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STUDIES ON A FACTOR CHEMOTACTIC FOR MACROPHAGES ELABORATED BY SAD/2 FIBROSARCOMA CELLS

by

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ABSTRACT

STUDIES ON A FACTOR CHEMOTACTIC FOR MACROPHAGES
ELABORATED BY SAD/2 FIBROSARCOMA CELLS

By

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Preliminary studies with serum-containing supernatant from the SAD/2 fibrosarcoma cell line grown *in vitro* determined that the supernatant was apparently chemotactic for macrophages when chemotaxis was assessed microscopically. Prior to isolation and purification of this tumor material, a rapid method for the assessment of chemotaxis utilizing ^{3}H-uridine labeled macrophages was developed. This method employed the use of double polycarbonate filters which separated the lower wells containing ^{3}H-uridine labeled macrophages in modified blind-well Boyden chambers. After incubation, cells on the lower filters were precipitated with trichloroacetic acid, and radioactivity was assessed by liquid scintillation spectrometry.

The radioactive method was compared to the microscopic method of quantitating chemotaxis. There was good correllation between microscopic and radioactive methods (10% difference in the two methods) when either endotoxin treated serum, a known chemotactant, or the tumor supernatant was used as a chemotactant.

Since good correllation existed between the two methods, the radioactive method was used to determine whether the tumor supernatant (TS) was a true chemotactant or a chemokinetic agent. Four sets of chambers were assembled. The first set contained cells suspended in medium in the top well, TS in the lower well. The second set contained cells suspended in TS in the top well, TS in the lower well. The third set

contained cells suspended in TS in the top well, medium in the lower well. The fourth set (control) contained cells suspended in medium in the top well, and medium in the lower well. A 5-10 fold increase in radioactivity of filters from the first set of chambers was observed. Radioactivity of the second and third sets of chambers were comparable to controls. The TS was considered a true chemotactant, since much greater radioactivity on filters of the second and third sets of chambers would be anticipated if the TS were merely chemokinetic.

Experiments were then begun to purify the chemotactant using the aforementioned assay to assess chemotactic activity. Methods used to isolate the tumor material labeled with ^{3}H-leucine were gel filtration through Sephacryl S200 and Sepharose 6B and gel electrophoresis. Serum-free supernatants from SAD/2 tumor cells were concentrated and chromatographed on Sephacryl S200. Fractions containing chemotactic activity were applied to a Sepharose 6B column, and then applied to gels for electrophoresis. Active fractions from the Sepharose 6B column contained two components on gels with and without sodium dodecylsulfate (SDS). The apparent molecular weights of the 2 components were 68,000 and 78,000 on SDS gels. Samples pretreated with 2 mercaptoethanol prior to SDS gel electrophoresis also contained two bands, which indicated single polypeptide chains. Trypsinized samples resulted in loss of the 2 bands on SDS gels, and the loss of chemotactic activity. Heat treatment of active Sepharose 6B fractions at 100 C for 15 minutes also resulted in loss of chemotactic activity.

Sepharose 6B active fractions were injected intraperitoneally into mice, and differential cell counts of the peritoneal cells were performed 48 hr later. These data were compared to cell counts from

control mice injected with phosphate buffered saline. There was a significant increase in the numbers of macrophages and a significant decrease in the number of lymphocytes in test animals compared to controls ($p < 0.01$). From these results it was concluded that the partially purified tumor material caused an inflammatory response *in vivo*. The potential implications of this tumor material in tumor-bearing animals was discussed.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LITERATURE REVIEW

The involvement of macrophages in protection of the host against tumors and tumor growth has received much attention by investigators in recent years. There is an extensive amount of literature on the effects of tumors and tumor cell products on macrophages, and on the effects of macrophages on tumor growth. Since the research reported in this thesis is limited to a study of a tumor product's affect on macrophage function, the literature review will be focused on the affects of tumors and tumor cell products on macrophage function. The second portion of the literature review will be devoted to chemotaxis and the methods used to study chemotaxis.

The literature concerning the affect of tumors on macrophage function is filled with contradiction as will become apparent during this literature review. A point to be remembered, however, is that investigators have worked with a variety of animal tumors in their research. Since all tumors are not the same, the host response as well as the *in vitro* response of inflammatory cells may be different depending on the tumor system.

The fact that large numbers of macrophages are found within the tumor mass of a variety of human and murine tumors has been established (10,21,22,54,55). The function of these macrophages in tumors has slowly become apparent. The suggestion that resident macrophages in tumors prevented metastasis was made by Evans (10) and Eccles & Alexander (8). These investigators found that a correlation existed between the number of macrophages within tumors and metastasis of tumors. This suggestion led Wood & Gillespie (55) to determine experimentally that when localized tumors were excised, trypsinized, and depleted of

macrophages, the tumors became metastatic when injected into syngeneic animals. Resident macrophages within tumors have also been reported to inhibit growth of tumor cells *in vitro* (11) or to be cytotoxic to tumor cells *in vitro* (12,39,40). Haskill (15) reported that a population of cells within tumors, probably of monocyte origin, was involved in antibody dependent cell mediated cytotoxicity of tumor cells *in vitro*.

Investigators have therefore observed large numbers of macrophages within tumors and have found them functional *in vitro* (11,12,15). These same investigators observed that many tumors continue to grow in spite of macrophage presence in the tumors. Consequently, investigators have become interested in the apparent failure of macrophages to control tumor growth.

Several investigators have observed that patients with tumors had impaired macrophage functions *in vitro* (3,47), and that surgical removal of the tumors restored macrophage function (50). These observations lead to the proposal that tumor cell products inhibit macrophage function. This depression of macrophage function has been measured in a variety of ways. First, tumor cells are reported to suppress macrophage migration *in vivo* and macrophage chemotaxis *in vitro* (25,47,48). Snyderman et al. (47,48) found a low molecular weight component (6-10,000 MW) obtained from the dialysate of tumor cell lysates that inhibited chemotaxis of macrophages *in vitro* when cells were pretreated with the component. When the lysate was injected into the flanks of mice, it inhibited an inflammatory response to PHA in the peritoneal cavity. Meltzer and Stevenson (26) also found that resident macrophages from tumors had depressed chemotaxis *in vitro*, but did not attempt to characterize the component which caused this affect. Secondly, inhibition of macrophage

phagocytosis has been associated with tumor growth. Otu et al (33) reported a marked depression of carbon clearance *in vivo* throughout the first 72 hr after tumor implantation in animals. Gallahon and Wood (14) found that macrophages isolated from small tumors had increased phagocytosis *in vitro*, but large tumors (>1cm^3) had a marked decrease in phagocytic function. Thirdly, impaired macrophage function has been measured by suppression of macrophage mediated resistance to infection with intracellular parasites (31). These investigators found that animals injected with tumor cells 24 hr prior to Listeria challenge had depressed rate of Listeria clearance from their livers. These same investigators were careful to observe in subsequent work however, that this impaired state was short-lived and was replaced by a state of greatly enhanced antibacterial resistance which closely paralleled a state of enhanced resistance to a second challenge of the tumor (32).

Depression of macrophage function has been attributed to a soluble product found in the serum *in vivo* (32,33), in tumor cell lysates (36, 47,48), and in supernatants of *in vitro* grown tumor cells (30,33). Most of these inhibitors have not been characterized, with the exception of the low molecular weight inhibitor obtained by dialysis of tumor cell lysates described by Snyderman and Pike (48).

Activation of macrophage function during tumor growth has also been of interest to investigators, since increased macrophage function has been associated with incidence of tumors in humans (24). These investigators reported that increased phagocytosis of radiolabeled aggregated serum albumin *in vivo* was correlated with resistance to tumor metastasis. Other researchers working with animal tumor systems have also observed

increased macrophage function. Stimulation of phagocytosis was observed in tumor-bearing mice by Meltzer and Stevenson (26). Meltzer, Tucker and Bruer (28) reported that peritoneal macrophages from tumor immunized BCG infected mice had increased chemokinetic response to tumor cells *in vitro*. Snodgrass et al., (44) and Schuller et al. (41) used pyran activated macrophages to obtain similar results. Blakeslee (2) reported that supernatants from tumor cells grown *in vitro* activated peritoneal macrophages to inhibit tumor growth when macrophages were co-cultured with tumor cells. Meltzer et al. (27) found a low molecular weight (15,000 MW) product in the supernatant of tumor cells grown *in vitro* that enhanced chemotaxis of normal and BCG infected macrophages. This material was produced in serum-containing medium. These investigators did not show whether this component was actively produced by the tumor cells or whether it was the result of the action of tumor cells on serum components in the medium.

The SAD/2 fibrosarcoma contains a large number of phagocytic cells (21). In addition, peritoneal macrophages from SAD/2 bearing animals were found to have an enhanced cytostasis of tumor cells *in vitro* (19). Since the tumor has a large number of macrophages, and its growth *in vivo* stimulated peritoneal macrophages, this was a convenient model to study tumor cell/macrophage interaction.

The research of this investigator has concentrated on purification of a macrophage chemotactant produced by the SAD/2 tumor cells grown *in vitro* in serum-free medium. A rapid method for assessment of chemotactic activity was developed to process large numbers of samples generated during the purification procedure. The second part of this literature review, therefore, will be devoted to recent methods used to

assess leukocyte chemotaxis. For more extensive reviews of chemotaxis, the reader is referred to Harris (17) and Wilkinson (53).

The word chemotaxis is derived from the Greek roots χυμο's (juice, liquid) and Ταξις (an arrangement, battle array), but has taken on the meaning of movement in biological contexts (53). This movement in leukocyte chemotaxis is a directional movement, toward or away from a concentration gradient. Chemotaxis toward a concentration gradient is important in inflammation. Inflammatory cells can respond to injury, infection, or tumor cells which may be the result of chemical gradients. The function of these inflammatory cells is to aid in repair of tissue, prevent the spread of infection, and possibly control tumor growth.

Most of the early research dealing with leukocyte chemotaxis employed implanting of capillary tubes containing test chemicals into animals (53). Observation of cells which migrated into the tubes after a suitable incubation period was used to assess chemotaxis.

The study of chemotaxis *in vivo* was modified by employing the skin window technique by Rebuck et al. (38). With this technique an area on the skin was scrapped, the test substance was applied to the lesion and the site was covered with a sterile coverslip. The cells which migrated into the site in response to the chemotactant attached to the coverslip, and they were stained and counted after an appropriate incubation period. Perillie and Finch (35) reported an improvement of the skin window technique which was quantitatively more accurate. A chamber containing physiological saline was placed over the lesion, and cells migrating into the chamber could be quantitated.

Recent research in leukocyte chemotaxis was stimulated by the introduction of a new *in vitro* technique for assessment of chemotaxis (4).

This system involved the use of a chamber containing two wells separated by a porous filter. The lower well contained the chemotactic substance and the upper one contained the cells. The cells then migrated through the pores toward a concentration gradient to the bottom surface of the filter. The filters were removed after incubation, and they were examined microscopically.

Various modifications of the Boyden chamber have been used to assess human (52) and murine (46) chemotaxis. These structural modifications essentially involve the same principles that Boyden used with either Millipore filters or polycarbonate filters separating the two wells.

The *in vitro* method of quantitation of chemotaxis originally utilized Millipore filters in chemotaxis chambers, followed by staining and counting polymorphonuclear leukocytes (PMN) which had migrated through the pores to the opposite side of the filter (4). Other investigators have used the leading front of cells within the pores to assess chemotaxis (56). This latter technique makes identification of cell type difficult when mixed cell populations are used in chemotaxis systems.

Another method used to quantitate PMN chemotaxis was described by Zigmond and Hirsch (56). This method involved placement of the chemotactant on a slide, allowing it to dry, and adding a drop of cell suspension to the streak. A coverslip was placed over the suspension, and the cells were observed for the formation of lamellipodium (the front of the cell has a broad smooth veil, the rear is marked by a constricted tail). Only cells with lamellipodium directed toward or away from the test line were quantitated.

Since mononuclear cells are also involved in inflammation, it was

desirable to devise a method for quantitation of mononuclear cell chemotaxis. Quantitation of mononuclear cell chemotaxis was made possible by the use of larger pores in the Millipore filter and longer incubation periods (20,51). Horwitz and Garrett (18) used a polycarbonate filter which was 12 μm thick (Millipore filters are 150 μm thick) and required less time for incubation than did the Millipore filters. These filters also made identification of cell type more accurate as cells were not trapped within pores of the filter.

Attempts to improve quantitation of chemotaxis with a rapid radiolabeling technique have been fairly successful. Gallin et al. (13) used ^{51}Chromium to label granulocytes followed by chemotaxis in Boyden chambers containing two filters. The second filter containing ^{51}Cr-labeled cells, was used for quantitation of chemotaxis. Although assessment of chemotaxis was more objective and rapid, the labeling procedure caused clumping and subsequent loss of cells when they were filtered through sterile gauze (13). This technique also has a high spontaneous release of label from the cells which makes the background counts high (1). The use of ^{99m}Technetium to label granulocytes (9) and human monocytes (34) presents an alternate label to ^{51}Chromium. However, this label is not readily available to researchers, and a short half-life (5.9 hr) necessitates corrections for decay if large numbers of samples are to be counted (9).

The agarose technique has also been used as a rapid method of quantitating leukocyte chemotaxis. This technique involved placement of guinea pig tissue explants or exudate cells in plastic petri dishes, and agarose containing serum or gamma globulin was poured over the preparation (55). Migration of cells occurred primarily between the agar

and petri dish, however, some cells migrated on the surface of the agar as well. Cutler (6) later used this technique to quantitate guinea pig neutrophil chemotaxis.

Nelson et al. (29) modified this technique by making wells in the agarose, and introducing chemotactant and cells in separate wells. Diffusion of chemotactant into the agarose caused a gradient to which human neutrophils and monocytes migrated. Migration of cells was primarily under the agarose. The agarose could be removed, and cells remaining adherent to the petri dish could be quantitated by measuring the distance the cells migrated toward the chemotactant. The agarose technique is reported to be a rapid and easily quantitated method for both neutrophils and monocytes. However, the method is not consistent when murine peritoneal macrophages are used (Tobi Jones, personal communication).

A RAPID METHOD FOR ASSESSMENT OF A MACROPHAGE CHEMOTACTANT PRODUCED BY SAD/2 FIBROSARCOMA CELLS GROWN *IN VITRO*

by

Louise Schaub Simon, Ronald Patterson, and Tobi L. Jones

INTRODUCTION

The role of macrophages in host defense against neoplasia has received much attention in recent years. Macrophages are found in large numbers in a variety of human (22,54) and animal tumors (8,10,15,16,21). Speculation that macrophages in tumors prevent metastasis (10) is supported by a report that tumors depleted of macrophages became metastatic *in vivo* (55). Macrophages isolated from tumors are reported to be cytostatic or cytotoxic *in vitro* (12,39,40).

Recent research has focused attention on modulation of macrophage function, in particular, macrophage chemotaxis, by tumor cells or tumor products. Snyderman and Pike (36,48) have isolated by dialysis a low molecular weight intracellular tumor product which inhibits macrophage chemotaxis *in vitro* and macrophage migration *in vivo*. Meltzer et al. (27) have reported enhancement of *in vitro* macrophage chemotaxis by a product found in supernatants of tumor cells grown *in vitro* in serum-containing medium.

In this report, we describe an *in vitro* macrophage chemotactant produced by fibrosarcoma tumor cells grown *in vitro* in serum-free medium. As a prelude to isolation and purification of this product we report a rapid method of assessing chemotaxis *in vitro* which utilizes [5,6-^{3}H]uridine labeled peritoneal macrophages. The usefulness of this method which will be used in processing large numbers of samples generated during purification procedures will be discussed.

MATERIALS AND METHODS

Media and Reagents. Sterile Eagle's minimal essential medium (MEM) was used for collection and preparation of peritoneal exudate cells for

chemotaxis. Tumor cells were cultured in CMRL 1066 medium containing 10% decomplemented fetal calf serum (d1066). Serum free 1066 (i1066) was used for the generation of supernatants from tumor cells. All media contained 50 U/ml penicillin, 50 μg/ml streptomycin, and was buffered with $NaHCO_3$. Four percent bovine serum albumin [(BSA) Sigma] was prepared in Hank's balanced salt solution and buffered with $NaHCO_3$. All components were obtained from Grand Island Biological Co., Grand Island, NY, and Microbiological Associates, Walkerville, MD.

Preparation of Mouse Peritoneal Macrophages for Chemotaxis. Two to six month old male DBA/2 mice (Jackson Labs, Bar Harbor, ME) were used in all experiments. Peritoneal exudate cells were stimulated according to the method of Snyderman and Pike (46) with some modifications. Mice were injected intraperitoneally (IP) with 1.5 ml of sterile 9% proteose peptone (Difco Laboratories, Detroit, MI) 3-4 days prior to harvest of peritoneal cells. Mice were sacrificed by cervical dislocation, and the peritoneal cavities were lavaged with 5 ml MEM. The peritoneal exudate cells (PEC) were counted, centrifuged at 163 x g, and resuspended in MEM to a concentration of 10^7/ml. The cells were labeled with 10 μCi [5,6-^{3}H]uridine (Amersham, 48 Ci/mmol)/10^7 PEC/ml MEM for 1 hr at 37C in 17 x 100 mm polystyrene tubes. Macrophages were kept in suspension during the labeling process with a small magnetic stiring bar. After labeling, the cells were washed 3 times in 10 volumes of cold MEM, and resuspended in MEM at 4.4 x 10^6 PEC/ml. An equal volume of 4% BSA was added to the cell suspension to give a final concentration of 2% BSA and 2.2 x 10^6 PEC/ml.

Chemotaxis of Mouse Peritoneal Macrophages. The method of Snyderman and Pike (46) was used for chemotaxis with modifications. Uridine

labeled PEC were employed, and two polycarbonate filters (Nucleopore) with 5 μm pores were placed in blind-well modified Boyden chambers. The lower well was filled with the chemotactant or control medium, and the two polycarbonate filters were placed on top. The top well was assembled and filled with 0.2 ml of the cell suspension. The chambers were incubated at 37 C in a humidified atmosphere of 5% CO_2 for 3.5-4 hr prior to preparation of the filters for quantitation of chemotaxis.

Evaluation of Chemotaxis with Radiolabeled Cells. After incubation of the Boyden chambers, the cells were carefully removed from the top chamber with a Pasteur pipette and the chambers were disassembled. The top filter was discarded, and the second filter containing only cells which had migrated through the first filter, was used for evaluation of chemotaxis. The cellular material on the filter was precipitated with approximately 3 ml 10% trichloroacetic acid (TCA) by suction filtration. The filters were air dried, placed in vials containing scintillation fluid, and counted by liquid scintillation spectrometry.

The following procedure was used to determine that increased CPM observed on chemotaxis filters was due to increased number of chemotaxing macrophages. Various concentrations of uridine-labeled cells were pipetted onto polycarbonate filters contained in glass petri dishes and incubated at 37 C in humidified 5% CO_2 atmosphere for 3.5 hr. Two filters for each concentration were stained in the same manner as described below for the visual chemotactic assay, and cells were counted microscopically at 1000 X. The mean number of cells per field from 20 fields was determined, and the number of cells per filter was calculated based on the area of the filter. Percent adherence was calculated as follows: $\frac{\#\text{cells calculated/filter}}{\#\text{cells placed on filter}}$ X 100. A parallel set of filters was

precipitated with TCA, dried, placed in scintillation fluid, and mean CPM per filter ± standard error was determined.

Evaluation of Chemotaxis by Visual Assay. A visual assay for chemotaxis was done in parallel using the same uridine labeled PEC population as for the radioactive assay. After incubation of Boyden chambers and removal of cells, duplicate lower filters were prepared for visual quantitation of chemotaxis by fixing in absolute alcohol, staining with Ehrlich hemotoxylin, and rinsing in tap water. Air dried filters were mounted in immersion oil on slides, and examined microscopically at 1000 X. The mean number of macrophages per field present in 20 oil immersion fields on both sides of duplicate filters was determined.

Chemotactants. Serum-free culture supernatants from the *in vitro* line of the SAD/2 methylcholanthrene-induced fibrosarcoma were tested for chemotactic activity. Confluent cultures were trypsinized with 0.1% trypsin (Sigma) in PBS at 37 C. The cells were centrifuged and resuspended in d1066 at a concentration of 1.5×10^6/ml, and 10 ml were plated in 250 cm^3 flasks (Corning). The cultures were incubated in a humidified atmosphere of 5% CO_2 at 37 C for 24 hr, washed 3 times with PBS, and 10 ml i1066 were placed on the washed cells. Supernatants were collected after an additional 48 hr incubation period. Following centrifugation at 163 x g to remove detached cells, the supernatants were tested for chemotactic activity as previously described.

Mouse serum was treated with endotoxin [(E. coli serotype 0261 B6) Sigma] to generate chemotactic complement components (45) as described by Snyderman and Pike (46). This endotoxin-treated serum (ETS) was included in each experiment as a positive control. The negative control consisted of i1066 in the lower chamber.

Mouse embryonic cells used to generate supernatants from normal cells were in the fourth passage of cells harvested from 15-18 day old DBA/2 embryos. Embryonic cells were grown in the same manner as tumor cells to generate supernatants.

Determination of True Chemotaxis or Chemokinesis of Tumor Supernatants. A modification of the method of Zigmond and Hirsch (56) was used to determine whether tumor supernatants were chemotactic (directional movement) or chemokinetic (random movement) for macrophages. Four sets of duplicate blind-well Boyden chambers were prepared. The lower wells of the first set contained tumor supernatants (TS) and the upper wells contained cells suspended in il066 with 2% BSA. The second set contained TS in the lower wells, and cells suspended in full strength TS in 2% BSA in the upper wells. The third set contained il066 in the lower wells, and cells suspended in full strength TS containing 2% BSA in the upper wells. The fourth set was the medium control. Upper and lower wells were separated by double polycarbonate filters, and the radioactive method was used to quantitate migration of macrophages as described previously. Parallel sets of chambers containing ETS as chemotactant in lower and upper wells were included in each experiment.

Statistical Evaluation. In both methods, chemotactic activity was determined in duplicate chambers. Twenty oil immersion fields were examined in the visual assay and data is reported as mean number of cells per oil immersion field on duplicate filters. The radioactive assay was reported as mean number of counts per minute per filter. Standard error was used as an estimate of variance.

RESULTS

Preliminary experiments which utilized a standard chemotaxis assay described by Snyderman and Pike (46) determined that culture supernatant from SAD/2 cells grown *in vitro* in serum-free medium was chemotactic for peritoneal macrophages. Since a chemotactic assay was to be used during the isolation and purification of the tumor material, we wished to modify the standard chemotaxis assay to facilitate assaying the large number of samples generated in purification procedures.

Initial experiments were set up to determine whether uridine labeled macrophages could be used for chemotaxis. Radiolabeled PEC were placed in the top well of a modified Boyden chamber, and were separated from the lower well containing tumor supernatant, ETS or supernatants from embryonic fibroblasts by double polycarbonate filters. When lower filters were precipitated with TCA, there was a 2-6 fold increase in CPM observed in chambers containing tumor supernatant or ETS in the lower well when compared to negative controls (Table I). Radioactivity on the second filter of chambers containing supernatants from the fourth passage of embroyonic cells was comparable to radioactivity of control filters. (Data not shown).

Determination of Increased Radioactivity Caused by Increased Numbers of Cells/Filter. Experiments were set up to determine whether increased radioactivity observed on filters placed over ETS represented increased numbers of cells. Various concentrations of cells were pipetted onto filters and incubated. Filters were prepared for liquid scintillation counting and for visual quantitation as described in materials and methods. Table II shows the correlation between visual counts of cells on each filter with the radioactivity of parallel filters. The results

indicate that as the concentration of cells increase, the radioactivity increases proportionally. The specific activity of the cells for each concentration was approximately the same, which indicated a homogeneous labeling of cells. The overall mean and standard error (SE) of the specific activity was 0.034 + 0.005 CMP/cell. An overall mean of adherence was 53.3 $\pm$ 8.9%.

Comparison of Visual and Radioactive Assays With Macrophage Chemotactants. Since increased numbers of cells correlated with increased radioactivity on the filters when cells were layered onto the filters, it was then possible to compare visual and radioactive methods of quantitating chemotaxis. Both tumor supernatants and ETS were used as chemotactants in this comparison. Each sample of chemotactant and control medium was set up in four Boyden chambers. After incubation, two filters were prepared for scintillation counting and two filters were prepared for microscopic observation. The results of this comparison in three separate experiments are shown in Fig. I. When either tumor supernatant or ETS was present, there was an increase in observed cell numbers on the lower filter that correlated with an increase in the precipitable CPM. Note that there was migration of macrophages to the lower filter of negative controls in both the visual and radioactive assay, but this migration represented random migration of cells in response to the medium. The migration of macrophages in response to TS and to ETS was greater than the random migration both in the visual and in the radioactive assay.

Determination of Chemotaxis or Chemokinesis of Tumor Supernatant. It is apparent from Fig. I that tumor supernatant caused greater migration of macrophages to the lower filter than did control medium.

Since it was uncertain whether the tumor material was chemotactic or chemokinetic for macrophages, experiments were set up to clarify this point. Chambers were assembled with TS in the lower well, cells in the top well; TS in both wells; and TS in the top well in contact with the cells and i1066 in the lower well. Assessment of macrophage migration to the lower filter by the radioactive method (Table III) showed that the greatest increase in radioactivity occurred when cells were in the top well and TS in the lower well. When TS was in contact with the cells and either TS or medium was in the lower well, the radioactivity observed was comparable to negative controls (cells in the top well, i1066 in the lower well), which indicated random migration.

DISCUSSION

The purpose of this study was to develop a rapid *in vitro* chemotaxis assay for mouse peritoneal macrophages. The method described used [5,6-^{3}H]uridine labeled PEC and a double polycarbonate filter in each blind-well Boyden chamber. Precipitation of the cellular material on the lower filter with TCA allowed assessment of cells which had migrated through the first filter while non-chemotaxing PEC on the first filter were discarded. The technique allowed one to process a large number of samples in a minimum amount of time. Contrary to the lengthy procedure of counting cells on filters, the results of this assay were available immediately after liquid scintillation counting.

These data indicate that radiolabeled macrophages could be used to assess chemotaxis for a known chemotactant (ETS) and it can also be used to test for chemotactic activity of other material such as TS

(Table I). When supernatants from the fourth passage of embryonic fibroblasts were tested for chemotactic activity, radioactivity of the lower filters were comparable to control filters (Data not shown). It was concluded, therefore, that normal cells with moderate growth rates do not produce a material comparable to the tumor material, or produce it in extremely low quantities not detectable by the assay system described.

We showed that increasing cell numbers adherent to the filter caused an increase in the CPM per filter (Table II). With the exception of the lowest concentration tested, the percent macrophages observed at each concentration were similar. The decrease in adherence of macrophages at the lowest concentration was observed in several experiments. Within the upper range of concentrations tested, however, varying the concentration of cells did not alter adherence of cells to the filters.

When the data from Table II were graphed on semi-log paper, a straight line graph was obtained. From such a standard curve, one could calculate the number of cells migrating to the second filter in the radioactive chemotaxis assay if desirable. When such determinations from several experiments were made, we observed that the radioactive method predicted approximately 10% more cells than were counted in the visual assay. Two reasons could account for this difference. First, only whole cells on the surfaces of the filters which were well spread were counted in the visual assay. No attempt was made to count cells within the pores of the filters. Second, physical disruption of cells occurred in separation of upper and lower filters. Portions of cells could be observed microscopically, but were not counted. It is

likely that the radioactive method detected labeled portions of disrupted cells remaining on the filter, as well as those cells within the pores.

When the visual and radioactive assays for chemotaxis were compared (Fig I) using a known chemotactant (ETS) and tumor supernatant (TS), the radioactive method was sensitive to the increased number of cells present on the lower filters of ETS and TS as compared to controls. The radioactive method did not distinguish between cell types migrating to the lower filter. However, visual assessment of cells on the lower filter determined that less than 2.5% of the cells observed were polymorphonuclear leukocytes. The remaining cells were all macrophages.

Various investigators (20,56) have described counting methods for determining random (chemokinesis) versus directional movement (chemotaxis) of cells toward a substance. The radioactive method described in this paper was useful in differentiation of chemokinesis and chemotaxis. The data from Table III established that TS was a chemotactant for macrophages. If TS merely increased random movement of macrophages, filters from chambers containing cells and TS in the top well and TS in the lower well should contain approximately the same radioactivity as those chambers containing cells in the top well and TS in the lower well. The same would be true for chambers containing cells and TS in the top well and il066 in the lower well. Since the radioactivity of filters from sets of chambers containing cells and TS in the top wells was comparable to the control, and the radioactivity of filters from chambers with cells in the top well and TS in the lower well was much greater, the TS stimulated directional movement (chemotaxis) of

macrophages.

The possibility that the tumor material chemotactic for macrophages was the result of degrading tumor cells in the serum-free medium was minimal. After 48 hr incubation in serum-free environment, the monolayer of cells remained intact. The viability of tumor cells which had detached from the surface of the flask was monitored when the supernatant was collected and centrifuged. Greater than 96% of the pelleted cells were viable based on exclusion of Trypan Blue dye.

The tritiated uridine method used to label macrophages in this research has advantages over the use of ^{51}Chromium and ^{99m}Technetium to label cells for chemotaxis assays (9,13). Use of ^{51}Cr to label cells causes clumping of leukocytes which necessitates an additional filtering through sterile gauze to remove clumps (13). Preparation of additional cells would therefore be necessary to compensate for loss of clumped cells. The inavailability and short half-life (5.9 hr) of ^{99m}Tc make this label difficult to work with. Also, corrections have to be made for the decay of the label if large numbers of samples are processed (9). Papierniak et al., (34) also report a high background on filters due to spontaneous release of label from the cells. These problems were not an issue when macrophages were labeled with ^{3}H-uridine. Cell clumping did not occur during the labeling process. The long half life of tritium and the availability and ease of incorporation of ^{3}H-uridine made it an ideal label for macrophages. Any unincorporated label which might be spontaneously released was eliminated either during the washing procedure or during the TCA precipitation of the filters.

The radiolabeling technique described herein has several advantages. It can be used to assess mouse peritoneal macrophage

chemotaxis objectively, and can be used to distinguish chemokinesis from chemotaxis. It is also very useful in processing large numbers of samples which would be generated in purification procedures of any chemotactant (43).

TABLE I

Use of ^{3}H-Uridine Labeled Macrophages to Assess Chemotaxis

Experiment	Control[a]	ETS[a]	Fold Increase Over Control For ETS	TS[a]	Fold Increase Over Control For TS
1	3404 ± 484	11106 ± 494	3.2	11213 ± 327	3.2
2	2413 ± 48	6688 ± 11	2.8	7722 ± 87	3.2
3	1623 ± 137	5731 ± 336	3.5	9251 ± 401	5.8
4	2998 ± 141	6650 ± 402	2.3	12291 ± 372	4.1

[a]Data represents average radioactivity ± SEM.

TABLE II

Correlation Between Number of Cells per Filter

And Radioactivity (CPM) observed per Filter

Number of Cells Plated On Filters	Mean Number of Cells Observed /filter ± SEM	CPM/filter ± SEM	Specific Activity CPM/Cell	Per cent Adherence
2×10^5	$9.92 \pm 0.09 \times 10^4$	4750.0 ± 596	0.038	60
1×10^5	$4.87 \pm 0.06 \times 10^4$	2274.3 ± 200	0.037	60
5×10^4	$2.16 \pm 0.04 \times 10^4$	722.0 ± 5	0.027	52
2.5×10^4	$8.32 \pm 0.11 \times 10^3$	369.4 ± 27	0.036	41

TABLE III

Determination of Chemotaxis of Peritoneal Macrophages Toward Tumor Supernatant

Chamber Number:	1	2	3	4
Top Well:	Cells	Cells + TS	Cells + TS	Cells
Bottom Well:	TS	TS	i1066	i1066
Experiment				
1	1259 ± 191 (10)[a]	277 ± 8 (2.2)	339 ± 170 (3.2)	125 ± 2
2	825 ± 4 (4.3)	292 ± 44 (1,5)	146 ± 21 (0.8)	192 ± 2
3	633 ± 40 (5.1)	98 ± 8 (0.8)	123 ± 7 (1.0)	123 ± 7

[a]Value in parenthesis represents fold increase over control

Figure 1. Comparison of visual and radioactive methods for quantitation of chemotaxis. ^{3}H-Uridine labeled macrophages were used in a chemotaxis system as descirbed in the text. Microscopic observation of filters was done in parallel with determination of precipitable radioactivity on filters. Data represent the mean for three experiments containing duplicate filters $\pm$ standard error of the mean.

 Visual Radioactive

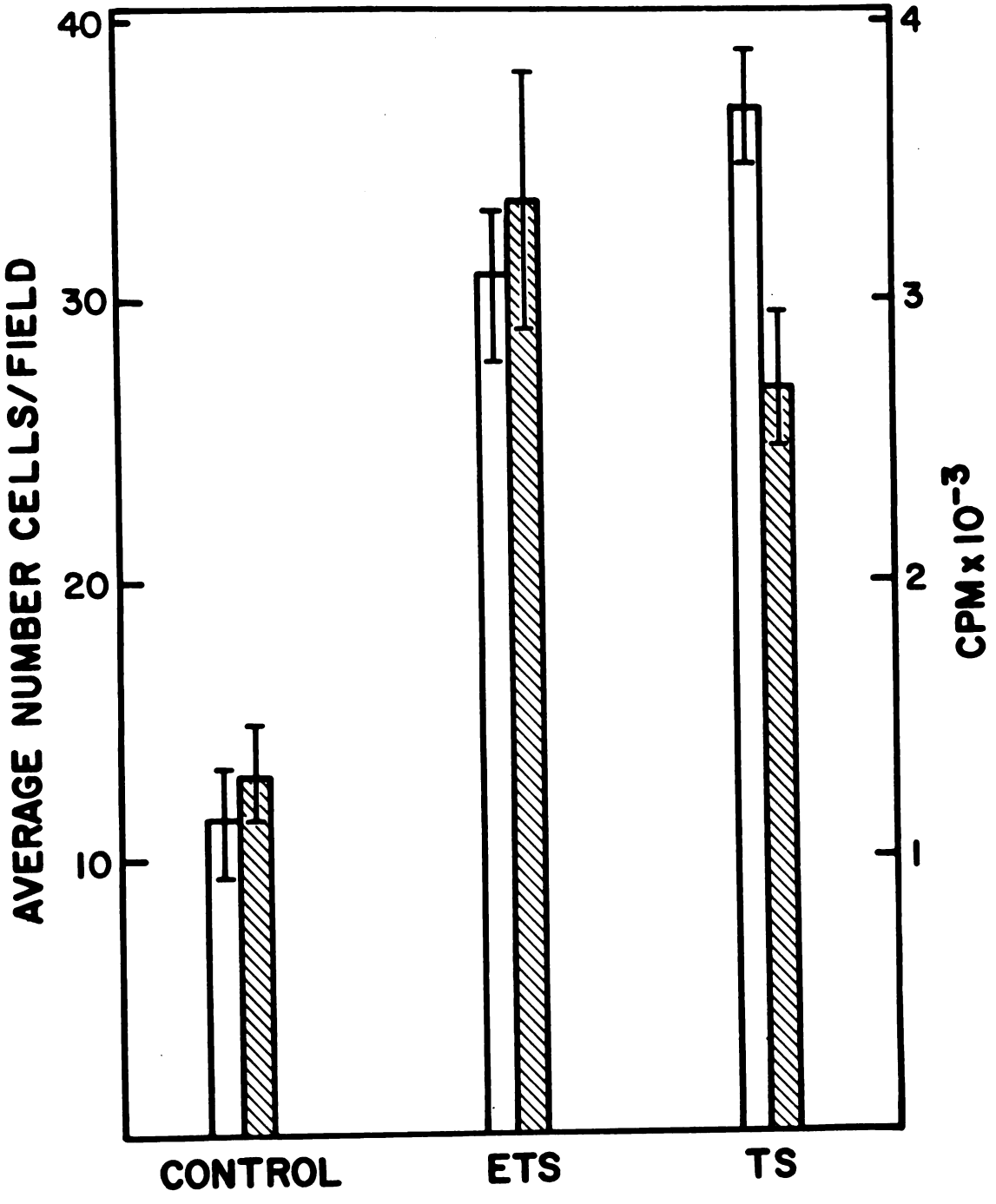
AVERAGE NUMBER CELLS/FIELD
40
30
20
10
4
3
2
1
CPM x 10⁻³
CONTROL
ETS
TS

Figure 1.

ISOLATION AND PARTIAL PURIFICATION OF A MACROPHAGE CHEMOTACTANT PRODUCED BY SAD/2 FIBROSARCOMA CELLS GROWN *IN VITRO*

by

Louise Schaub Simon, Ronald Patterson, and Tobi Jones

INTRODUCTION

The effect of tumor cell products on macrophage chemotaxis has been the subject of much research in recent years. Several researchers have reported that patients with cancer have depressed monocyte chemotactic responses *in vitro* (3,50). Murine syngeneic neoplasms have also been reported to depress macrophage migration *in vivo* and chemotaxis *in vitro* (36,47,48). These investigators have isolated by dialysis a 6-10,000 MW factor from tumor cells which depressed macrophage chemotaxis (36,48).

Stimulation of macrophage function by tumor cell products is also reported in the literature. Snodgrass et al. (44) reported that tumor cells produce a soluble product which stimulates macrophage chemokinesis *in vitro*. Meltzer et al. (27) have reported that culture supernatants of several *in vitro* tumor lines contain a 15,000 MW component which is chemotactic for macrophages.

We reported previously (42) that growth medium from the murine SAD/2 fibrosarcoma line contained material chemotactic for macrophages *in vitro*. Similar chemotactic activity was not obtained from the growth medium of fourth passage mouse embryonic fibroblasts. In the present report, the isolation and partial purification of radiolabeled chemotactic products from culture supernatants of SAD/2 cells is described. In addition, the ability of the partially purified material to cause an inflammatory response in mice is assessed.

MATERIALS AND METHODS

Media. Sterile Eagle's minimal essential medium (MEM) was used for collection and radiolabeling of peritoneal exudate cells. Tumor cells were maintained in CMRL 1066 medium containing 10% heat-inactivated

fetal calf serum (d1066). Serum-free CMRL 1066 (i1066) was used to generate culture supernatants from tumor cells. Leucine-free MEM (LMEM) prepared according to Eagle (7), was used instead of i1066 when the supernatant products of tumor cells were to be labeled with tritiated leucine. All media contained 50 U/ml penicillin, 50 µg/ml streptomycin, and were buffered with $NaHCO_3$. All components were obtained from Grand Island Biological Co., Grand Island, NY, and Microbiological Associates, Walkerville, MD.

Cells Used. An *in vitro* line of the DBA/2 methylcholanthrene induced fibrosarcoma SAD/2 (obtained from Jackson Labs, Bar Harbor, ME) was used as the source of macrophage chemotactant according to Simon et al., (42).

Macrophage Chemotaxis. Chemotactic activity of tumor cell products was measured as previously described (42). Two to six month old male DBA/2 mice (Jackson Labs, Bar Harbor, ME) were used as a source of peritoneal macrophages for all *in vitro* chemotaxis studies. Peritoneal exudates were induced in these mice by intraperitoneal (IP) injection of proteose peptone (Difco Laboratories, Detroit, MI). Mice were sacrificed by cervical dislocation, and exudate cells were collected by lavage of the peritoneal cavity with 5 ml MEM. The cells were counted, centrifuged at 163 x g, and resuspended cells were labeled with 10 µCi [5,6-^{3}H]uridine (Amersham, 48 Ci/mmol)/10^7 PEC/ml MEM for 1 hr at 37 C. The cells were washed three times, and resuspended in MEM containing 2% bovine serum albumin [(BSA) Sigma] at a concentration of 2.2 x 10^6 PEC/ml.

Blind-well modified Boyden chambers were used in this study. Two polycarbonate filters (Nucleopore Corporation, Pleasonton, CA) with

5 μm pores separated the lower well containing control medium or chemotactant from the upper well containing 4.4 x 10^5 uridine labeled PEC in a 0.2 ml volume. Following incubation of the chambers at 37 C in a humidified atmosphere of 5% CO_2 for 3.5-4 hr, chemotaxis of macrophages was quantitated by trichloroacetic acid (TCA) precipitation of the cells which migrated onto the lower filter followed by liquid scintillation spectrometry.

Serum-free supernatants collected from SAD/2 tumor cells previously shown to be chemotactic for macrophages (42), and column fractions prepared from these supernatants, were used as chemotactants. In all experiments, endotoxin (Sigma)-treated mouse serum, prepared as described by Snyderman and Pike (46) was included as a positive control for chemotaxis. When crude tumor supernatants were tested for chemotactic activity, the negative control was il066 placed in the lower well. Eluates from the Sephacryl S200 and Sepharose 6B columns taken prior to application of samples to the columns were used as the negative controls when fractions from these columns were tested for chemotactic activity.

Radiolabeling of Tumor Supernatants. The chemotactant from SAD/2 cells was labeled in the following manner. Tumor cells were cultured in dl066 for 24 hr, washed with PBS, and 10 ml of LMEM containing 10 μCi/ml L-[4,5-^{3}H]leucine (48 Ci/mmol, Amersham) were placed on the cells. The cells were cultured an additional 48 hr, and the supernatants were collected and subjected to chromatographic and electrophoretic procedures as described below.

Chromatography of Tumor Supernatants. SAD/2 culture supernatants collected after 48 hr of incubation were concentrated 25-30 times by ultrafiltration with a UM 20 filter (Amicon Corporation, Lexington,

MA). Five ml of the concentrated supernatant were placed on a Sephacryl S-200 column (2.5 x 59 cm bed) equilibrated with phosphate buffered saline (PBS). Elution buffer was 0.15 m PBS, pH 7.2. Flow rate was 33 ml/hr, and was controlled by a peristaltic pump (Pharmacia). Absorbance was read at 280 nm. Fractions (3.2 ml) were collected and were assayed in duplicate for chemotactic activity. Those fractions which had chemotactic activity were pooled, concentrated by ultrafiltration 4-6 fold and placed on a Sepharose 6B column (2.5 x 63.5 cm bed) equilibrated with 0.15 M PBS. Flow rate was 18.8 ml/hr and was controlled by a Mariott flask (Pharmacia). The absorbance and collection of fractions were the same as for the Sephacryl column. Fractions were assayed in duplicate for chemotactic activity.

Fractions from the Sepharose 6B column which contained chemotactic activity were applied to a DEAE Sephacel column (0.80 x 11.6 cm bed) which was equilibrated with 0.15 M PBS. Salt elutions were made with 0.15 M, 0.3 M and 0.6 M NaCl in 0.004 M phosphate buffer. Eluates were brought to physiological saline conditions by dilution with 0.004 M phosphate buffer, concentrated to the original volume by ultrafiltration, and assayed for chemotactic activity.

Gel Electrophoresis. Ten percent polyacrylamide gels containing 0.1% sodium dodecylsulfate (SDS) were prepared as described by Porzio and Pearson (37). Native gels were prepared in the same manner, except that SDS was omitted in preparation of the gel. All ingredients for the gels were purchased from Bio Rad Laboratories, Richmond, CA. The buffer used for SDS gel electrophoresis was 200 mM Tris/glycine (pH 8.5) with 0.1% SDS. The same buffer without SDS was used for electrophoresis of native gels. Fifty µl containing approximately

10 µg protein (determined as in Lowry et al., 23) of the chemotactively active Sepharose 6B fractions were placed on the gel and allowed to electrophorese at 1 mA per tube for approximately 2 hr. Gels were fixed, stained with Coomassie Brilliant Blue, and destained according to Porzio and Pearson (37). Gels were scanned at 540 nm to obtain protein profiles. When radiolabeled tumor material was electrophoresed, the gels were cut into approximately 1 mm slices with a gel slicer (Bio Rad). Two adjacent slices were placed in 5 ml toluene based scintillation fluid containing 5% NCS tissue solubilizer and 1% 4N NH_4OH and incubated at 37 C overnight. Radioactivity was determined by liquid scintillation spectrometry and was expressed as counts per minute (CPM).

Marker proteins and their molecular weights used to determine molecular weight of the tumor material on SDS gels were α actinin, from myofibril protein (gift of M.A. Porzio) 102,000, BSA (Sigma) 68,000, Soybean trypsin inhibitor (Sigma) 22,000, hemoglobin (Schwarz Mann) 15,500, and lysozyme (Sigma) 13,000.

Trypsin Treatment of Chemotactant. Sepharose 6B column fractions with chemotactic activity were subjected to 5% insoluble trypsin (Sigma) treatment for 50 min at 37 C. Trypsin-bearing beads were removed by centrifugation, and the supernatant was applied to SDS gels as previously described. This supernatant was also tested for chemotactic activity. In one experiment the Sepharose 6B active fractions were trypsinized overnight at 15 C prior to application to SDS gels.

In Vivo Inflammatory Response. Groups of five female (DBA/2 x C57Bl/6) F_1 mice were injected IP with 0.5 ml of 4 fold concentrated Sepharose 6B fractions which had chemotactic activity. Groups of five control mice were injected with the same volume of PBS. Forty-eight

hours after injection, mice were sacrificed by cervical dislocation, and peritoneal cells from each mouse were collected by lavage of the peritoneal cavity with 5 ml MEM. The volume obtained from each mouse was recorded and the total number of peritoneal cells obtained from each mouse was calculated from hemocytometer counts. The cells were centrifuged at 163 x g and resuspended in 0.2 ml MEM. Cells were pipetted onto duplicate slides, air dried, fixed in methanol, and stained with Giemsa stain. Differential cell counts on preparations from each animal were determined by counting 100 cells on each slide at 1000 X under oil.

Statistical Evaluation. Chemotactic activity was expressed as the mean CPM of duplicate filters $\pm$ standard error (SE). For the inflammatory response studies, the mean number of recovered peritoneal cells and the mean number of each cell type observed in the differential counts from control and experimental animals was compared by the Student's t test.

RESULTS

Chromatography of Tumor Supernatants. Experiments were conducted to determine whether chemotactic material contained in culture supernatant from SAD/2 cells (42) could be purified by gel filtration. Chemotactic activity was retained when crude supernatants were concentrated with an Amicon UM 20 filter which retains molecules of greater than 20,000 MW. Crude supernatants which had been concentrated were applied to a Sephacryl S-2000 column. The protein profile and corresponding chemotactic activity of fractions from this column are depicted in Fig. IA. Greatest chemotactic activity was found in fractions (12-15) immediately following the void volume. When fractions with greatest

chemotactic activity were pooled, concentrated and applied to a Sepharose 6B column, two major protein peaks were obtained (Fig. IB). Chemotactic activity (fractions 15-23) was found to correspond with the second protein peak (Fig. IB). A 4-5 fold increase in radioactivity occurred when Sepharose 6B active fractions were compared to crude supernatant in the chemotaxis system.

In order to determine whether the chemotactant was actively synthesized, tumor cells were radiolabeled with ^{3}H-leucine and the supernatants were chromatographed on Sephacryl S 200 and Sepharose 6B. Those fractions with chemotactic activity were also found to be labeled with ^{3}H-leucine (Fig. IB).

Several attempts to purify Sepharose 6B active fractions with DEAE Sephacel resulted in loss of biological activity when 0.1 M 0.3 M and 1 M NaCl eluates were tested for chemotactic activity. Fractions obtained when a gradient elution (0-1 M NaCl) was used also contained no chemotactic activity.

Gel Electrophoresis. The fractions from the Sepharose 6B column which had greatest activity corresponded to a single protein peak (Fig IB). Since attempts to further purify these fractions on DEAE Sephacel were unsuccessful, the active fractions were analyzed by gel electrophoresis to determine whether this peak consisted of one or several proteins. SDS gels were used to assess whether the active peak contained more than one protein separable by electrical charge.

The results of SDS gel electrophoresis of crude tumor supernatant compared with Sepharose 6B fractions (50 µl containing 10 µg protein applied to each gel) which had chemotactic activity are shown in Fig. 2, A and B. Sepharose 6B fractions contained two Coomassie Blue positive

bands on SDS gels. Treatment of active Sepharose 6B fractions with 2 mercaptoethanol (2ME) prior to electrophoresis resulted in gels with 2 bands (Fig. 2C).

To confirm that these bands were protein, the Sepharose 6B fractions which were chemotactively active were trypsinized, and 50 µl were applied to SDS gels. Fig. 2D shows a diminished amount of Coomassie Blue positive bands after 50 min trypsin treatment. When active fractions were treated with trypsin overnight prior to application to gels, there were no Coomassie Blue positive bands after staining of gels. Trypsinization of chemotactively active Sepharose 6B fractions also resulted in loss of chemotactic activity when tested *in vitro* (data not shown).

Radiolabeled Sepharose 6B fractions with good chemotactic activity were applied to SDS gel electrophoresis. Results of these experiments (Fig. 3) show that the two radioactive peaks correspond to the two protein peaks found on SDS gel scans.

The apparent molecular weights of the proteins in the Sepharose 6B active fractions were 68,000 and 78,000 on SDS gel electrophresis (Fig. 3). A shift of apparent molecular weights to 74,000 and 81,500 respectively occurred when active fractions were pretreated with 2ME before application to SDS gels. Native gels contained 2 bands (Fig.4) which supported data (Fig. 3) obtained from SDS gels.

Attempts were made to retrieve biological activity from protein bands in native gels. Slices were made of the gels, and they were extracted overnight with PBS while dialyzing against PBS. Extracts tested had no chemotactic activity.

Experiments were carried out to determine whether loss of

chemotactic activity was due to toxicity of the gel. Freshly prepared gels were sliced and extracted overnight similar to the gels containing protein bands. Extracts from these gels were used as a diluent in the preparation of ETS in place of PBS. This preparation was tested in parallel with ETS containing PBS diluent in a chemotaxis system. The chemotaxis of macrophages toward ETS containing gel-extract diluent was reduced by 70% compared to ETS containing PBS diluent.

Inflammatory Response. The partially purified tumor material was chemotactic for peritoneal macrophages *in vitro*, and tests were conducted to determine whether it exhibited activity *in vivo*. To determine whehter chemotactically active Sepharose 6B fractions would elicit an inflammatory response *in vivo*, 0.5 ml of the active fractions were injected IP into mice. Total cell yield and differential counts from the peritoneal wash of individual mice were determined (Table I). Although the total cell number of peritoneal cells obtained from experimental animals was greater than that obtained from control animals, the values were not statistically significant. Results of the differential cell count, however, demonstrated a significant increase in the number of macrophages, and a significant reduction in the number of lymphocytes of experimental animals when compared to controls. The number of basophils, polymorphonuclear neutrophils, and eosinophils was not significantly different in experimental and control mice.

DISCUSSION

Previous research from this laboratory has determined that SAD/2 tumor cells grown *in vitro* in serum-free medium produce a material that is chemotactic for peritoneal macrophages *in vitro* (42). Purification of this material was desirable in order to investigate its

biological properties *in vivo*. If tumor cells produce such a material *in vivo*, this could account for the accumulation of large numbers of macrophages which have been reported in this tumor *in vivo* (21).

The methodology used to evaluate chemotaxis using radiolabeled cells was described in a previous paper (42). This technique greatly facilitated the assessment of numerous fractions obtained in gel filtration of crude tumor supernatant.

Our isolation procedures utilized two gel filtration columns. The Sephacryl S200 column eliminated many low molecular weight components found in the crude supernatant. Sepharose 6B was utilized to separate higher molecular weight components from the proteins associated with chemotactic activity.

Serum-free tumor supernatant was used in this research, and made interpretation of results easier for two reasons. First, most of the serum components were eliminated from gel beds making serum contaminants in the active fractions less likely. Secondly, without serum in the medium in which the tumor cell products were generated, there was less chance that the chemotactant was a cleavage product of serum generated by the action of tumor cells on the serum.

To show that the proteins in chemotactic Sepharose 6B fractions were synthesized by the cells, attempts were made to radiolabel the proteins associated with chemotactic activity. Results of these experiments showed that radioactivity was associated with both proteins found in chemotactic fractions (Fig. 1B and Fig 3). We concluded from this data that the tumor material was actively produced by the cells and was not a cleavage product of residual serum components.

Identification of which of the two proteins in Sepharose 6B

active fractions was the chemotactant was complicated by loss of biological activity when samples were applied to DEAE Sephacel. Sepharose 6B active fractions were therefore applied to native gels and retrieval of chemotactic activity from gel slices was attempted. These attempts were unsuccessful because of the toxicity of the gel in our system. We rechromatographed the active fractions on Sepharose 6B and applied individual fractions associated with the protein peaks to gel electrophoresis. Two bands were always observed in each individual fraction, which indicated they had similar molecular weights. The molecular weights of the two proteins found in these active fractions were 68,000 and 78,000 based on SDS gel electrophoresis. Pretreatment of active fractions with 2ME followed by SDS gel electrophoresis resulted in a shift of apparent molecular weight to 74,000 and 81,500. The reason for this shift is not known at this time but could be due to breaking of intrachain sulfhydryl bonds and subsequent unfolding of the molecule making it more accessible to SDS. The proteins were apparently composed of a single polypeptide chain since no additional bands were observed in gels containing samples treated with 2ME (Fig. 2C).

Although data from gel electrophoresis would suggest that the tumor material contained only 2 proteins, this does not eliminate the possibility that a small molecular weight species might adhere noncovalently to the larger molecular weight species. This possibility, however, is minimal, since treatment with SDS should dissociate the small molecular weight species and make it apparent as a separate band farther down the gel. This was never observed even when concentrated crude supernatants or Sepharose 6B fractions were applied to

SDS gels for electrophoresis (Fig. 2A and 2B).

There is reason to believe one or both of these proteins, obtained from the Sepharose 6B column cause chemotaxis of macrophages. First, trypsinization of these active fractions caused disappearance of chemotactic activity. Second, the same trypsinized sample resulted in loss of bands on SDS gels (Fig. 2D). Thus there was a correlation between the loss of biological activity by trypsinization and the disappearance of bands on SDS gels.

The effect of these partially purified samples on macrophage migration *in vivo* was tested by IP injection into mice. When peritoneal cells (PC) were harvested 48 hr later, a significant increase in the number of macrophages in the peritoneal cavity had occurred. Although there was no significant difference in the total cell number harvested from control and experimental mice, this could be explained by preliminary experiments in our laboratory which indicate that when PC are pretreated with Sepharose 6B fractions, the *in vitro* adhesiveness of peritoneal macrophages is increased (Unpublished data).

The data in this paper is different from the data of other researchers (36,48,49). These researchers indicate that tumor cell products depress macrophage chemotaxis. However, they attribute depressed macrophages migration *in vivo* and macrophage chemotaxis *in vitro* to a low molecular weight (10,000 MW) component extracted from various tumor cell lines. Meltzer et al. (27) reported that a low molecular weight (15,000 MW) chemotactant from serum-containing tumor supernatants stimulated macrophage migration *in vitro*. However, these researchers did not determine whether this product was due to alteration of serum components by the tumor cells, or whether it was actively synthesized

by the cells (27).

Our research indicates that two large molecular weight components (68,000 and 78,000 MW) found in growth medium of tumor cells cause enhanced macrophage chemotaxis *in vitro* and an inflammatory response *in vivo*. The enhancement of inflammatory response *in vivo* by tumor cell products could be one reason why numerous macrophages are found in this tumor (21).

One reason for the differences we observed with other researchers findings is the tumor types. The fibrosarcoma used in this research is different from the tumor lines used by other researchers (27,48). The SAD/2 tumor line produces material which is chemotactic for macrophages *in vitro* and elicits an inflammatory response *in vivo*. It is possible that *in vivo* the tumor cells produce this material and may be one reason why macrophages are selectively drawn into the tumor and remain localized there. The role of macrophages in tumors was suggested by Eccles and Alexander (8) and by Evans (10) to be control of metastasis. Wood and Gillespie (55) have shown that when tumors were depleted of macrophages prior to injection, metastasis of the tumor resulted.

Recent research has indicated that a soluble product is involved in cytotropic responses of macrophages to tumor cells *in vitro* (44). The argument for a macrophage chemotactant produced by tumor cells *in vivo* is made more plausible by these researchers findings, and our own findings presented in this paper. If tumor cells produced a macrophage chemotactant *in vivo*, this could explain how macrophages are attracted to the tumor site, and why they remain localized within the tumor mass.

Figure 1. Chromatograph of Serum-free Tumor Supernatant. A. Protein profile (——) and chemotactic activity (0——0) of fractions obtained when tumor material was applied to the Sephacryl S200 column. B. Protein profile (——) chemotactic activity (0——0) and radioactivity of fractions (●——●) obtained when biologically active Sephacryl S200 fractions were applied to the Sepharose 6B columns. Arrow=phenyl red marker.

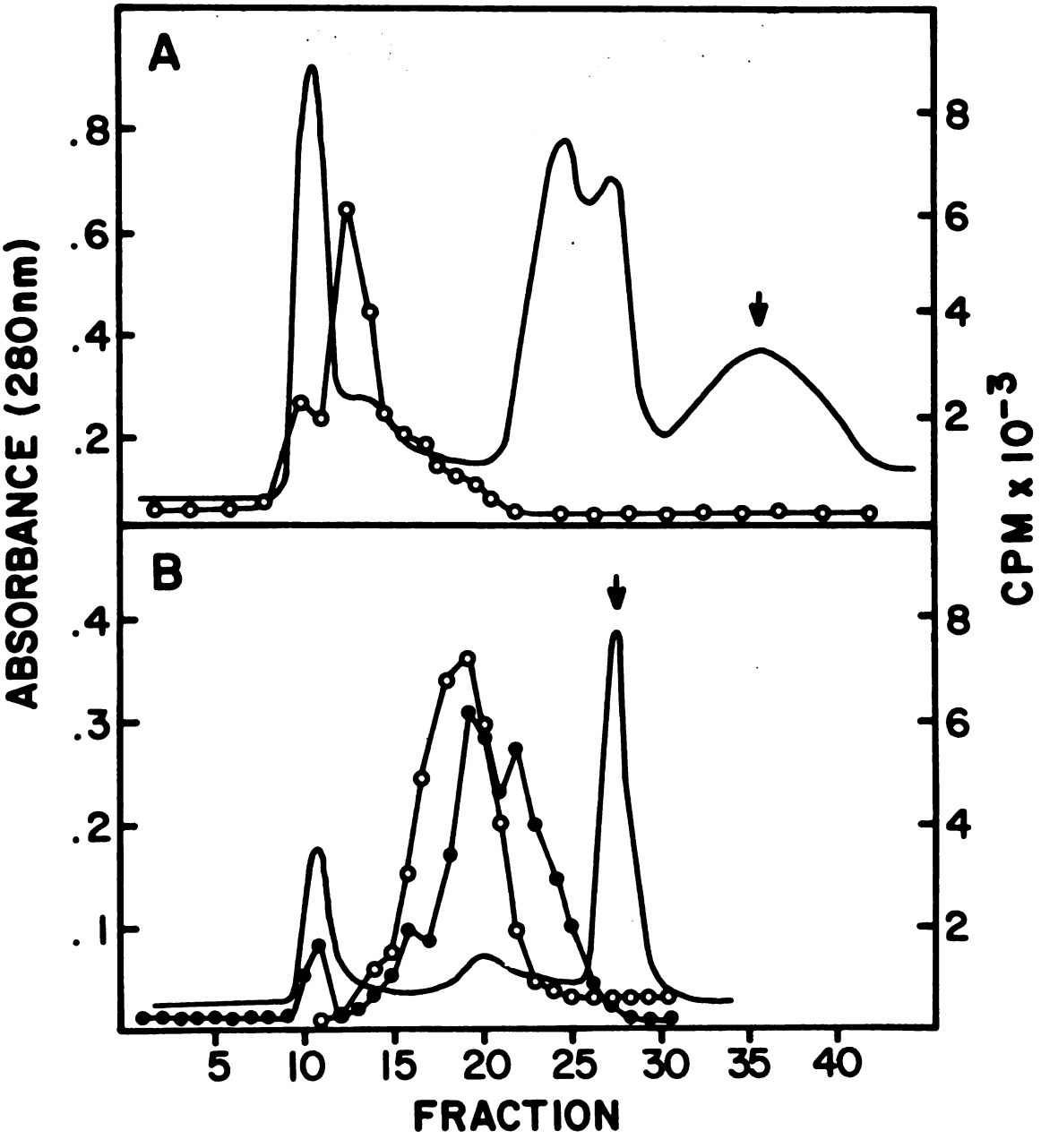
A
B
ABSORBANCE (280nm)
CPM x 10⁻³
.8
.6
.4
.2
.4
.3
.2
.1
8
6
4
2
8
6
4
2
5
10
15
20
25
30
35
40
FRACTION

Figure 1.

Figure 2. SDS gel electrophoresis of crude tumor supernatant and chemotactively active fractions from the Sepharose 6B column. A. Crude tumor supernatant. B. Sepharose 6B active fractions without 2ME. C. Sepharose 6B active fractions with 2ME. D. Trypsinized Sepharose 6B active fractions. E. Myofibril standards. F. Other standards as described in the text.

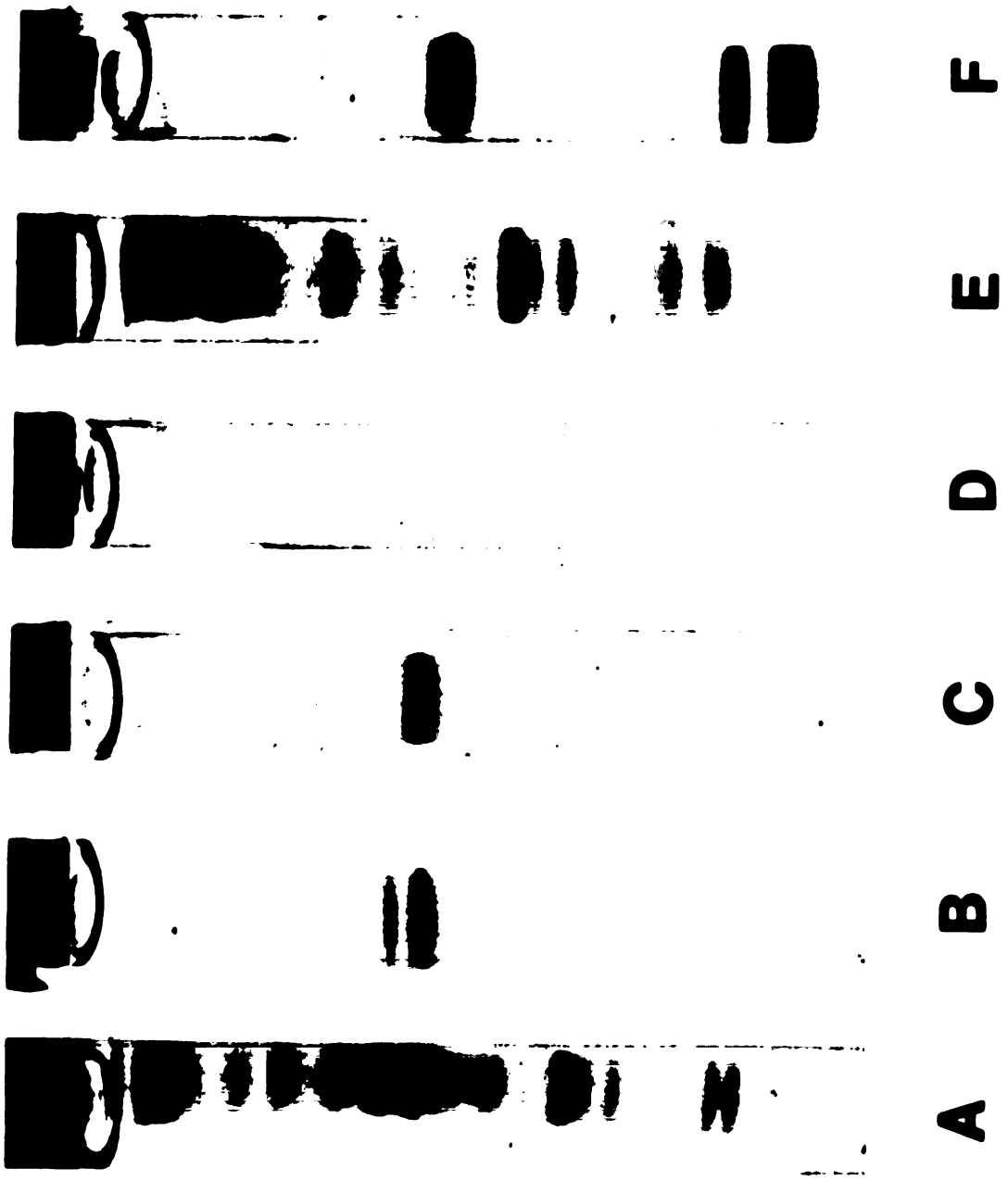

Figure 2.

Figure 3. Protein profile of radiolabeled Sepharose 6B active fractions on SDS gel electrophoresis. Protein profile (——) and radioactivity of Radiolabeled Sepharose 6B active fractions (●——●) on SDS gel electrophoresis. Molecular weight standards were: A. α actin (102,000 MW), B. Bovine serum albumin (68,000 MW), C. Soybean trypsin inhibitor (22,000 MW), D. Hemoglobin (15,500 MW) and lysozyme (13,000 MW). TD=tracking dye.

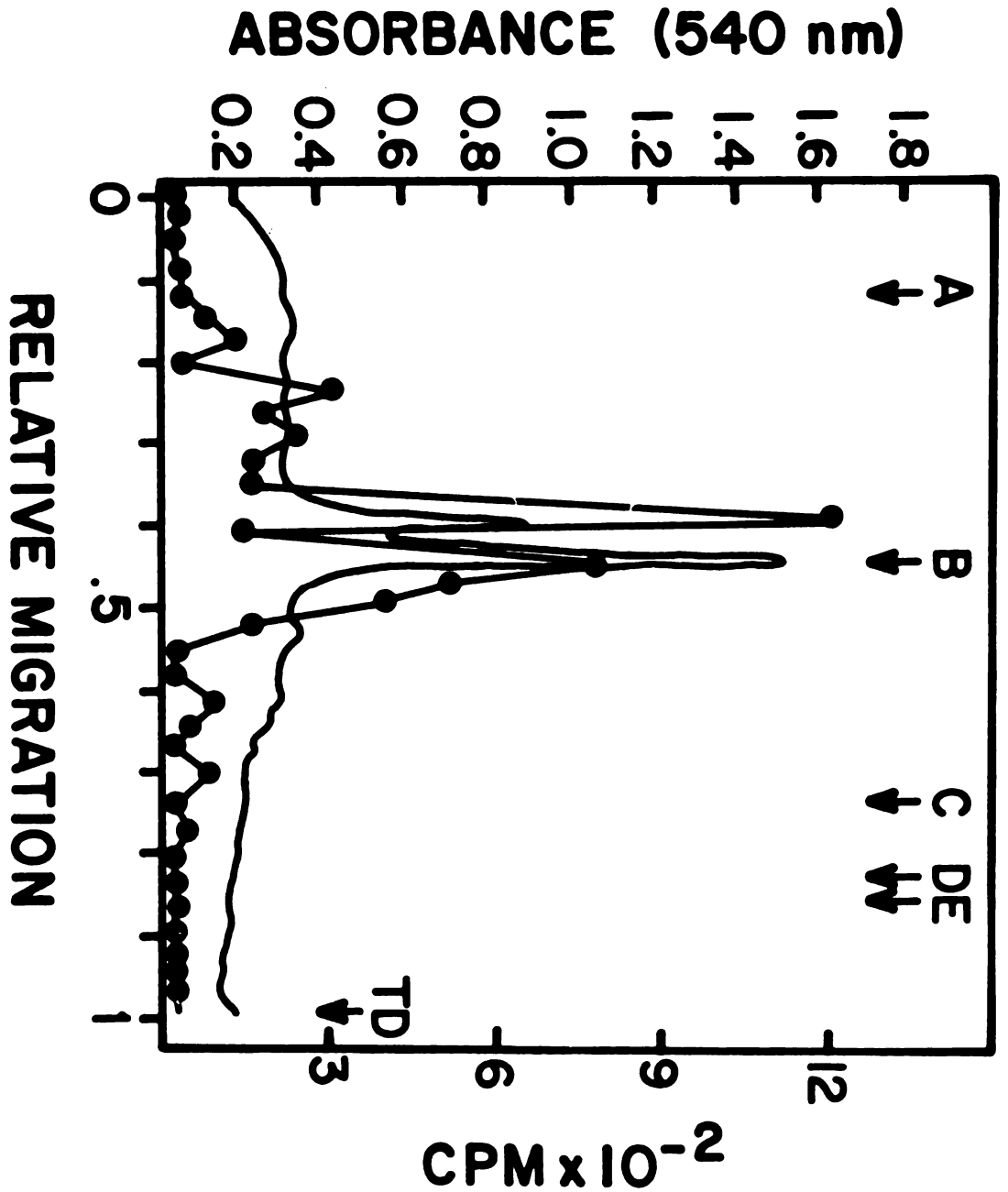
ABSORBANCE (540 nm)
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
RELATIVE MIGRATION
0
.5
1
A
B
C
DE
W
TD
3
6
9
12
CPM x 10⁻²

Figure 3.

Figure 4. Protein profile of active Sepharose 6B fractions on native gel. TD=tracking dye.

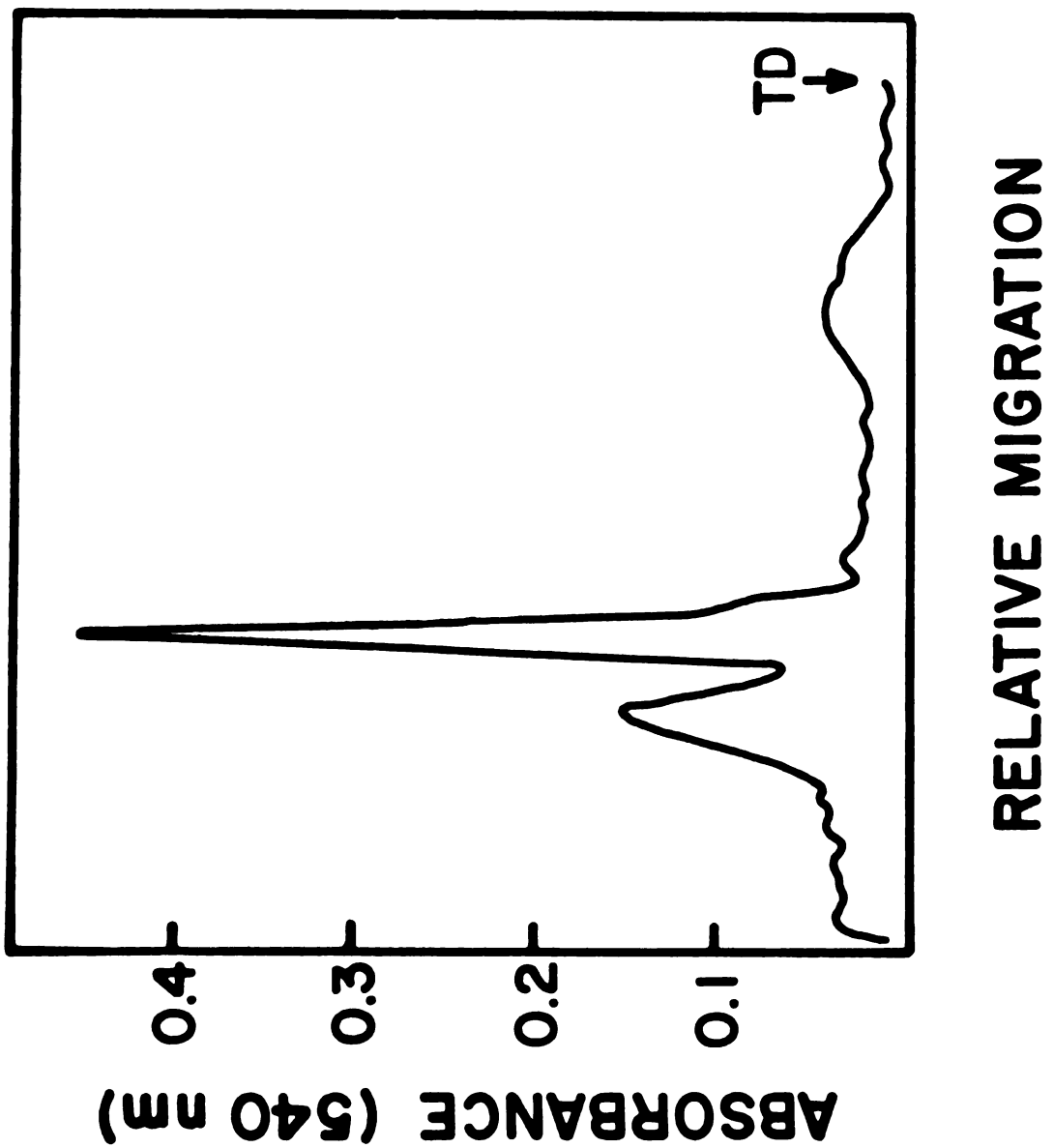

Figure 4.

TABLE I

Measurment of Inflammatory response *In Vivo* to

Partially Purified Tumor Material

	Experiment 1		Experiment 2	
Treatment	6B Injected	Controls	6B Injected	Controls
Average Number PC/Mouse	1.1 ± 0.1	0.94 ± 0.1	1.1 ± 0.1	0.81 ± 0.1
Differential Cell Counts[a]				
Macrophages[b]	78.8 ± 4.0	50.6 ± 2.3	79.1 ± 2.3	50.7 ± 2.8
Lymphocytes[b]	17.0 ± 4.7	48.2 ± 2.3	18.4 ± 2.8	47.9 ± 2.9
Basophils	1.6 ± 0.5	0.9 ± 0.4	1.1 ± 0.4	0.4 ± 0.2
Neutrophils	0.9 ± 0.6	0	0.8 ± 0.4	0.9 ± 0.9
Eosinophils	1.3 ± 0.8	0	0.6 ± 0.4	0

[a]Data represents % of each cell type per 100 cells counted.

[b]$p < 0.01$ when compared to control values.

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