettes (most of them incom-

Table 1
Rosetting of antibody-sensitized tumor cells with (anti-Ig)Ab-E

Cell line	Inhibitor[a]	Sensitizer[b] (rabbit serum)	(Anti-Ig) Ab-E[c]	% RFC[d] at following dilution of sensitizer			
				1:10	1:30	1:90	1:270
Line 10		Anti-line 10[e]	Anti-b4	100	100	100	81
Line 10		Normal	Anti-b4	2	0	0	0
Line 10		Anti-line 10[f]	Anti-b4	85	59	37	32
Line 1		Anti-line 10[f]	Anti-b4	35	21	15	5
Line 10		Anti-line 10[f]	Anti-b5	0	0	0	0
Line 10	Line 10 antigen	Anti-line 10[f]	Anti-b4	0	0	0	0

[a] Crude preparation of the soluble line 10 antigen, 50 μl, was added to the cells before sensitization.
[b] The sensitizers had the *b4* allotpic specificities (carried by 90% of Ig).
[c] Sheep Ab-E specific for the b4 or b5 Ig allotype.
[d] Percentages of rosetted tumor cells (RFC). Tumor cells were incubated with the dilutions of the sensitizers at room temperature for 60 min, washed 4 times, and mixed with the (anti-Ig)Ab-E.
[e] The anti-line 10 antiserum was unadsorbed.
[f] The anti-line 10 antiserum was adsorbed with spleen and liver (but not with line 1) cells.

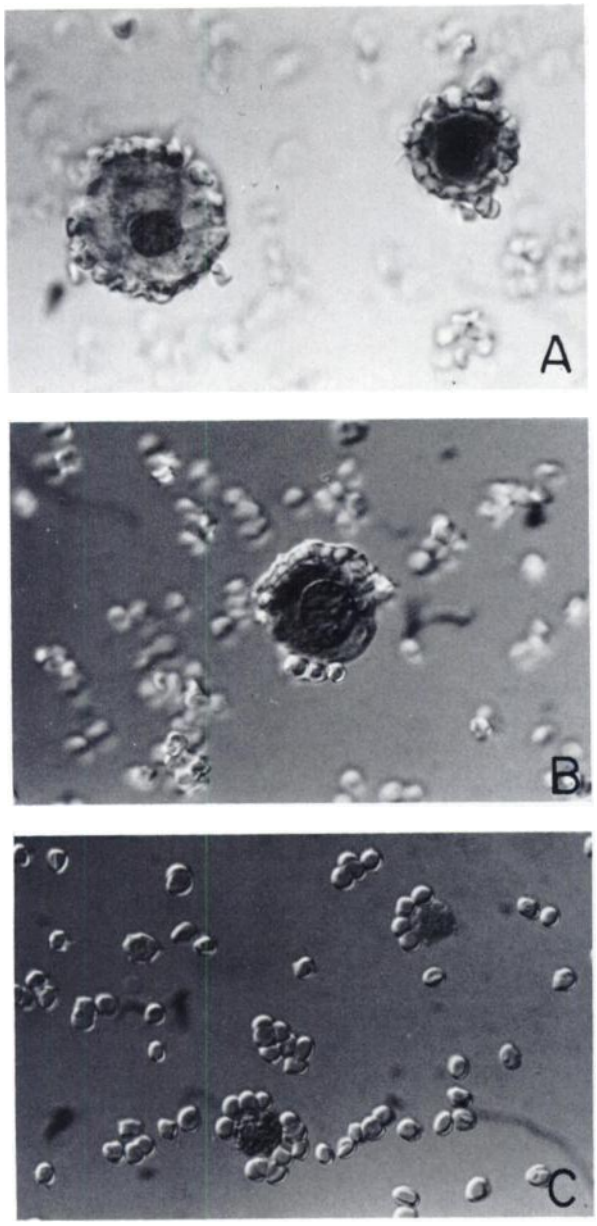

Fig. 1. Tumor cells sensitized with rabbit anti-line 10 antiserum and rosetted with anti-rabbit-Ig-coated erythrocytes. × 500. The antiserum had been adsorbed with spleen, liver, and line 1 cells. *A*, line 10 cells forming complete rosettes after sensitization with 1:10 dilution of anti-line 10; *B*, line 10 cell forming incomplete rosette after sensitization with 1:270 dilution of anti-line 10; *C*, line 1 cell forming incomplete rosette after sensitization with 1:10 dilution of anti-line 10.

plete) with the (anti-Ig)Ab-E (not shown); whereas tumor cells formed high percentages of complete rosettes as exemplified for line 10 cells (Table 1, Line 3). Thus, after adsorptions, the antisera sensitized many tumor cells to form complete rosettes but sensitized only a few hepatocytes, just enough to form incomplete rosettes.

TSA on Line 1 Cells. Line 1 and line 10 cells were sensitized with serial dilutions of anti-line 1 antiserum adsorbed with normal spleen and liver cells. When sensitized with a 1:10 dilution of the antiserum, more than 70% of line 1 cells rosetted, whereas less than 10% of line 10 cells formed rosettes (incomplete) (Chart 1). Thus, the adsorbed anti-line 1 antiserum sharply distinguished line 1 cells from line 10 cells.

TSA on Line 10 Cells. Line 1 and line 10 cells were sensitized with serial dilutions of anti-line 10 antiserum adsorbed with normal cells. Line 10 cells rosetted, but line 1 cells also rosetted significantly (Table 1, Lines 3 and 4). Therefore, the anti-line 10 antiserum was adsorbed twice with line 1 cells. When sensitized with a 1:10 dilution of this adsorbed antiserum, more than 60% of line 10 cells rosetted whereas less than 10% of line 1 cells formed rosettes (incomplete) (Fig. 1*C*; Chart 1). Thus, the anti-line 10 antiserum, adsorbed with spleen and liver cells, identified antigens common to line 10 and line 1 cells. After further adsorptions with line 1 cells, the antiserum sharply distinguished line 10 from line 1 cells.

By this new immunocytoadhesion method, we showed that line 1 and line 10 hepatoma cells may be distinguished from normal liver cells and from each other. By *in vivo* and *in vitro* methods, it has been previously shown that these 2 diethylnitrosamine-induced hepatomas bear antigenically different TSA. Thus, strain 2 guinea pigs, immunized with line 10 cells, showed delayed cutaneous hypersensitivity to line 10 (but not to line 1) cells and resisted the growth of line 10 (but not of line 1) cells (13). Peritoneal exudate cells from animals bearing line 10 tumors incorporated [^{3}H]thymidine (lymphocyte transformation) and did not migrate (macrophage migration inhibition) when treated with line 10 (but not with line 1) soluble antigen (3, 5). Rabbits immunized with line 10 (or with line 1) cells produced antibodies specific for line 10 (or for line 1) cells as shown by C1 fixation, transfer test, immunofluorescence, and complement-dependent cytotoxicity methods (1). Also, strain 2 guinea pigs, immunized to (or bearing a growing) line 10 hepatoma, had serum antibody to line 10 (but not to line 1) cells as shown by indirect immunofluorescence (12). We confirmed this antigenic difference of TSA on line 1 and line 10 cells by our rosetting method with (anti-Ig)Ab-E.

This rosetting was mediated by antibody, since line 10 cells, (*a*) treated with normal rabbit serum having the *b*4 allotype (a genetic marker of κ light chain of rabbit Ig) did not rosette with anti-b4 allotype Ab-E; (*b*) treated with anti-line 10 antiserum (made in a rabbit having the b4

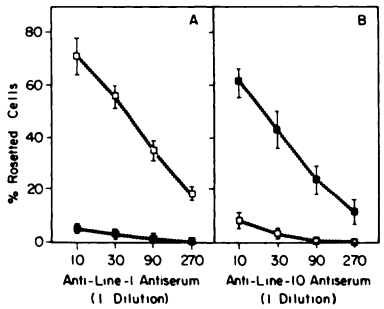

Chart 1. Titration curves of rabbit antisera against line 1 cells (*A*) and line 10 cells (*B*). Percentages (mean ± S.D.) of rosetted line 1 (□) and line 10 (■) cells. Both cell lines were sensitized with either antiserum and rosetted with (anti-Ig)Ab-E. The anti-line 10 antiserum was adsorbed with spleen, liver, and line 1 cells.

allotype) rosetted with anti-b4 Ab-E but not with anti-b5 Ab-E; and (*c*) incubated with anti-line 10 antiserum in the presence of a soluble extract from line 10 cells did not rosette with the anti-b4 Ab-E (Table 1).

This rosetting of antibody-sensitized tumor cells with (anti-Ig)Ab-E is simpler and probably more sensitive and reliable than the previously described rosetting of antibody-sensitized cells with anti-Ig antibody and Ig-antigen-coated erythrocytes (mixed anti-globulin reaction) (2). We have recently shown that direct rosetting of Ig-bearing cells with Ab-E is more sensitive than rosetting by the mixed antiglobulin reaction (6). The Ab-E, having many antibody molecules on the membrane, is probably very avid for the complementary antigen. The method is also simpler than other serological methods such as C1 fixation, immunofluorescence, or cytotoxicity. Finally, the method appears to be a general method for detecting cell surface antigens. In principle, any other surface antigen should be detectable with the appropriate antiserum by this method. This method is also being used to compare the TSA of polyoma virus-transformed cells to the TSA of cells transformed by other means (G. A. Molinaro, E. O. Major, G. Bernhardt, S. Dray, and G. di Mayorca, manuscript in preparation).

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