Detection of Tumor-Specific Antigens in an Equine Sarcoid Cell Line

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Indirect immunofluorescence and lymphocyte cytotoxicity experiments demonstrated the presence of a tumor-specific antigen(s) on the surface of cells from an equine sarcoid cell line (Mc1). Autologous serum (taken from the horse from which the Mc1 cells were derived) and sera from three other sarcoid-bearing horses revealed a similar membrane immunofluorescence when reacted with Mc1 cells, indicating the existence of cross-reacting antibodies. Results of serum colony inhibition experiments indicate that these antibodies are not cytotoxic. Incubation of Mc1 cells with autologous lymphocytes resulted in a toxicity to Mc1 cells, thereby demonstrating a cell-mediated immune reaction to autologous tumor cells.

Many tumors, tissue culture cells derived from tumors, and cells transformed in vitro contain tumor-specific antigens which differ from normal tissue antigens of the animal from which the tumor or transformed cells were derived. There are several different types of tumor antigens: (i) virus-specific tumor antigens (4, 7, 12, 18, 20); (ii) T antigens (5, 6, 11, 21, 25, 29); and (iii) tumor-specific transplantation antigens (TSTA's) (1, 2, 8, 10, 14, 16). Other, less common types of tumor antigens have been reported to occur in specific types of neoplasia or transformed cells (3, 27). One or more tumor antigens may be found within a tumor or transformed cell.

Tumor-specific antigens are most frequently found in tumors or transformed cells induced with either oncogenic viruses or chemical carcinogens. Only a few spontaneous animal tumors have been found to contain tumor antigens. Reported studies concerning equine sarcoids (spontaneous connective tissue tumors occurring on the skin of horses) have not included the detection of tumor-specific antigens. This report concerns the detection of these antigens in an equine sarcoid cell line by immunofluorescence and in vitro lymphocyte cytotoxicity techniques.

MATERIALS AND METHODS

Cell cultures. An equine sarcoid cell line (Mc1 cells) (28), and a normal equine dermis cell line

(NBL-6 from the American Type Culture Collection, Rockville, Md.) were used in this study.

Indirect immunofluorescent techniques. Test sera included serum taken from the horse from which the Mc1 cells were derived (Mc1 serum), as well as serum taken from three other horses bearing equine sarcoids (i.e., Val serum, Tuc serum, and Ttl serum). Pooled normal equine serum (NES) was used as a serum control.

Normal equine globulin (NEG) was fractionated from pooled NES using the ammonium sulfate precipitation method of Spendlove (24). The protein content of the NEG was determined using the method of Lowry and co-workers (17), and then adjusted to give 15 mg of protein per ml of 0.85% NaCl. Rabbit anti-normal equine globulin (RANEG) was produced in five rabbits by intravenous inoculation of NEG. A 0.1-ml amount of NEG was injected on day 1, followed by 0.25 ml on day 4, 0.5 ml on day 8, and 1.0 ml on days 12, 16, 20, 24, and 28. Then 30 days later rabbits were given 2.0 ml of NEG intramuscularly. Animals were bled 6 days after the intramuscular injection, and the serum was harvested and stored at −20 C.

The method of Spendlove (24) was used to conjugate RANEG with fluorescein isothiocyanate (FITC). The conjugated antiserum (RANEG-FITC) was then used to stain cells in suspension according to a modification of the method of Pearson and co-workers (19). Tissue culture cells were trypsinized off the surface of culture vessels using phosphate-buffered saline (PBS) containing 0.5% trypsin plus 0.025% ethylenediaminetetraacetic acid. After washing the cells three times with PBS, cell viability was determined by the trypan blue exclusion test. Approximately 5.0×10^6 viable cells were suspended in 0.05 ml of the appropriate test or control sera. This suspension was incubated for 45 min in a 37 C water bath. After washing the cells three more times in PBS, the cells were incubated in the dark for 45 min at 37 C

with 0.05 ml of 1:5 RANEG-FITC. Unreacted, conjugated antiserum was removed by washing the cells three times with PBS. The stained, packed cells were suspended in 1 or 2 drops of buffered glycerol. The cell suspension was placed on a clean microscope slide, after which a cover slip was applied and sealed with fingernail polish.

A blocking test was conducted prior to the indirect fluorescent antibody (FA) technique by adsorbing the unlabeled primary sera (i.e., Mc1, Val, Tuc, and Ttl sera) with Mc1 cells to remove specific antibodies to tumor antigens. One milliliter of each of the primary sera was incubated with 10^7 Mc1 cells overnight at 4 C. The cells were removed by centrifugation and adsorption was repeated for 1 h at 37 C by using fresh Mc1 cells. The suspension was centrifuged, and the adsorbed sera were pipetted off and used in place of unadsorbed primary sera in the indirect FA technique.

A Zeiss Universal microscope with a 12-V 100-W tungsten-halogen quartz illuminator and a darkfield ultra-condenser for blue light fluorescence was used. Photomicrographs were taken with a Zeiss 35-mm camera and Kodak Tri-X-Pan film (ASA 400).

Colony inhibition techniques. The same sera which were used in the indirect FA test were also used in the colony inhibition (CI) technique (26). These sera were incubated with Mc1 cells in culture in an attempt to detect tumor-specific antigens on Mc1 cells. The inhibition of colony formation by the test sera was based on a comparison with normal equine or fetal bovine sera. Experiments were also conducted in which the guinea pig complement was either omitted or heat-inactivated before use.

Lymphocyte cytotoxicity techniques. A modification of the techniques of Sinkovics and co-workers (22, 23) and Kurth and Bauer (15) were used in an attempt to detect lymphocytes which were cytotoxic to Mc1 cells. Lymphocytes were separated from the blood of normal horses and from the horse from which the Mc1 cells were derived (Mc1 horse) by using the technique of Joel (13).

Suspensions of Mc1 cells in growth medium were delivered into two-well chamber slides (Lab-Tek Products, Maperville, Ill.) at a concentration of 10^4 cells per well, and incubated for 24 h at 37 C in an 80% humidified atmosphere of 5% CO_2 in air. The cells which had adhered to the surface were then washed three times with PBS. The normal and test lymphocytes were washed five times in minimal essential medium (MEM), after which they were suspended in growth medium and delivered into the wells containing Mc1 cells to give a concentration of 100 lymphocytes per Mc1 cell. After 36 h of incubation, the culture medium was discarded and the cells were washed twice with MEM. The attached cells were stained for 20 min with 1% crystal violet in 20% methanol, and washed several times with PBS. In each well, the adherent Mc1 cells were counted in 10 random fields; the average count per field was calculated; comparisons were made between cells treated with normal equine lymphocytes and cells treated with lymphocytes derived from the Mc1 horse.

RESULTS

Indirect FA test. Indirect immunofluorescence studies using Mc1 sera and RANEG-FITC revealed specific membrane immunofluorescence on Mc1 cells of all passages tested (subcultures 42, 97, and 141). The degree of fluorescence varied somewhat with different serum samples taken from the Mc1 horse. Some Mc1 sera samples revealed very little immunofluorescence (21/250 or 9%), whereas others revealed over 85% (213/250) of the cells with very intense membrane staining (Fig. 1).

The cell membrane was the only portion of the cell which fluoresced. The membrane never exhibited a complete ring of fluorescence, but instead had discrete patchy areas of fluorescence scattered over the entire membrane. By focusing up and down on the suspended cells, areas of speckled fluorescence were detected at all levels of the cell membrane. When NES was used in place of Mc1 sera, there were a few cells which retained the stain; however, less than 10% exhibited the membrane immunofluorescence.

When sera from other sarcoid-bearing horses (Tut, Val, and Ttl horses) were used in place of Mc1 sera in the indirect FA technique, approximately 10 to 50% of Mc1 cells exhibited specific membrane immunofluorescence (Table 1). The pattern of the staining was identical to that described for the Mc1 cells which were reacted with Mc1 sera and RANEG-FITC (Fig. 2). NES and normal equine dermis cell controls exhibited little or no membrane immunofluorescence.

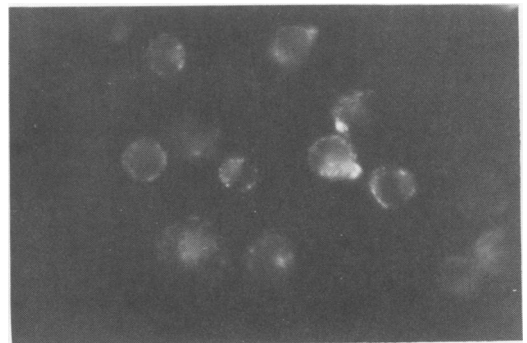When Mc1 sera were incubated with Mc1 cells prior to the FA technique, as described for

FIG. 1. *Specific immunofluorescence of tumor-specific antigens on the membrane of 141st passage Mc1 cells. Mc1 serum was the primary serum used on the indirect FA technique. Rabbit anti-normal equine globulin, conjugated to FITC, was the secondary serum. ×685.*

the blocking technique, there was a decrease in both intensity of fluorescence and the number of cells exhibiting specific immunofluorescence. Parallel tests with Mc1 sera which were not blocked revealed approximately 25 to 50% more cells which retained the fluorescent dye than tests with the blocking serum (Table 1).

CI tests. Table 2 shows results of several CI tests. The reactions of NES with Mc1 cells resulted in the formation of an average of 31 colonies (colonies were counted only if they contained more than 10 to 12 cells). When Mc1 sera were incubated with Mc1 cells prior to seeding, there was no significant CI regardless of whether or not active guinea pig complement was used. Furthermore, there was no colony inhibition when Tut, Val, or Ttl sera were incubated with Mc1 cells.

Lymphocyte cytotoxicity experiments. Lymphocyte cytotoxicity studies resulted in a destruction of Mc1 cells with a subsequent decrease in the number of cells attached to the culture vessel surface after 36 h of incubation. The supernatant tissue culture media often contained clusters of floating lymphocytes and Mc1 cells. Each floating cluster contained an average of 8 to 10 lymphocytes and 1 to 3 larger rounded-up Mc1 cells. An average count of 10 random fields of Mc1 cells revealed 39 adherent cells per field. In addition, some Mc1 cells, which were still adherent to the surface, had autologous lymphocytes attached to them (Fig. 3). About 10% of the Mc1 cells had attached lymphocytes, ranging from 1 to 5 lymphocytes per cell.
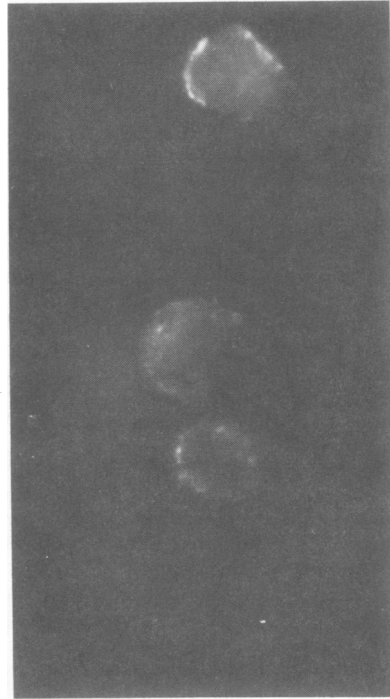

FIG. 2. *Specific immunofluorescence of tumor-specific antigens on the membrane of 141st passage Mc1 cells. Homologous serum from a sarcoid-bearing animal was the primary serum. Rabbit anti-normal equine globulin, conjugated to FITC, was the secondary serum.* ×685.

Incubation of normal equine lymphocytes with Mc1 cells revealed a greater number of Mc1 cells adherent to the surface of the culture vessel than in the test system described above. The supernatant tissue culture media also contained floating lymphocytes and Mc1 cells, but not in the large clusters seen with Mc1 cells and autologous lymphocytes. An average count of 10 random fields revealed 64 adherent cells as compared with the test system. There were occasional Mc1 cells (approximately 1 out of 20) that had lymphocytes attached to their surface; however, there were usually only one or two lymphocytes per cell.

DISCUSSION

Indirect FA techniques using Mc1 sera and RANEG-FITC revealed specific membrane immunofluorescence on all Mc1 cells tested, denoting a permanent antigenic change on the surface of Mc1 cells. Mc1 sera should not have reacted with Mc1 cells, due to their autologous relationship, unless the cells contained a "for-

TABLE 1. *Comparison of the number of fluorescent Mc1 cells after treatment with homologous or autologous, blocked or nonblocked sera in the indirect FA technique*

Serum source	Number of fluorescent Mc1 cells[a]	
	Blocked sera	Nonblocked sera
Autologous		
Mc1	103	191
Homologous[b]		
Val	40	53
Tuc	82	107
Ttl	67	84
NES	5	3

[a] The number of fluorescent Mc1 cells (97th subpassage) out of 250 counted.

[b] Val, Tuc, and Ttl are horses with equine sarcoids; NES is serum pooled from several normal horses without sarcoids.

TABLE 2. *Growth of Mc1 cells in the presence of autologous or normal equine sera, with or without complement*

Antisera	Antigen[a]	Complement	No.[b] of colonies
Mc1[c]	Mc1 (P$_{55}$)	−	20
Mc1	Mc1 (P$_{55}$)	+	31
Mc1	Mc1 (P$_{124}$)	−	35
Mc1	Mc1 (P$_{124}$)	+	30
NES[d]	Mc1 (P$_{55}$)	−	23
NES	Mc1 (P$_{55}$)	+	27
NES	Mc1 (P$_{124}$)	−	39
NES	Mc1 (P$_{124}$)	+	34

[a] Mc1 cells in either the 55th or 124th passage.

[b] Averages of at least three replicates of each test system.

[c] Sera taken from the same horse from which the Mc1 cells were derived.

[d] Pooled normal equine sera.

eign" antigen(s) on their surface. The cell membrane was the only portion of the cell which fluoresced, indicating that the antigens detected were possibly TSTA's.

The reason for the varying degrees of fluorescence when different Mc1 serum samples were utilized remains unclear. Samples taken within a week or two of each other often revealed similar degrees of fluorescence, whereas an interim of several months between collections often resulted in varying degrees of fluorescence. This might have been due to actual changes in the amount of circulating antibody. The number of sarcoid cells available to react in antigen-antibody complexes with sarcoid-specific antibody may vary with growth, immunological destruction, or surgical removal of tumor cells. As the number of available tumor cells varies, so may the amount of free circulating antibody (9). The amount of circulating antibody would then directly affect the degree of immunofluorescence observed with different Mc1 serum samples.

The sera from three other sarcoid-bearing horses revealed a similar membrane immunofluorescence when reacted with Mc1 cells, although the degree of staining was almost always less than when Mc1 serum was used. This finding allows inference that there are similar antibodies in other sarcoid-bearing horses which react with Mc1 cell tumor antigens, and, in turn, other equine sarcoids must then have some similarity in antigenic expression on their cell surfaces. The reason the fluorescence was not as intense as with Mc1 sera was probably due to slight antigenic differences in Mc1 cells as compared with other sarcoid cells.

When Mc1 sera were adsorbed with Mc1 cells prior to the indirect FA technique, as described for the FA blocking procedure, there was a decrease in both intensity of fluorescence and the number of cells exhibiting specific immunofluorescence. The blocking procedure apparently removed many of the antibodies to equine sarcoid tumor antigens, thereby demonstrating the immunological specificity with which the unblocked serum reacts with Mc1 cells.

The colony inhibition test for serum cytotoxicity did not demonstrate an immunological reaction between test sera and Mc1 cells. This does not necessarily contradict the immunological specificity for Mc1 cells noted in these sera by the indirect FA technique. The antibodies reacting in the indirect FA procedure do not need to be cytotoxic, whereas antibodies detected by the colony inhibition techniques are perforce cytotoxic. Antibodies to tumor-specific antigens were probably present in the sera of sarcoid-bearing horses, and these antibodies probably reacted with surface antigens of Mc1 cells in the colony inhibition test; however, due to their noncytotoxicity they were not detected.

Lymphocyte cytotoxicity experiments have been used in the past to demonstrate a cell-mediated immune response to autologous tumor cells (22, 23). Incubation of Mc1 cells with autologous lymphocytes resulted in a decrease
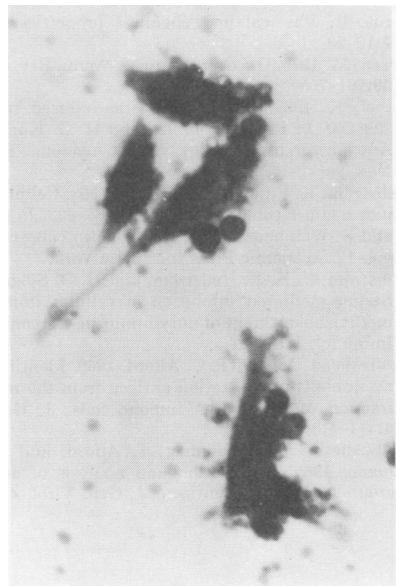

FIG. 3. *Lymphocyte cytotoxicity experiment showing Mc1 lymphocytes attached to 142nd passage Mc1 cells. ×500.*

in the number of viable Mc1 cells as compared with control trials in which normal homologous lymphocytes were used. In addition, many Mc1 cells, which were still attached to the vessel surface, had autologous lymphocytes attached to them, giving evidence of an immunological reaction between Mc1 cells and autologous lymphocytes, which presumably resulted in cytotoxicity. This reaction should not occur unless foreign antigens are present on Mc1 cells. Consequently, this experiment gives further evidence of the presence of TSTA's on Mc1 cells.

ACKNOWLEDGMENTS

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