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Mastocytoma-Induced Suppression of in Vitro Antibody Formation[1] (38653)

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Mastocytoma tumor cells derived initially from mast cells of DBA/2 mice are highly malignant when transplanted to syngeneic mice (1). These cells can be readily cultivated *in vitro* and have been widely used in cellular immunology as a "target" for *in vitro* assay of cell-mediated immunity (2, 3). For example, when chromium 57 labeled mastocytoma cells are incubated with spleen cells from an allogeneic mouse strain sensitized to DBA/2 transplantation antigens there is a rapid release of the chromium, indicative of specific target cell lysis (3). During studies in this laboratory utilizing this model system to test cell-mediated immune responses to tumor and virus antigens it was noted that the expected immune response of normal spleen cells to sheep erythrocytes *in vitro* was markedly depressed when mastocytoma cells were present in the cultures. In the present report we describe the immunosuppressive properties of mastocytoma cells and a cell-free extract derived from these cells.

Methods and Materials. Experimental animals. Young adult DBA/2 mice obtained from Jackson Memorial Laboratories, Bar Harbour, Maine were used for these experiments. The animals were approximately 18–20 g in weight when used and were fed Purina mouse chow and water *ad libitum*.

Tumor cells. Mastocytoma tumor cells, designated P815, were obtained originally from the American Type Culture Collection. The tumor cells were maintained by serial passage through DBA/2 mice. For this purpose 10 million viable nucleated tumor cells, standardized by hemocytometer count, were injected intraperitoneally (ip) into normal mice. Ten to 15 days later ascitic fluid was collected from the peritoneum of the mice and the cells separated by centrifugation at 3000 rpm for 10 min at 4°. Packed cells were resuspended in an equal volume of sterile culture medium. Supernatants of cell-free homogenates were prepared by repeated freezing and thawing of the ascites cells and centrifugation at 3000 rpm for 10 min.

Antigen. Sheep erythrocytes (SRBC) obtained in Alsever's solution and washed three times in culture medium were resuspended to a concentration of 2×10^7 erythrocytes per ml medium. For *in vitro* immunization 0.1 ml of the freshly washed RBC suspension was added per culture.

Spleen cell cultures. Pooled spleens from normal DBA/2 mice were minced with scissors in a sterile Petri dish containing sterile medium. The cell suspensions were clarified by passage through 26 gauge needles and then transferred to 15 ml plastic conical tubes. Aggregates were allowed to settle for 3–5 min at 4° and the tubes were then centrifuged at 500 rpm for 30 sec. The cell suspensions were transferred to other tubes, which were centrifuged at 1000 rpm for 10 min. The cell pellets were then resuspended in appropriate volumes of culture medium. Erythrocytes were removed by incubation of the cells with 0.84% ammonium chloride for 10 min at room temperature. Viability counts were then determined by the trypan blue stain technique using a hemocytometer.

In vitro culture. Marbrook culture vessels (Bioresearch Company, Vineland, NJ), were used for *in vitro* culture (4). Five million spleen cells were placed into 0.5 ml fresh culture medium consisting of Hanks' MEM medium fortified with MEM and fetal calf serum (Grand Island Biological Company, NY). The splenocytes were placed on the dialysis membrane in the inside chamber and 11 ml culture medium were placed in the outside chamber. The chambers were in-

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TABLE I. EFFECT OF GRADED NUMBERS OF MASTOCYTOMA TUMOR CELLS ON HEMOLYTIC ANTIBODY PLAQUE RESPONSE OF SPLEEN CELLS FROM NORMAL MICE IMMUNIZED *in vitro* WITH SHEEP ERYTHROCYTES

Mastocytoma cells added per culture[a]	PFC per culture[b]	Percent of control response
None	5350 ± 475	—
10^3	5076 ± 348	95
10^4	2189 ± 175	41
10^5	386 ± 53	8
10^6	100	2

[a] Indicated number of mastocytoma cells added to cultures of 5×10^6 spleen cells from normal DBA/2 mice *in vitro*.

[b] Average PFC response (±sd) of 5–6 cultures 5 days after *in vitro* immunization with 2×10^6 sheep RBCs.

cubated in a 37° humidified incubator with an atmosphere of 5% CO_2.

Antibody plaque assay. Five days after culture initiation aliquots of spleen cells from three or more culture chambers were tested for hemolytic antibody plaque forming cells (PFC) using the plaque assay in agar gel essentially as described by Jerne *et al.* (5, 6). The number of hemolytic plaque forming cells per million splenocytes cultured or per whole chamber tested was determined for control cell suspensions and for splenocytes incubated with mastocytoma cells or mastocytoma cell supernatants.

Experimental Results. Incubation of normal spleen cells with sheep erythrocytes *in vitro* resulted in the rapid appearance of hemolytic plaques. When 5 million normal splenocytes were incubated with varying numbers of mastocytoma cells there was a marked alteration in the number of PFCs. As can be seen in Table I, 10^3 tumor cells resulted in a slight depression of the number of PFC appearing in the cultures of normal spleen cells incubated with sheep RBCs. Ten times more mastocytoma cells resulted in a 50–60% suppression in the number of PFCs, whereas 10^5 tumor cells caused a 95% suppression. This depression was readily apparent when the number of such PFCs was calculated per whole chamber. There was even a greater suppression when PFCs were calculated per million splenocytes since there was a marked reduction in the number of recoverable spleen cells from culture vessels containing 10^4 or 10^5 mastocytoma cells. The mastocytoma cells appeared to rapidly over grow the splenocytes during the 5-day incubation period.

In order to eliminate the possibility that immunosuppression in this system was due merely to a "crowding" out of normal spleen cells additional cultures were prepared with supernatants from tumor cells to determine if cell-free extracts from the mastocytoma cells would influence the immune response of the spleen cells to SRBC. For this purpose, supernatants from mastocytoma cell homogenates were added in graded dilutions to cultures containing five million splenocytes from normal DBA/2 mice. As can be seen in Table 2, immunosuppression occurred when 0.1 ml of a 1:1 to 1:25 dilution of the supernatant was added to the cultures. Those vessels containing undiluted supernatant showed relatively few PFCs *in vitro*. The fivefold greater dilution resulted in a 70–75% suppression, whereas the 1:25 dilution resulted in a 40–50% suppression. A 1:50 dilution had no affect. There was essentially no difference in the viability of the splenocytes in cultures containing the different concentrations of the supernatants as compared to those cultures with medium alone.

As a control, mastocytoma supernatants were heated at 56° for 30 min. This abolished the suppressive activity of the supernatant (Table II). In most cases the 1:1 dilution of heated supernatant usually caused a slight to moderate enhancement of the PFC response. The 1:5 and 1:25 dilution did not alter the expected responses. As additional controls, cell-free extracts prepared from spleens or peritoneal cell exudates from normal DBA/2 mice were added to cultures of normal spleen cells immunized *in vitro* with SRBC; no suppressive affect occurred in terms of PFC formation to the SRBC by the normal splenocytes.

Discussion. The results of this study indicate that mastocytoma cells, as well as a cell-free extract prepared from these tumor

TABLE II. IMMUNOSUPPRESSIVE EFFECT OF SUPERNATANTS OF MASTOCYTOMA CELL HOMOGENATES ON PFC RESPONSE BY NORMAL SPLEEN CELL CULTURES *in vitro* AND LABILITY TO HEAT.

Mastocytoma supernatant added to cultures[a]	PFC per culture[c]
None	5170 ± 602
Nonheated	
1:1	976 ± 205
1:5	1420 ± 314
1:25	2083 ± 518
1:50	4950 ± 535
Heated[b]	
1:1	5975 ± 750
1:5	5240 ± 683
1:25	5055 ± 705

[a] 0.1 ml of indicated dilution of supernatant from mastocytoma cell-homogenate added to cultures containing 5×10^6 spleen cells from normal DBA/2 mice.

[b] Supernatant heated for 30 min at 56°.

[c] Average response (±sd) of 5–6 cultures 5 days after *in vitro* immunization with sheep RBCs.

cells, can markedly depress the normal *in vitro* immune response of splenocytes to sheep erythrocytes. There have been no previous reports indicating that mastocytoma cells, which are extensively used in cellular immunology as a "target" cell for cell-mediated immune reactions *in vitro* (2–4), have immunosuppressive properties. The results of the present study clearly show that this tumor cell, when incubated *in vitro* with normal splenocytes, can markedly affect the normal immune responsiveness of the immunocytes.

It seems likely that immunosuppression is due to a product or factor associated with the mastocytoma cells and not merely to the physical "crowding" out or overgrowth of the normal splenocytes by the rapidly growing tumor cells. Others have reported that a number of tumor cells appear to secrete "factors" which are immunosuppressive, both *in vivo* and *in vitro* (7–10). For example, Ehrlich ascitic tumor cells or a soluble product derived from such cells adversely influence antibody formation. Similarly, it is now widely recognized that leukemic cells, especially those induced by murine leukemia virus, are immunosuppressive, both *in vivo* and *in vitro* (11, 12).

Recent studies in this laboratory indicated that a cell-free homogenate prepared from Friend virus leukemic splenocytes, even though rich in infectious virus particles, could not influence the antibody response of normal splenocytes immunized *in vitro* with sheep erythrocytes (13). Only leukemic splenocytes *per se*, and not cell-free homogenates, were immunosuppressive. In contrast, the results of the present study indicate that not only viable mastocytoma cells, but also cell-free extracts derived from these tumor cells can suppress the immune response of normal spleen cells *in vitro* to sheep erythrocytes. It seems unlikely that the active factor in the soluble extract from the mastocytoma cells is a tumor virus or a large macromolecule since heating at 56° for 30 min completely abolished the suppressive effect of the cell-free extract. An additional possibility explaining immunosuppression would take into account the possible presence of tumor specific transplantation antigens on the mastocytoma cells. Such antigens could influence the immune response of normal DBA/2 spleen cells to sheep erythrocytes by means of "antigenic competition". However, it seems unlikely that such transplantation antigens, if they exist, would be completely inactivated by heating at 56°.

These observations that cell-free supernatants prepared from mastocytoma cells are immunodepressive *in vitro* should permit further physicochemical analysis of the factor(s) and evaluation of the significance of immunodepression associated with this tumor cell product to the general area of tumor immunology.

Summary. Mastocytoma cells in vitro suppressed the immune response of normal syngeneic spleen cells to sheep erythrocytes. The immune response of dispersed spleen cells from normal mice *in vitro* was inhibited by simultaneous incubation with relatively small numbers of mastocytoma cells or a cell-free extract prepared from these cells. Inhibition occurred when diluted cell-free extracts from the mastocytoma cells or as few as 10^4 or 10^5 tumor cells were incubated with 5 million normal splenocytes. The supernatant factor from mastocytoma cells was labile to heating at

56° for 30 min. Detailed examination of the physicochemical properties of the mastocytoma cell extract capable of suppressing immune responses to sheep red cells should be of value.

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