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Characterization of a Dialyzable Immunosuppressive Fraction from Mastocytoma Culture Supernatants (39901)

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Generalized immunosuppression often accompanies the oncogeneic process. For example, tumor-bearing individuals, either experimental animals or man, may evince a state of generalized immunological impairment as shown by suppressed responsiveness to a wide variety of antigens (1-4). A number of mechanisms have been proposed for immunosuppression in animals bearing experimental tumors; these include the presence of suppressor T lymphocytes or suppressor macrophages. Tumor-inducing viruses and/or tumor-associated antigens, including alpha fetoprotein, have been reported to impair normal immunocompetence (5). "Blocking" factors, thought to be complexes of tumor antigens and antibody, also are known to interfere with immune responsiveness, both *in vivo* and *in vitro* (6).

Earlier studies in this laboratory with mastocytoma cells showed that this tumor cell line, which is widely used in experimental immunology as the "target" cell for assessing cellular or humoral immunity *in vitro,* has the ability to markedly depress immune responsiveness of normal splenocytes immunized *in vitro* with sheep erythrocytes (7-9). Cell-free extracts of mastocytoma cells, as well as extracts of splenocytes from mastocytoma-bearing mice or ascites fluids from these animals, also are immunosuppressive. In the present study mastocytoma cells cultured *in vitro* in serum-free medium were found to release a relatively low molecular weight dialyzable factor with immunosuppressive properties. This dialyzable factor was much smaller than many of the subcellular immunosuppressive substances which have been prepared from a variety of tumor cells (10-12), but relatively similar in nature to a polypeptide moiety reported present in serum of some cancer patients, but not normal individuals (13).

Methods and materials. For these studies mastocytoma cells (designated PB815X) were initially obtained from the American Type Culture Collection (Rockville, Maryland) and have been passaged in this laboratory *in vitro* for over 1 year (7-10). The cell line was maintained by weekly transfer of 5×10^5 cells in 75-cm^2 plastic tissue culture flasks (Falcon Co., Oxford, California), in RPMI 1640 medium with fetal calf serum (FCS). For preparation of culture supernatants, cells were first cultured in medium containing FCS for 2-3 days. The cultures were washed with medium without FCS and then recultured in 4 ml of FCS-free RPMI 1640 for 24 hr in a humidified CO_2 incubator. The supernatants were obtained after centrifugation at 1000*g* for 30 min. For dialysis 50 to 60 ml of pooled culture fluids were placed in dialysis bags (Arthur H. Thomas Co., Philadelphia, Pennsylvania, Catalog No. 3787-D32) and dialyzed against 20 vol of demineralized water for 24 hr. The dialysates, as well as the residue within the dialysis bags, were concentrated 20- to 30-fold by pervaporation. These were then subjected to gel filtration on Sephadex columns (Pharmacia Fine Chemicals, Uppsala, Sweden), using G10 and G25 Sephadex beads which were swollen in demineralized water overnight and then poured into appropriate columns. One-milliliter eluates were obtained from the columns using demineralized water at a rate of 3 ml/hour. Optical density at 280 μm was determined for each fraction.

The culture supernatants, dialysates, and individual and pooled Sephadex fractions were tested for effects on antibody formation using an *in vitro* culture system (14). For this purpose 5×10^6 viable splenocytes from normal DBA/2 mice were cultured in glass-bottom vials (Arthur H. Thomas Co., Philadelphia, Pennsylvania, Catalog No. 9710B24) exactly as described earlier by Kamo *et al.* (7-9) as a modification of the Mishell-Dutton culture system (15). Each

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culture was stimulated *in vitro* with 2×10^6 washed sheep erythrocytes (SRBC) and the number of hemolytic antibody plaque forming cells (PFCs) was determined 5 days later as described earlier, using the standard Jerne hemolysis in agar gel plaque assay (16).

Results. Supernatants of mastocytoma cells incubated for 24 hr in FCS-free medium markedly suppressed the PFC response of normal splenocytes (Table I). Addition of 0.5 ml of a supernatant to 1.5 ml of culture medium resulted in a 50% or greater depression in the number of PFCs. Such suppression was essentially similar to that observed when 5×10^6 mastocytoma cells were added directly to the same number of splenocytes (7, 8).

Suppressive activity was present mainly in the dialyzable portion of the supernatants, since addition of concentrated dialysates from clarified supernatants was just as effective as the undialyzed supernatant. The residual material within the dialysis bags had little or no suppressive effects (Table I). Sephadex fractionation indicated that the suppressive factor in the dialyzable material was readily recoverable in the early postvoid volume and thus appeared to have a molecular weight between 1000 and 5000 (Table II). Fractions obtained after elution of the columns with demineralized water were only slightly suppressive, if at all.

The active immunosuppressive fractions from the Sephadex column were stable to heating at 56° for 30 min, but were readily inactivated by heating at 80° for 30 min, or by boiling for 5–15 min (Table II). Furthermore, incubation of 10 to 20 μg of the active Sephadex fraction with 10–25 μg of trypsin abolished immunosuppressive activity. Similar incubation with even higher concentrations of DNase or RNase had no inhibitory effect on the suppressive material.

Discussion. These results indicate that a low molecular weight dialyzable material secreted by mastocytoma cells grown in serum-free tissue culture medium for 24 hr has marked immunosuppressive properties in terms of inhibiting the expected PFC responsiveness of normal splenocytes immunized *in vitro* with SRBC. The active factor appeared to have a molecular weight smaller than 5000 but greater than 1000 as determined by Sephadex gel filtration assays. Furthermore, the active material was always present in ninhydrin-positive fractions and could be inactivated by treatment with trypsin *in vitro* but not by DNase or RNase treatment.

Further characterization studies should permit a more detailed evaluation of the physicochemical nature of this suppressive factor secreted by mastocytoma cells. It seems unlikely that the material is merely salt-free amino acids or other simple constituents of the mastocytoma cells or the culture medium. On the other hand, it is possible that this relatively low molecular weight material found in supernatants of mastocytoma cultures is related to the immunosuppressive material extractable directly from mastocytoma cells growing in DBA/2 mice, as well as splenocytes or ascites fluid from

TABLE I. EFFECT OF DIALYZABLE SUPERNATANT FROM SERUM-FREE MASTOCYTOMA CULTURES ON HEMOLYTIC ANTIBODY PLAQUE RESPONSES OF NORMAL SPLEEN CELLS IMMUNIZED *in vitro* WITH SHEEP ERYTHROCYTES.

Material added to splenocyte cultures[a]	Spleen cell culture response[b]		
	PFC/culture (± SE)	Percentage of control	Cell viability (± SE)
None (control)	9940 ± 179	–	46.0 ± 1.5
Mastocytoma supernatant[c]	4013 ± 584	40.5	48.0 ± 1.2
Supernatant heated[d]	9260 ± 230	93.2	49.2 ± 2.2
Dialyzate[c]	4000 ± 210	40.2	49.5 ± 1.5
Dialyzate heated[d]	9830 ± 236	98.9	48.1 ± 1.8
Dialyzate residue	9375 ± 154	94.3	49.5 ± 2.1

[a] Cultures of 5×10^6 normal DBA/2 mouse spleen cells in 2.0 ml of medium in flat-bottomed culture chambers incubated with 0.5 ml of indicated material or same volume of medium only.

[b] Average response of five to six cultures 5 days after *in vitro* immunization with 2×10^6 RBCs.

[c] Supernatant obtained from 24-hr cultures of mastocytoma cells and used untreated or after dialysis.

[d] 80° for 30 min.

TABLE II. EFFECT OF SEPHADEX FRACTIONS PREPARED FROM DIALYZED CONCENTRATES FROM MASTOCYTOMA SUPERNATANTS ON HEMOLYTIC ANTIBODY RESPONSIVENESS OF NORMAL SPLEEN CELLS IMMUNIZED *in vitro* WITH SHEEP ERYTHROCYTES.

Sephadex fraction tested[a]	PFC/culture[b] ± SE	Cell viability[b] ± SE
None (control)	7650 ± 158	52.1 ± 1.8
Fraction A		
Untreated	3341 ± 260	51.3 ± 2.2
Heated 56°, 30 min	3150 ± 210	50.9 ± 2.0
Heated 80°, 30 min	6950 ± 225	50.5 ± 1.6
Trypsin-treated	7100 ± 176	53.2 ± 1.9
DNase-treated	3831 ± 230	50.3 ± 2.0
RNase-treated	3610 ± 159	51.9 ± 2.6
Fraction B untreated	7230 ± 265	50.3 ± 1.9
Fraction C untreated	7410 ± 167	51.2 ± 1.7

[a] Sephadex fractions obtained from G-10 columns (1 × 58 cm) used for treatment of cultures (0.5 ml/2.0 ml of culture medium) containing 5 × 10^6 normal DBA/2 spleen cells. Fraction A comprised the postvoid volume fractions with immunosuppressive activity pooled and used before and after heating or treatment with 10–20 μg of enzyme for 20 min; Fraction B consisted of pooled void volume fractions which showed little or no suppressive activity; and Fraction C consisted of pooled eluate fractions.

[b] Average response of three to five cultures 5 days after *in vitro* immunizations with 2 × 10^6 SRBCs.

such tumor-bearing animals. Earlier studies had indicated that extracts from the tumor cells per se, as well as spleen cells from the tumor-bearing animals, were nondialyzable (8, 9). Nevertheless, the active factor did not appear to be a large macromolecule since activity was not sedimentable by centrifugation at 100,000*g* for 90 min. In this regard, mastocytoma cells cultured in serum-containing medium also resulted in an immunosuppressive supernatant which was nondialyzable (unpublished data). Therefore, the active material secreted by mastocytoma cells *in vivo* may indeed be a relatively low molecular weight peptide which, in the presence of serum or other macromolecules in tumor-bearing animals or serum-containing culture medium, forms a nondialyzable complex with larger molecules.

The presence of a dialyzable low molecular weight immunosuppressive substance in supernatants of mastocytoma cells grown in serum-free medium suggests that the active substance responsible for immunologic impairment due to mastocytomas might be readily purified so as to permit further immunochemical analyses. Furthermore, the relationship of this factor to similar low molecular weight suppressive substances in the serum of cancer-bearing animals and patients certainly should be investigated. It would also be of interest to relate this low molecular weight factor with similar supernatant factors recently found in cultures of other mastocytoma cell lines as well as cultures of other tumor cell lines which depress cell-mediated immunity *in vitro* (11–15).

Summary. Immunosuppression induced by mastocytoma cells is related to a soluble factor present both in the supernatants of tumor cell cultures and in tumor cell extracts and ascites fluid of tumor-bearing animals. In the present study culture supernatants of mastocytoma cells growing in serum-free medium contained an immunosuppressive material which was readily dialyzed. The concentrated dialysates from the culture supernatants were fractionated by Sephadex gel filtration. The postvoid volume fraction from Sephadex G-10 columns contained most of the immunosuppressive activity present in the dialysates. The material in the active fractions had a molecular weight in the range of 1000 to 5000. The immunosuppressive activity of the culture supernatants, the concentrated dialysates, and the postvoid volume Sephadex fractions were stable to heating at 56° for 30 min but was inactivated when heated at 80°. Furthermore, the immunosuppressive activity was inhibited by treatment with trypsin *in vitro* but was resistant to treatment with DNase or RNase. These results suggest that a relatively small molecular weight dialyzable material, possibly polypeptide in nature, is responsible for the immunosuppressive activity of mastocytoma cells.

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