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Antibody Mediated Neutralization of Immunosuppression Induced by Mastocytoma Ascites Fluids (40179)

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Mastocytoma tumor cells are widely used in cellular immunology as a target for either antibody or T-cell mediated cytolysis in vitro. These tumor cells also are markedly immunosuppressive, both *in vivo* and *in vitro* (1–6). Cell-free extracts derived from mastocytoma cells have marked immunosuppressive effects, both *in vivo* and *in vitro*. Furthermore, culture fluids from mastocytoma cells grown in vitro may similarly impair antibody formation. However, the nature and mechanism of such immunosuppression is still largely unknown. It seems likely that the immunosuppressive activity of cell-free extracts or culture fluids derived from mastocytoma cells may be directly related to immunosuppression induced by intact tumor cells *per se*. In order to ascertain whether there is an immunologic relationship between the mastocytoma suppressive factor present in cell-free extracts and culture supernatants of these tumor cells, antisera were prepared in the present study by appropriate immunization of rabbits. For this purpose intact mastocytoma cells grown *in vivo* or *in vitro*, as well as ascitic fluid from tumor-bearing animals, cell-free extracts of tumor cells *per se* and culture supernatants were used to immunize the rabbits. The resulting antisera were examined for their ability to neutralize the immunosuppressive activity of cell-free ascites fluid from mastocytoma-bearing mice.

Experimental animals. Young adult DBA/2 mice, weighing 18–20 g each, were obtained from Cumberland View Farms, Clinton, Tennessee. They were housed in groups of 6–8 in plastic mouse cages and fed Purina mouse pellets and water *ad libitum*.

Tumor cells. Mastocytoma cells, designated P815-X2 were initially obtained from the Wistar Institute, Philadelphia, PA, and have been passaged for several years in this laboratory by intraperitoneal (ip) injection of 1×10^6 cells every 2 weeks, as described elsewhere (1–4).

Tumor cell preparation and extracts. Mastocytoma cells were harvested from the peritoneum of mice, washed several times in medium RPMI 1640 (Grand Island Biological Co., Grand Island, NY) and cell-free extracts prepared by repeated freezing and thawing (6–10 times) of 50% suspensions of the tumor cells followed by centrifugation at 5000*g* for 30 min at 4°.

Ascites fluid. The ascites were harvested from mice bearing mastocytoma tumors 15–30 days after ip injection with the cells. The cells were separated from the fluid by low speed centrifugation (500*g*) at 4°. The supernatants were then further centrifuged at 100,000*g* for 90 min and the supernatant used for experiments.

Mastocytoma cultures. Mastocytoma cells (P815-X2) were maintained by weekly transfer of 5×10^5 cells in 75-cm^2 plastic tissue culture flasks (Corning Glass Work, Corning, NY), in RPMI 1640 medium with 10% fetal calf serum (Grand Island Biological Co., Grand Island, NY). For preparation of culture supernatants, 5×10^6 tumor cells were first cultured in medium containing FCS for 72 hr on the bottom of plastic tissue culture flasks. The confluent monolayers of mastocytoma cells were washed two times with Hanks' solution and the cells incubated an additional 24 hr at 37° in Hanks' solution in a moist chamber with 5% CO_2, 95% air. The culture supernatants were recovered by centrifugation at 100,000*g* for 90 min at 4°.

Immunization and serum preparation. Adult New Zealand white rabbits, 2–3 kg each, were immunized by intramuscular injections of 0.5 ml of one of the following preparations: (a) Intact tumor cells (5×10^6 viable cells); (b) cell-free mastocytoma extracts (10.0 mg/ml); (c) cell-free ascites fluid (10.0 mg/ml); and (d) culture fluids (1.0 mg/ml). Each of the preparations was emulsified in an equal volume of incomplete Freund's adjuvant (Difco Laboratories, Detroit, Mi). The rabbits

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were injected twice per week with each preparation and serum samples were obtained by weekly bleeding starting 3 weeks after the first immunization. All sera were absorbed for 3 hr at room temperature with an equal volume of packed suspensions of normal DBA/2 spleen cells, and, after clarification by centrifugation, heat-inactivated at 56° for 30 min. The absorbed serum samples were stored at −20° until used for testing.

Assay for in Vitro antibody formation. 5×10^6 Spleen cells from normal adult DBA/2 mice were cultured in 1.0 ml medium in individual vials exactly as described elsewhere (7). Cultures were immunized by the addition of 0.1 ml of a freshly washed suspension of sheep erythrocytes (2×10^6 RBCs). The numbers of antibody plaque forming cells (PFCs) developing in these cultures were determined by the direct hemolytic plaque assay without a supporting matrix exactly as described elsewhere (8). Three to four determinations were made for each cell suspension and the numbers of plaques on the slides were counted and averaged.

In Vitro immunosuppression and neutralization. Cultures of normal splenocytes were incubated *in vitro* with 0.2 ml ascites fluid from mastocytoma bearing mice. For neutralization 0.1 ml of a rabbit antiserum, in the appropriate dilution, was first added to a 1:10 dilution of the ascites fluid prior to incubation with normal splenocytes being immunized in vitro with SRBC. The tubes were incubated at 37° for 30 min and the incubation mixture centrifuged at 5000*g* for 30 min at 4°. The supernatants were removed with a Pasteur pipette and examined for residual immunosuppressive activity against antibody forming spleen cells.

Immunoelectrophoresis. Immunoelectrophoretic assays with the various rabbit antisera and ascites fluid were performed exactly as described elsewhere by standard technique (9).

Experimental results. Immunization of spleen cells from normal mice with sheep erythrocytes *in vitro* resulted in appearance of relatively large numbers of PFCs (Table I). Treatment of these cultures with ascitic fluid from mastocytoma bearing mice, but not from control mice, markedly depressed the antibody response. However, if the ascitic fluid was first incubated with serum from rabbits immunized with ascitic fluid from mastocytoma-bearing mice, neutralization of immunosuppression occurred. Furthermore, antiserum prepared against extracts of tumor cells present in the ascites, as well as extracts of solid mastocytoma cells, neutralized the immunosuppressive activity (Table I). In addition, antisera prepared by immunizing rabbits with cell-free culture supernatants from the mastocytoma cells grown in vitro also neutralized the immunosuppressive activity of the ascites fluid. In all cases rabbit antiserum against normal mouse serum or sera from rabbits immunized with other tumor cells had no neutralizing activity against the immunosuppressive ascitic fluid from the tumor-bearing mice.

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TABLE I. EFFECT OF ANTISERA TO CELL-FREE EXTRACTS AND CULTURE SUPERNATANTS OF MASTOCYTOMA CELLS ON ANTIBODY RESPONSIVENESS OF NORMAL SPLEEN CELLS IMMUNIZED *in vitro* WITH SHEEP ERYTHROCYTES.

Culture group	Addition to normal DBA/2 spleen cell cultures[a]	PFC/10^6 spleen cells[b] ± SD	Percent of control	Neutralization of Immunosuppression
A	None (Control)	1,154 ± 257	—	—
B	Ascites fluid no addition	510 ± 56	42.7	—
C	plus normal rabbit serum	504 ± 62	42.2	No
D	plus antisera to ascitic fluid	1,412 ± 91	118.3	Yes
E	to ascitic cell homogenate extract	1,508 ± 396	126.3	Yes
F	to mastocytoma homogenate extract	1,132 ± 319	94.8	Yes
G	to culture supernatant	1,207 ± 419	101.1	Yes
H	to normal mouse serum	496 ± 183	41.5	No

[a] 5×10^6 Spleen cells from normal DBA/2 mice immunized *in vitro* with 2×10^6 SRBC and tested for PFC 5 days later; treated cultures incubated with ascites fluid from mastocytoma bearing mice plus indicated rabbit serum on day of culture initiation.

[b] Average PFC response of 5–8 cultures per group.

ascitic fluid from the tumor bearing mice was not neutralized with rabbit antiserum against normal mouse serum, the rabbit antiascitic fluid serum was absorbed by normal DBA/2 mouse sera, since the ascitic fluid of tumor bearing mice contained normal serum components. These sera were then utilized for immunoelectrophoretic analyses.

As can be seen in Fig. 1, the absorbed serum from rabbits immunized with ascites fluid from the mastocytoma-bearing mice reacted with the ascitic fluids by forming precipitin bands in the immunoelectrophoretic assay. One line of precipitation occurred. When normal rabbit serum was similarly reacted with the ascitic fluid, there was no precipitation. Furthermore, normal DBA/2 serum or immunoglobulin, as well as normal DBA/2 tissue suspension, including spleen cells, did not remove this precipitin reactivity of the antiascites fluid serum after absorption, indicating that the absorbed antiascites fluid serum was not reacting with a normal serum protein or a material antigenically related to serum or tissue protein present in ascitic fluid of tumor bearing mice.

Discussion. Mastocytoma cells and cell-free extracts derived from these cells, as well as culture supernatants from tumor cells growing *in vitro*, are markedly immunosuppressive (1–6). Addition of relatively small numbers of tumor cells *per se*, as well as ascitic fluid or cell-free culture supernatants to normal spleen cell cultures markedly impaired antibody formation to SRBCs. As shown in the present study, sera prepared in rabbits immunized with either cell-free extracts of the tumor cells, cell-free ascitic fluid or culture supernatants were equally effective in neutralizing the immunosuppressive activity of the cell free ascitic fluids from the tumor bearing mice. Thus it is likely that the immunosuppressive substance present either in intact tumor cells *per se*, or in cell-free extracts or culture supernatants are antigenically related, even if not identical. Since neutralization of suppressive activity of ascitic fluid was achieved even with antibody prepared in rabbits immunized with cell-free culture fluids, it is evident that the mastocytoma cells growing *in vitro* release a factor(s) which induces antibody capable of neutralizing the suppressive activity of ascitic fluid *per se*. These findings support the concept that the immunosuppressive material present in ascitic fluid from mice bearing *in vivo* implanted tumor

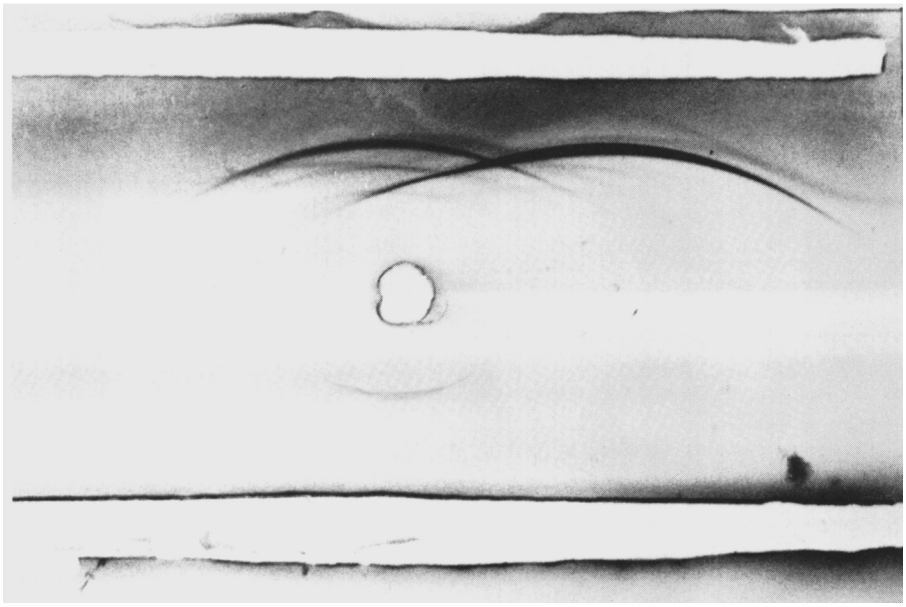

FIG. 1. Immunoelectrophoretic analysis of ascitic fluid. Upper trench contained rabbit antiserum against ascitic fluid; well contained ascitic fluid; lower trench contained rabbit antiserum against ascitic fluid which was absorbed by normal DBA/2 mouse serum. (Cathode towards left).

cells and cell-free culture supernatants from *in vitro* growing tumor cells are immunologically related.

It should be noted that recent studies in other laboratories indicated that immunosuppressive activity derived from serum of tumor bearing individuals could be neutralized by allogeneic serum with specificity towards immunoglobulins (10). Other studies in this laboratory, however, have shown that absorption of the rabbit antiserum with normal mouse immunoglobulins or DBA/2 tissue did not remove the precipitating antibody as detected by gel diffusion analysis and also did not affect the immunosuppressive activity. Furthermore, recent studies in this laboratory indicated that Sephadex G-200 fractionation procedures (using sodium phosphate buffer, pH 7.0) with the ascitic fluid from mastocytoma-bearing mice resulted in three major peaks. When fractions under the peaks were tested by immunodiffusion reactions against the absorbed rabbit antisera to the ascitic fluid, the first fractions resulted in a positive precipitin reaction. The other fractions did not react with this antiserum. When the three major peaks were examined for immunosuppressive activity by the hemolytic plaque assay, activity was detected only for the first fraction. When ascitic fluid from the mastocytoma-bearing mice was subjected to Sephadex G-200 filtration in sodium acetate buffer adjusted to pH 3.6, three peak fractions were also obtained, similar to that at neutral pH. However, the last peak showed a significant increase in 280 nm absorbing material. When the peak fractions were assayed for PFC inhibitory activity after adjustment to pH 7.0, it was found that the immunosuppressive activity was present exclusively in the last peak. The molecular weight of the last fraction was estimated by Sephadex G-200 gel filtration, using standard proteins of known molecular weights. The results with the Sephadex G-200 gel filtration analysis showed that the material with the immunosuppressive activity had a molecular weight in the range of 5000 or less. These results indicate that the immunosuppressive material present in the ascitic fluid of mastocytoma-bearing mice may be a small molecular weight peptide which normally complexes with relatively larger molecular weight carrier proteins (presumably alpha globulins).

Summary. Immunosuppression induced in vitro by cell-free ascites fluid from mastocytoma bearing mice was examined in regards to neutralization by antiserum prepared in rabbits. Serum from rabbits immunized with mastocytoma cell extracts or culture supernatants readily neutralized ascitic fluid from tumor-bearing animals in regards to immunosuppression of normal mouse spleen cells immunized *in vitro* with sheep red blood cells. Rabbit serum to normal mouse immunoglobulins had no neutralizing effect on the immunosuppressive ascitic fluid. Antisera to ascites fluid resulted in a sharply defined precipitin arc after immunoelectrophoresis which could not be abolished by absorption with normal DBA/2 serum. Thus the immunosuppressive factor present in ascites fluid, as well as in cell-free extracts and culture fluids of mastocytoma cells, appeared to be antigenically distinct from mouse immunoglobulins and readily neutralized by serum from rabbits immunized with various mastocytoma preparations.

1. Kamo, I., Kateley, J., and H. Friedman, Proc. Soc. Exp. Biol. Med. **148,** 883 (1975).
2. Kamo, I., Patel, C., Kateley, J., and Friedman, H., J. Immunol. **114,** 1749 (1975).
3. Kamo, I., Patel, C., Patel, N., and Friedman, H., J. Immunol. **115,** 382 (1975).
4. Kamo, I., Patel, C., and Friedman, H., J. Nat. Cancer Inst. **56,** 333 (1976).
5. Friedman, H., Specter, S., Kamo, I., and Kateley, J., Ann. N.Y. Acad. Sci. **276,** 417 (1976).
6. Kamo, I., and Friedman, H., Progr. Cancer Res. **25,** 271 (1977).
7. Kamo, I., Pan, S. H., and Friedman, H., J. Immunol. Methods **11,** 55 (1976).
8. Cunningham, A. J., and Szenberg, A., Immunology **14,** 599 (1968)
9. Tozawa, H., Watanabe, M. and Ishida, N., Virology **55,** 242 (1973)
10. Levy, J. G., Smith, A. G., Whitney, R. B., McMaster, R., and Kilburn, D. G., Immunology **30,** 565 (1976).

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