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Cytolytic Antibodies to Methylcholanthrene-Induced Sarcomas Elicited by Immunization of Syngeneic Mice*

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Abstract—*Cytolytic antibodies were tested in sera of C3H/HeHa mice grafted twice with solid syngeneic sarcoma or immunized with an MC-sarcoma converted into an ascitic form. These sera reacted with syngeneic tumor as well as with normal and malignant allogeneic cells. Absorption experiments suggested that these antisera contained two populations of antibodies. One population combined with antigen(s) shared by the MC-sarcomas of C3H/HeHa mice, by normal and malignant cells of C3H/St mice, and by normal and malignant cells of C57BL/6 mice. The other antibody population failed to react with cells of C57BL/6 mice but it reacted with C3H/HeHa MC-sarcoma and with normal and malignant cells of C3H/St origin.*

INTRODUCTION

CHEMICALLY-induced tumors show individual antigenic specificity as determined by rejection tests in syngeneic animals [1]. However, cross-reactions between various tumors have been described [2–3].

The search for antibodies in the sera of mice bearing chemically induced tumors has produced negative results. However, the presence of antibodies against the tumor specific antigens in sera of animals immunized against chemically induced syngeneic tumors has been demonstrated by different methods. Studies on antigenic specificities detected by such antisera on the surface of these tumor cells, have shown either individual specificity or antigens common to different tumors [4–9]. The presence of cross-reactive antigens has been widely demonstrated in murine leukemias and lymphomas [10] and can be explained in terms of viral antigens expressed on the surface of such cells [11].

The purpose of this study was to analyze the specificity of the antibodies produced by immunizing syngeneic animals with chemically induced tumors and the reported cross-reactivity of these tumors with tumors of different histogenetic origin such as lymphomas.

MATERIAL AND METHODS

Mice used in this study were males and females of strains C3H/HeHa, C3H/St and C57BL/6, 6–12 weeks old. They were supplied by West Seneca Lab. (Buffalo, N.Y.) and Jackson Laboratories (Bar Harbor, Maine) and were maintained in our animal facilities. Food and water were supplied *ad libitum*.

Solid sarcomas MC-22, MC-25 and MC-26 were induced in our laboratory by injecting C3H/HeHa mice subcutaneously with 1 mg of 20-methylcholanthrene (Sigma Chemical Company, St. Louis, MO.) dissolved in olive oil. The tumors were transplanted by the trocar into the subcutaneous tissue of the back of the mice.

The ascitic MC1M was obtained from Dr. Theodore Hauschka (Roswell Park Memorial Institute, Buffalo, N.Y.). This tumor was induced with 3-methylcholanthrene in C3H/HeHa mice and was originally classified as a rhabdomyosarcoma. A solid methylcholanthrene-induced sarcoma of

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C3H/HeHa mice, which was obtained from Dr. Richmond Prehn (Institute for Cancer Research, Philadelphia, PA), was converted in this laboratory into an ascitic form by intraperitoneal passage in syngeneic mice. This ascitic tumor is referred to as MAT. The lymphosarcoma 6C3HED was supplied by Dr. Theodore Hauschka (Roswell Park Memorial Institute, Buffalo, N.Y.). This tumor was induced by estrogen in C3H/St mice. The C57BL/6 lymphocytic leukemia, EL-4, was obtained from Dr. Robert Schwartz (New England Medical Center, Boston, Mass.). All ascites tumors were maintained by weekly transfers in the peritoneal cavity of syngeneic mice.

Ascitic tumor cell suspensions for use as target cells in cytolysis tests were obtained by aspirating fluid from the peritoneal cavity of tumor bearing mice.

Spleen, thymus, liver, kidney, brain and testis cell suspensions were prepared from organs from mice killed by cervical translocation or ether intoxication. The organs were minced in medium 199, pressed through a 400-mesh stainless steel screen and the cells were washed before use.

Sera of "tumor-bearing" mice were obtained by grafting C3H/HeHa mice with MC-25. To this end the mice were injected subcutaneously with minced MC-25 tumor using a 14-gauge trocar. For the first inoculation a 10 mm long plug of tissue was administered. When the tumors reached a size of 1–2 cm in diameter, they were removed surgically and the animals were inoculated a second time with 1/2–3/4 of the amount of tumor tissue used for the first inoculation. Tumors were again surgically removed when they reached a size of 1–2 cm, and the mice were bled 4 days later.

C3H/HeHa anti-MAT serum was produced by subcutaneous inoculation of C3H/HeHa mice with ascitic MAT cells. When the size of the tumors reached a diameter of 1–2 cm, they were surgically removed. The mice were then restimulated by injecting intraperitoneally 2×10^7 living ascitic tumor cells. The mice were bled 10–14 days after the injection. The sera from these mice were pooled, dispensed in small aliquots and stored at −70°C before use.

The cytolysis in agar gel procedure used was that described by Fuji *et al.* [12]. In brief, a 1.5% cell suspension in agarose-dextran solution was spread on a microscope slide and the serum dilutions to be tested were placed as 5 μl droplets on the agar. The slides were then incubated for 1½ hr at 37°C in a wet chamber. At the end of this period the slides were rinsed in phosphate buffered saline solution and then incubated with a commercial preparation of rabbit complement (Difco Laboratories, Detroit, Mich.) diluted 1/10. Controls with similarly prepared slides incubated with heat-inactivated rabbit complement were always included. The slides were incubated for 1 hr at 37°C and then they were rinsed in saline, air-dried and fixed in 95% ethanol for 30 min. Finally, the slides were rinsed in tap water and air-dried. The reactions were read and they were graded 1–4+ depending upon the intensity of lytic reaction.

Dextran hemagglutination was performed according to a technique described by Gorer and Mikulska [13]. Serial dilutions of the antisera to be tested were prepared in 1.5% dextran (mol.wt 115,000, Glaxo Laboratories, Ltd., Greenford, England) in phosphate-buffered saline (PBS), pH 7.2.

Absorptions were performed using a packed cell sediment prepared by centrifugation of cell suspension at 1800 ***g*** for 5 min. In most experiments 0.1 ml of antiserum at 1/10 dilution was absorbed three times; for each absorption 0.05 ml of packed cells was used. The serum-cell mixture was incubated at room temperature for 30 min and at 4°C for 30 min. The mixture was then centrifuged at 1800 ***g*** for 5 min and the absorbed specimen was removed.

RESULTS

Sera of C3H/HeHa mice that were grafted twice with syngeneic solid sarcoma and surgically freed of tumor were tested first for antibodies to cells of MC-25. To this end, cytolysis in agar gel was performed in which dispersed cells of MC-25 were used as target cells. Numerous experiments along these lines were negative or at the best doubtful. This was, however, ascribed to a technical failure. Previous experience in this laboratory clearly showed that with the exception of the thymus, solid tissues cannot be readily used as target cells for cytolytic tests. Accordingly, subsequent experiments were performed with the cells of lymphoma 6C3HED which originated from C3H/St mice. We expected that the substrain difference of the tumor origin would not play any significant role in detection of tumor antibodies and indeed we found in the very first experiments that sera of the mice under investigation lysed 6C3HED cells up to

Table 1. Cytolysis in gel. Serum from a C3H/HeHa mouse grafted with MC-25 sarcoma tested against normal and malignant cells of C3H/St and C57BL/6 mice

Antiserum dilution 1 to:	6C3HED cells	EL-4 cells	Lysis of: C57BL/6 thymocytes	C3H/St thymocytes	C3H/HeHa thymocytes
5	+++	+++	++	++	−
10	+++	+++	+	+	−
20	+++	++	+	+	−
40	++	++	+	±	−
80	++	++	±	−	−
160	+	+	−	−	−
320	−	−	−	−	−

Table 2. Cytolysis in gel. reactions of pooled C3H/HeHa anti-MAT serum with MAT, 6C3HED and EL-4 cells as well as with C3H/St and C3H/HeHa thymocytes

Antiserum dilution 1 to:	MAT cells	6C3HED cells	Lysis of: El-4 cells	C3H/St thymocytes	C3H/HeHa thymocytes
10	++++	++++	+++	++	−
20	++++	+++	+++	++	−
40	+++	+++	++	+	−
80	+++	++	++	+	−
160	++	++	+	−	−
320	++	+	+	−	−
640	+	+	−	−	−
1280	+	−	−	−	−
2560	−	−	−	−	−

a titer of 160. However, to our surprise, we noted that normal thymocytes of C3H/St origin were also lysed by these sera. The results obtained with sera of C3H/HeHa mice grafted with a syngeneic MC-sarcoma are exemplified in Table 1. As seen in this table, the sera under investigation reacted also with EL-4 lymphoma cells of C57BL/6 origin but, significantly, they did not lyse normal syngeneic thymocytes of C3H/HeHa origin.

Subsequently, an ascitic form of MC-sarcoma of C3H/HeHa mice was obtained. This tumor, designated MAT, was used as a target antigen and it was lysed by sera of tumor-grafted C3H/HeHa mice; it was also used as an immunizing antigen.

Pooled serum from C3H/HeHa mice immunized with the ascitic tumor, MAT, were then tested for cytolytic activity against MAT, 6C3HED and EL-4 cells. The reactions of one such pool are presented in Table 2. The pooled antiserum exhibited strong reactions with MAT and 6C3HED cells showing titers of 1280 and 640, respectively. The antiserum also gave moderately strong reactions with EL-4 cells. As expected, no reactions were obtained with C3H/HeHa thymocytes, but the antiserum did react with C3H/St thymocytes. When this antiserum was tested for agglutinating activity against C3H/St and C57BL erythrocytes, no reactions were observed.

Absorption experiments presented in Table 3 showed that lytic activity of the C3H/HeHa anti-MAT serum against all three target cells was removed by MAT and 6C3HED cells. Significantly, it was also removed by normal C3H/St tissue, but not by normal C3H/HeHa tissue. Absorptions with EL-4 cells and normal C57BL/6 tissue removed all lytic activity against EL-4 cells and reduced but did not completely remove activity against either MAT or 6 C3HED cells.

In order to study further the cross-reactions of the C3H/HeHa anti-MAT serum with C57BL/6 tissues, experiments were performed, the results of which are summarized in Table 4. As can be seen, three absorptions with either 6C3HED cells or C3H/St kidney completely removed lytic activity against 6C3HED

Table 3. Cytolysis in gel. Reactions of C3H/HeHa anti-MAT serum with murine tumor cells after absorption with neoplastic and normal murine tissues

Antiserum	Titer with: MAT cells	6C3HED cells	EL-4. cells
Unabsorbed	640	640	320
Absorbed with:			
MAT	<10	<10	<10
6C3HED	<10	<10	<10
EL-4	160	160	<10
C3H/HeHa kidney	320	320	160
C3H/St spleen, thymus, or kidney	<10	<10	<10
C57BL/6 kidney	160	160	<10

Table 4. Cytolysis in gel. Reactions of the C3H/HeHa anti-MAT serum with 6C3HED cells. Effect of stepwise absorptions with neoplastic and normal murine tissues

Antiserum	Titer with 6C3HED cells
Unabsorbed	640
Absorbed:	
3 × with 6C3HED or C3H/St kidney	<20
3 × with EL-4 or C57BL/6 kidney	160
3 × with EL-4 or C57BL/6 kidney and 1 × with:	
6C3HED or C3H/St kidney	<20
EL-4 or C57BL/6 kidney	160

cells. However, moderate cytolytic activity remained after the same number of absorptions with either EL-4 cells or C57BL/6 kidney. This residual activity could be removed by an additional absorption with 6C3HED cells or C3H/St kidney, but not by an additional absorption with EL-4 cells or C57BL/6 kidney. Similar results were obtained in experiments using MAT cells instead of 6C3HED cells.

The results of these experiments indicate that the serum under investigation contains two populations of antibodies. One population was directed against an antigen which is shared by C3H/St and C57BL/6 mice and is present in both normal and malignant tissues. The other population of antibodies was directed against an antigen which is present in normal and malignant C3H/St tissues but not in C57BL/6 tissues.

The specificity of these antisera for other MC-induced sarcomas was studied by absorption experiments using solid MC-sarcomas induced in C3H/HeHa mice. As can be seen in Table 5, absorption with sarcomas MC-22, MC-25 and MC-26 considerably reduced cytolytic activity of the C3H/HeHa anti-MAT serum against MAT and 6C3HED cells. Absorption with MC1M cells, the ascitic form

Table 5. Cytolysis in gel. Reactions of C3H/HeHa anti-MAT serum with MAT and 6C3HED cells after absorption with solid MC-sarcomas

Antiserum	Titer with: MAT cells	6C3HED cells
Unabsorbed	1280	1280
Absorbed with:		
MC-22	160	160
MC-25	160	80
MC-26	160	160
MC1M	640	640
C3H/HeHa thymocytes	640	640

of a MC-rhabdomyosarcoma of C3H/HeHa mice and C3H/HeHa thymocytes, had no significant effect on cytolytic activity of this antiserum. These experiments indicate that an antigen present in C3H St tissue is also found in MC-sarcomas induced in C3H/HeHa mice, but is not present in normal C3H/HeHa tissue or MC-rhabdomyosarcoma.

Further studies were performed to learn whether the original solid MC-sarcoma, from which MAT was obtained, is capable of conveying resistance to MAT, which could be anticipated on the basis of results of serological studies. To this end, the solid tumor was grafted into C3H/HeHa mice and was extirpated after it attained a size of 1–2 cm in diameter. The mice were then challenged with living ascitic MAT. In this experiment we were able to demonstrate that mice which had been grafted with MC-sarcoma, and subsequently challenged with MAT tumor, survived for more than 90 days. Similarly prepared mice that were challenged with MC1M cells died within 20 days and untreated C3H/HeHa mice inoculated with MAT or MC1M cells died within 21 days.

DISCUSSION

The purpose of this study was to establish *in vitro* reactions between tumor-specific antigens of MC-induced murine sarcomas and their corresponding antibodies. The method used for this purpose was cytolysis in agar gel.

Sera from C3H/HeHa mice grafted with syngeneic solid MC-sarcoma as well as sera of C3H/HeHa mice immunized with MAT cells produced reactions with MAT and 6C3HED cells. The antibodies responsible for these reactions could be removed by absorption with cells of either tumor and, significantly, with normal C3H/St tissue. Results of the absorption studies showed that these antibodies behaved like antibodies directed against antigens present in C3H/St but absent from normal C3H/HeHa tissues. The possibility that MAT, though induced in C3H/HeHa mice, acquired C3H/St ("St") antigens as a result of malignant transformation was considered.

In order to obtain further data concerning the possible appearance of normal "St" antigens in C3H/HeHa tumors, we performed absorptions of the C3H/HeHa anti-MAT serum with solid MC-sarcomas of C3H/HeHa origin. All three MC-sarcomas showed absorbing activity towards the antiserum under study even though they did not remove antibodies completely. Considering that normal C3H/HeHa tissues were incapable of absorbing these antisera, the conclusion that MC-sarcomas of C3H/HeHa mice acquire "St" antigens in the course of neoplastic conversion appeared justified. Since it has been suggested that the tumor-specific antigens expressed on the surface of tumor cells represent modified histocompatibility antigens [14–17] it is possible that "St" antigens represent an alloantigen present in some normal mice and acquired as a result of malignant transformation by other mice.

Furthermore, sera of C3H/HeHa mice grafted with MC-sarcoma and C3H/HeHa anti MAT sera contained antibodies lytic for EL-4 leukemia cells of C57BL mice. In some instances, reactions with EL-4 were stronger and more frequently observed than reaction with either MAT or 6C3HED. This feature most likely depended on the better lysability of EL-4 cells. Absorption experiments showed that reactions with El-4 should be considered a cross-reaction in that MAT or 6C3HED removed all antibodies reacting with EL-4, but EL-4 only removed a portion of antibodies combining with MAT or 6C3HED. Furthermore antibodies for EL-4 could be completely removed by normal C57BL tissue.

The presence of cross-reactive antigens, as detected by antibodies, in cells of chemically induced sarcomas, has been described previously [9, 14, 18]. Likewise, the fact that antisera against sarcoma cells cross-react with cells of different histogenetic origin, such as leukemia and lymphoma, has also been reported [19]. This common reactivity of cells from different tumors has been interpreted as being due to tumor antigens or to associated antigens, such as viral antigens, (i.e. MuLV, FeLV) or fetal antigens [11, 20]. Cross-reacting antigens have not only been determined through reactivity with antisera, but also by means of blastogenic stimulation of lymphocytes by tumor cell extracts, cytotoxicity and differential absorption tests [20, 21].

Experimental results obtained suggested that C3H/HeHa mice immunized with syngeneic MC-sarcomas formed two antibody populations. One population reacted with antigen shared by the MC-sarcomas of C3H/HeHa mice, by normal and malignant cells of C3H/St mice, and by normal and malignant cells of C57BL/6 mice. It is conceivable that these antibodies might have been directed against antigenic determinants of virus of the murine leukemia–sarcoma

complex (MuLV). This contention is supported by studies in which it was shown that chemical induction of tumors (methylcholanthrene induced sarcomas or lymphomas) is accompanied by the expression of antigenic determinants of the murine leukemia viruses. MuLV antigens have also been detected on EL-4 cells [18, 22, 24]. The other antibody population failed to react with cells of C57BL/6 mice but it reacted with C3H/HeHa MC-sarcoma and normal and malignant cells of C3H/St origin. These antibodies were apparently directed against an alloantigens "St" which appear in the C3H/St mice as normal tissue alloantigens, but in C3H/HeHa appear only as a result of malignant cell transformation. The possibility that "St" antigens are tumor-specific antigens and represent histocompatibility antigens, other than H-2, produced by genetic derepression or are modified forms of the products of any of the loci of the histocompatibility system, is being studied.

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