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Heterogeneity of KCl-solubilized Antigens of Chemically Induced Sarcomas[1] (Immunology) (40052)

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When tested by transplantation techniques chemically-induced tumors appear to carry tumor-unique, noncross reacting transplantation antigens (1–3). In contrast, *in vitro* assays such as microcytotoxicity (4, 5), lymphocyte proliferation (6, 7) and antibody-mediated cytotoxicity (8) show that these tumors also share common antigens in addition to their unique transplantation antigen. Evidence of cross-reactivity between chemically-induced tumors has also been demonstrated through delayed hypersensitivity testing (9, 10) and in certain circumstances by transplantation tests (1, 6, 11).

In previously reported studies from this laboratory (6), solubilized tumor membrane preparations were found to contain antigens which crossreacted among several fibrosarcomas as well as antigens which were unique to each individual tumor. Lymphocyte proliferation assays (LPA) demonstrated these same preparations also contained antigenic activity which, when given with adjuvants, provided tumor specific protection in transplantation tests. Studies presented in this paper show that the biologically active material in such membrane preparations are heterogeneous in molecular size, and that components of different size may either stimulate or inhibit proliferation in lymphoid cell subsets found in the tumor-bearing animal.

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Material and methods. Animals. Female, C57Bl/6J mice were purchased from the Jackson Laboratories, Bar Harbor, Maine. The animals were 6 weeks old upon receipt and were used in experiments when 9–10 weeks old. Animals were fed a diet of Mouse Chow (Purina Co.) and water *ad libitum*.

Tumors. Methylcholanthrene (0.5 mg in 0.1 ml olive oil) was injected into ten animals at four intramuscular sites. Fibrosarcomas become apparent in all mice within 6–12 weeks and were frozen following one passage in syngeneic mice (C57Bl/6J) or were passaged in syngeneic mice for not more than ten passages. Two of these tumors (M3 and M4) were utilized in this study. These tumors were tested extensively and shown to have unique (non cross protective) transplantation antigens, but shared antigens as detected in the LPA (see below). The tumors utilized were not selected on the basis of any known attribute but represented a random selection among those tumors available.

Preparation of solubilized tumor antigens and fractions. A modification of the procedure of Reisfeld (12) as described by Forbes (6) was used to solubilize antigens from sarcomas. The amounts added to cultured lymphocytes were graded by dilution of the crude or fractionated material, and indicated in microliters. Since the character of the activity had not yet been defined chemically, it was not appropriate to express it in terms of protein content. Solubilized tumor membrane preparations were fractionated by ultrafiltration through Amicon filters of varying pore size. Fractions obtained are designated in this report by the molecular weight range contained in the fraction. For example, fraction 50,000–100,000 contained material which passed an XM50 membrane (exclusion limit 50,000 daltons). Fraction <30,000 contained all material which passed a PM30 membrane (exclusion limit 30,000 daltons). That mate-

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rial retained above a membrane represented material which remained after extensive "dialysis" with large volumes of buffer (phosphate buffered saline, pH 7.2). The solubilized tumor antigen preparations were referred to by a tumor number designation followed by the bracketed suffix [s]. The Amicon membranes used in these procedures included XM300, XM100, XM50, and PM30. Membranes were used according to the manufacturers instructions and were only used one time. The molecular size performance of such membranes is well documented, and confirmed independently in this laboratory in nonconcurrent experiments.

Preparation of lymphoid cells. Spleen or lymph node cells were removed from exsanguinated mice under aseptic conditions. After mincing with scissors in RPMI-1640 (Gibco) the cells were passed through a 60 mesh stainless steel screen followed by sequential passage through 20 ga, 22 ga, and 25 ga needles. Single cell suspensions were washed three times in RPMI-1640 and were enumerated for viable cells by the trypan blue exclusion procedure.

Assay of tumor antigen stimulation of lymphoid cells. The lymphocyte proliferation assay (LPA) employed was based upon that described previously (6), but modified to use microplates and automated cell harvesting as described by Hartzman (13). Five hundred thousand lymphocytes per well in 0.2 ml RPMI containing 100 μg/ml streptomycin, 100 nm/ml penicillin and 5% heat inactivated human serum per ml, were distributed in round bottomed Linbro (≠IS-MRC-96) microplates. The antigens or dilutions thereof were added in a volume of 20 μl. The cultures were incubated at 37°C in an atmosphere of 5% CO_2. Tritiated thymidine (0.5 μCi) was added to each well after 48 hr incubation. Following 18 hr additional incubation the cultures were harvested on glass fiber filters with the harvester. The samples were then processed and counted in a Beckman LS250 liquid scintillation counter. Data presented represented the mean cpm values plus or minus one standard error (SE) of four replicate cultures at each dose tested.

In each experiment lymphocytes of tumor-bearing animals were taken from the animals 3 weeks following an injection of 1×10^5 viable tumor cells intramuscularly. Tumor cells for injection were prepared as previously described (14). Normal lymphocytes were taken from normal, age matched mice.

Results. Chemical characterization of lymphocyte stimulating activity in KCl solubilized tumor membranes requires initial purification of large volumes of material. Ultrafiltration through selected membranes provided a means to separate large volumes into fractions which were suitable for further characterization. In addition, these fractions were found to have selective effects on lymphocyte subpopulations. Unfractionated 3 *M* KCl solubilized tumor preparations of murine fibrosarcomas are often viscous, difficult to filter to assure sterility, and inactive in a lymphocyte proliferation assay, possibly due to content of polymeric DNA. It was found initially that a single purification step passage through ultrafilters excluding material over 300,000 daltons usually yielded a nonviscous fraction which contained the material stimulatory in the LPA. However, some lots of antigen were inactive after such a preliminary step (see Fig. 2A).

This note describes an extension of this approach in which fractions which were or were not inactive after this initial step were further fractionated and then tested for a capacity to stimulate spleen of tumor-bearing animals. Data from a representative experiment are shown in Figs. 1 and 2. Spleen cells were stimulated in a double peak pattern by unfractionated solubilized material (Fig. 1A). A major, broad peak occurs at the high (10 μl) dose and a second peak is found in the low (1 μl) dose range. The second peak was characterized both by a narrow range of effective dose and smaller magnitude, as compared with the high dose peak. Fractions containing material under 100,000, 50,000, or 30,000 daltons, respectively, gave reduced capacity to stimulate spleen cells especially that below 30,000 (Fig. 1D). In some experiments, the low dose peak persisted in this latter fraction (see Fig. 3, for example) suggesting that the active fraction has a lower limit of molecular size on the order of 30,000.

Preparations inactive after passage through the XM300 membrane were further fractionated by extensive dialysis on XM100 and XM50 membranes, which yield preparations

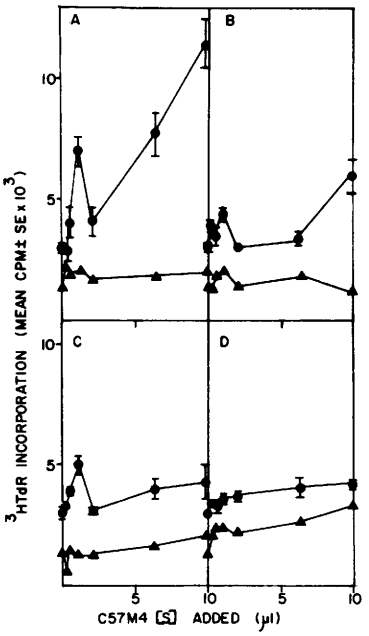

FIG. 1. LPA data from culture of spleen cells taken from normal (▲) or M4-bearing (●) animals, to which varying amounts of: (a) Unfractionated M4-[s]. (b) M4-[s], <100,000. (c) M4-[s], <50,000. (d) M4-[s], <30,000, had been added. Data represents one means of four replicates ± SE.

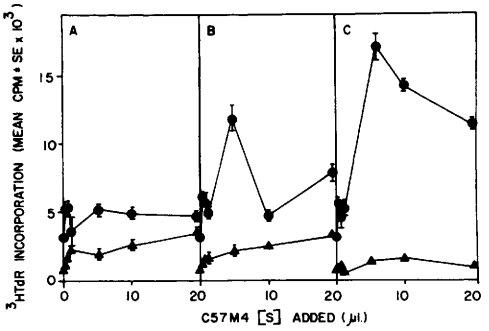

FIG. 2. LPA data from spleen cells taken from normal (▲) of M4-bearing (●) animals to which had been added varying amounts of (a) M4-[s], <300,000. (b) M4-[s], 100,000–300,000. (c) M4-[s], 50,000–100,000.

having both upper and lower limits of molelcular size. Activity in such preparations were greatest in the 50,000–100,000 dalton fractions. A representative experiment is shown in Fig. 2.

Unfractionated solubilized tumor membranes derived from syngeneic tumors contained lymphocyte stimulating activities shared by some but not all syngeneic MCA

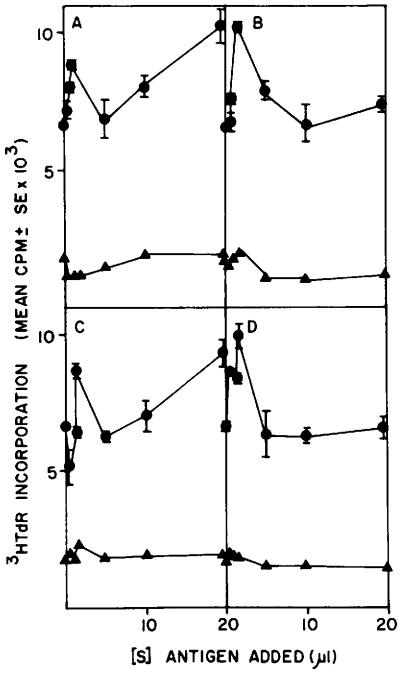

FIG. 3. LPA data from spleen cells taken from normal (▲) or M4-bearing animals (●), to which had been added varying amounts of (a) M4-[s], 30,000–300,000. (b) M4-[s], <30,000. (c) M3-[s], 30,000–300,000. (d) M4-[s], <30,000.

tumors (6, 7). It seemed possible that such crossreacting antigens might segregate into fractions of differing molecular size, and thus permit identification of a tumor-unique antigenic moiety. Figure 3 represents data from experiments in which fractions from two tumors were tested simultaneously. That containing the higher (30,000–300,000 daltons) or lower (<30,000 daltons) molecular weight molecules derived from the M3 or M4 tumor gave virtually identical patterns of stimulation of spleen cells taken from animals bearing the M4-tumor. Thus, no differences in the molecular size spectrum difference of the antigenic material was detected by this approach.

Discussion. The data presented here define further some characteristics of the lymphocyte stimulating material present in 3 *M* KCl-solubilized methylcholanthrene-induced fibrosarcomas. Application of a graded filter technique showed that stimulatory moieties involved are of two general categories in molecular size. One is about 30,000 daltons and

the other 50,000–100,000 daltons. The latter is effective at a high dose level; the crude 3M KCl solubilized membrane preparations also appear to contain both high (>300,000) and low (<50,000) molecular weight inhibitory substances. Whether these instances were specific or nonspecific was not determined. The concomitant presence of both antigens which stimulate lymphoid cells and inhibitors of unknown character obviously complicates the interpretation of any experimental data obtained with unfractionated material.

Quantitative aspects of the dose response kinetics, the two peak stimulatory behavior, may have at least two explanations. For example, two specific lymphocyte subpopulations may be responding to the high and low molecular weight fractions. On this premise, low molecular weight stimulation gives a sharp peak, with inhibition at higher dose levels, reminiscent of *in vitro* T-cell responses to mitogens and antigens (15, 16). The high dose stimulation peaks are sustained over a broader range of dose, or show progressively larger magnitude. The latter patterns suggest cumulative activation of multiple cell subsets, or nonspecific polyclonal activation of B-cell subsets by B-cell mitogen (17). It is established that both T- and B-cell subsets are stimulated by solubilized tumor preparations in the lymphocyte proliferation assay (unpublished), and that a third, non-T, non-B cell, nonmacrophage subset ("null cells") is also stimulated by such antigens. This explanation is thus viable, but not established by any data given herein.

A second explanation might be that the low dose stimulatory material is present in crude preparations in a concentration significantly higher than that giving high dose stimulation. Possibly responsible for the latter could be that the material is in polymeric complexes.

The presence of inhibitors of uncertain specificity is an observation of considerable practical importance in refining the LPA into a more useful experimental and clinical (18) tool. These data include that a relatively simple purification step can yield stimulatory tumor antigen or more uniform quality.

Summary. Solubilized antigens of murine methylcholanthrene-induced fibrosarcomas prepared by treatment of tumor tissue with hypertonic KCl were separated into fractions depending upon exclusion by molecular weight by selected ultra-filters. These studies showed that material of both high molecular weight (>300,000) and low molecular weight (<30,000) inhibited ^{3}H-thymidine incorporation by lymphoid cells taken from tumor-bearing animals. When the inhibitor were removed, the material remaining stimulated proliferation in such lymphoid cells over a wide range of molecular weights (<30,000 daltons to >100,000 daltons).

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