

## PRODUCTS OF ACTIVATED LYMPHOCYTES

### I. THE USE OF RADIOLABELING TECHNIQUES IN THE CHARACTERIZATION AND PARTIAL PURIFICATION OF THE MIGRATION INHIBITORY FACTOR OF THE GUINEA PIG

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It is clear that the specific immunological information for delayed-type hypersensitivity reactions is carried by sensitized lymphocytes, probably thymus (T)<sup>1</sup> cells, yet the molecular mechanisms by which they mediate the various manifestations such as resistance to infection, tumor and allograft rejection, and cell cooperation in vivo are unknown. Since their discovery, the products of activated lymphocytes (PALs) have held a special fascination by virtue of their possible role as biochemical mediators of the cell-mediated immune response in vivo, and more generally, as macromolecules which can produce profound biological effects in vitro and in vivo in minute quantities. Because of the small amounts produced by antigen-stimulated lymphocytes, the factors have been extraordinarily difficult to purify and characterize by conventional biochemical techniques. However, as a result of the painstaking efforts of Remold et al. (1, 2, 16) and Dumonde et al. (3), using large pools of material, the migration inhibitory factor of the guinea pig (MIF) appears to be within a molecular weight range of 35–82,000, a density range of 1.352–1.452, of greater electrophoretic mobility in acrylamide gels than serum albumin and labile to neuraminidase, suggesting a glycoprotein nature. The difficulty is how to proceed from this work to the total isolation, purification, and characterization, in chemical terms, of macromolecules such as MIF, available in such limited quantities.

Our goal was to develop methods which would: (a) require fewer lymphocytes than previous ones, e.g. less than one guinea pig equivalent; (b) permit physical chemical characterization of molecules in terms of intrinsic molecular properties; (c) permit quantitative chemical assays ultimately to replace the rather variable bioassays presently in use; and (d) be sufficiently general to be applicable to a whole range of cell constituents and products. Our approach has been to adapt sensitive isotopic labeling

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<sup>1</sup> Abbreviations used in this paper: Con A, concanavalin A; MEM, Eagle's minimal essential medium; MIF, migration inhibitory factor of the guinea pig; NGPS, normal guinea pig serum; PALs, products of activated lymphocytes; PHA, phytohemagglutinin; *pI*, isoionic point; PPD, purified protein derivatives; T cells, thymus cells.

methods to deal with this problem. The double-labeling technique, so useful for the study of phage and animal virus protein synthesis (4, 5), has provided the basis for this approach. In this method, nonstimulated control lymphocytes are labeled, for example with [ $^{14}\text{C}$ ]leucine, and cultures of the same pool of sensitized cells stimulated with antigen or mitogen are labeled with [ $^3\text{H}$ ]leucine. Although many species of proteins may be labeled, by comparing the ratio of  $^3\text{H}/^{14}\text{C}$  or absolute differences in labeling patterns, it is possible to identify and follow those proteins synthesized either *de novo* or in increased amounts by stimulated lymphocyte cultures. For convenience we shall designate these as "radiolabeled peaks." Rosenberg and Levy (6) have recently used this technique to determine that the earliest increase in labeling ratio in phytohemagglutinin (PHA)-stimulated lymphocytes was associated with the nuclear fraction.

The present work will indicate that the radiolabeling techniques reveal significant increases in purified protein derivative (PPD)- or concanavalin A (Con A)-stimulated cultures of cell-associated and cell-released labeled proteins. The double-labeling profiles of supernatants on Sephadex G-75 columns and then on isoelectric focusing were analyzed. The characterization of MIF in these studies was completed by testing for biological activity after each fractionation step. With these techniques it has been possible to recover MIF activity associated with a single radiolabeled peak from as few as  $6 \times 10^8$  lymphocytes.

#### *Materials and Methods*

*Animals.*—Hartley guinea pigs were sensitized to tuberculin as described previously (7). The National Institutes of Health guidelines for handling of experimental animals were followed in all experiments.

*Macrophage Migration Assay.*—Supernatants and purified fractions derived from them were tested for their ability to inhibit the migration of normal peritoneal exudates as described in detail previously (7). The exudates were induced by intraperitoneal injection of 20 ml of light mineral oil (Marcol 52; Humble Oil & Refining Co., Brooklyn, N.Y.) and Drakeol 6VR (Pennsylvania Refining Co., Butler, Pa.) 1:1. Peritoneal cells were harvested 72 h after injection of the paraffin oil in Hanks' solution containing 0.5 U/ml heparin, washed twice with Eagle's minimal essential medium (MEM) (Grand Island Biological Company, Grand Island, N.Y.), and resuspended to a cell concentration of  $30 \times 10^6$  viable cells/ml in MEM containing 15% normal guinea pig serum (NGPS) (North American Biologicals, Inc., Rockville, Md.), or in fractions reconstituted in the same medium. Migration areas were traced and measured after 24 and 48 h. For these experiments all migration percent inhibition was calculated relative to medium controls, i.e., the average migration of at least four replicate capillaries containing normal exudate cells migrated in fresh NGPS-MEM. This medium control is defined as 100% migration in these experiments.

*Lymphocyte Cultures and Production of MIF.*—Lymph node cells were obtained from tuberculin-sensitive guinea pigs 3–5 wk after sensitization and 7–10 days after skin testing with 10  $\mu\text{g}$  of tuberculin PPD (Ministry of Agriculture of Fisheries and Food, Central Veterinary Laboratory, Weybridge, England). The lymphocytes were teased in Hanks' solution containing 5% NGPS plus penicillin (100 U/ml) and streptomycin (100  $\mu\text{g}$ /ml) and strained to remove tissue fragments as described previously (7). The normal guinea pig serum used for these experiments was heat inactivated at 56°C for 30 min. After washing twice in serum-free medium, the cells were resuspended at a concentration of  $15 \times 10^6$  viable cells/ml in MEM

supplemented with antibiotics and cultured in Falcon tissue culture flasks (catalog no. 3024; Falcon Plastics, Div. of B-D Laboratories, Inc., Los Angeles, Calif.) at a density of  $8 \times 10^6$  viable cells/cm<sup>2</sup>, using 40 ml of cell suspension per flask. For production of MIF, lymphocytes were stimulated either with PPD (25  $\mu$ g/ml) or concanavalin A (Calbiochem, Los Angeles, Calif.; purified by the method of Agrawal and Goldstein [8]) at a concentration of 1  $\mu$ g/10<sup>6</sup> cells. The cultures were gassed and incubated in an atmosphere containing 10% CO<sub>2</sub>, 7% O<sub>2</sub>, and 83% N<sub>2</sub>. After incubation for 20 h, the cells were removed by centrifugation at 500 g for 15 min.

For most studies, the cell-free supernatants were concentrated quickly to 5 ml with dry Sephadex G-25 (coarse grade; Pharmacia Fine Chemicals, Uppsala, Sweden) and desalted. An example of the procedure is as follows: to 40 ml of supernatants is added 13 g of dry Sephadex until all available fluid has been absorbed. The slurry is then transferred to a Boerner centrifuge filter (Arthur H. Thomas, Philadelphia, Pa.; catalog no. 4578-F25) and centrifuged at 1000 g for 5 min. In this step the solution is concentrated to approximately  $\frac{1}{3}$  of the initial volume. After an additional concentration step, the 5-ml concentrates were then immediately desalted on a G-25 (superfine) Sephadex column (2.5  $\times$  25 cm; running buffer 0.01 M sodium phosphate, pH 7.5). The excluded peak was collected, frozen in dry ice methanol, and lyophilized.

When it became clear that the migration inhibitory activity was soluble in high salt concentrations while many other supernatant proteins were not, supernatants were directly lyophilized, dissolved in 5% original volume of 0.01 M HCl as described previously (9), mixed with label marker, and centrifuged to remove salted-out proteins (1000 g, 15 min). The concentrated soluble material was then applied immediately to the Sephadex G-75 columns.

*Gel Filtration on Sephadex G-75 Column.*—Sephadex G-75 was boiled in 0.01 M phosphate buffer, pH 7.5, for 1–3 h. After cooling, the slurry was poured into a 1.5  $\times$  125 cm column, previously sterilized by autoclaving. Upward flow chromatography was carried out using adapters (Glenco Scientific, Houston, Tex.). The running buffer, 0.01 M phosphate buffer, pH 7.5, was autoclaved for use.

For fractionation of supernatants, desalted lyophilized supernatants, either control or MIF containing, were dissolved in 0.5 ml of sterile distilled water and mixed with 0.5 ml of a sample of a radioactive control supernatant as a marker. Occasionally, a small precipitate formed and was removed by centrifugation. The sample was then applied to the Sephadex G-75 column and fractions of 1 ml were collected at a flow rate of 5 ml/h. For establishing the labeled protein marker profile, 0.1 ml of each fraction was counted in a scintillation counter. For further fractionation in migration inhibition studies, every two tubes were pooled, dialyzed in sterilized dialysis bags (Union Carbide Corp., Chicago, Ill.) against 12 liters of distilled water overnight, and lyophilized.

For calibration of the columns, molecular weight standards employed were human gamma globulin, mol wt 140,000; human serum albumin, mol wt 67,000; ovalbumin, mol wt 45,000; chymotrypsinogen A, mol wt 25,000; and cytochrome *c*, mol wt 12,400 (Schwarz/Mann Div., Becton Dickinson & Co., Orangeburg, N.Y.). *K<sub>d</sub>* values were established using gamma globulin to indicate the exclusion volume and tritiated H<sub>2</sub>O to indicate the total column volume.

*Incorporation of Labeled Amino Acid into Cell-Associated and Cell-Released Protein.*—For studying the incorporation of amino acid into total protein released by stimulated lymphocytes into the supernatants, lymph node cells from individual guinea pigs were cultured at a density of  $10 \times 10^6$  cells/1 ml in Leighton tubes (Bellco Glass, Inc., Vineland, N.J.). To one series of control cultures and another of PPD-stimulated cultures were added 1  $\mu$ Ci of [<sup>14</sup>C]leucine (specific activity > 250 mCi/mM; New England Nuclear Corp., Boston, Mass.). At various times one tube of each set was cooled in ice, and the contents were transferred to centrifuge tubes and centrifuged at 250 g for 10 min. The supernatants were precipitated with 4 ml of 5% trichloroacetic acid (TCA). The cell pellet was washed in MEM, centrifuged at 250 g, dis-

solved in 0.5% sodium deoxycholate, and precipitated with 4 ml of 5% TCA. The precipitates were heated for 10 min on a steam bath, cooled, trapped in a fiber glass filter (Whatman GF/A, 25 mm), and washed with approximately 5 cc of 5% TCA. The filters were dried and counted in a scintillation mixture (1 vol toluene and 2 vol Triton X-100 [Rohm and Haas Co., Philadelphia, Pa.] containing 4 g/liter 2,5-diphenyloxazole [K & K Laboratories, Inc., Plainview, N.Y.] at 75%.

*The Double-Labeling Technique.*—The basis of the technique is labeling of control cultures with leucine containing one isotope and the experimental with another, such that when the two are combined at a fixed ratio and fractionated, slight differences in synthesis of even small amounts of proteins can be detected as an increase in ratio in the stimulated to control samples. For these studies, lymph node cells from tuberculin-sensitive guinea pigs were washed and cultured in Joklik-modified MEM Spinner medium *minus leucine* at a density of  $40 \times 10^6$  cells/ml or  $8 \times 10^6$  cells/cm<sup>2</sup>. 5 ml of each suspension was cultured in a Falcon plastic culture flask (25 cm<sup>2</sup>, no. 3012). Into one culture, for example the control culture, is added 100  $\mu$ Ci [<sup>14</sup>C]leucine ( $> 250$  mCi/mM) and into an experimental culture, i.e. a PPD-stimulated or Con A-stimulated culture, is added 500  $\mu$ Ci [<sup>3</sup>H]leucine (30–50 Ci/mM). Experimental cultures were stimulated with PPD at 25  $\mu$ g/ml or concanavalin A at 1  $\mu$ g/ $10^6$  cells. The flasks were gassed and cultured in a Dutton-type sealed plastic chamber (Norbo Machine Co., Huron, S. Dak.) in an atmosphere of 10% CO<sub>2</sub>, 7% oxygen, and 83% nitrogen (Matheson Co., Inc., East Rutherford, N.J.). After 20 h of incubation, the cells were centrifuged at 500 *g* for 10 min. To the supernatants was added 0.2 ml of 40 mM cold leucine solution, after which they were ultracentrifuged at 100,000 *g* for 1 h in a Spinco ultracentrifuge (Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.). The labeled supernatants were desalted, and free labeled leucine was removed by passage through a G-25 Sephadex column (2.5  $\times$  25 cm). The column was monitored by counting 50- $\mu$ l aliquots of each 3 ml fraction. The excluded protein peak was collected and lyophilized.

It was convenient to have an internal marker or labeled standard of unstimulated supernatant for further fractionation procedures. For this purpose, lymphocytes were cultured in medium as described above in the presence of 50  $\mu$ Ci [<sup>14</sup>C]leucine, and the labeled marker supernatant was desalted and lyophilized as above.

*Gel Filtration of Double-Labeled Supernatants.*—To prepare the double-labeled mixture, lyophilized, desalted <sup>14</sup>C- and <sup>3</sup>H-labeled supernatants were dissolved in 0.5 ml of sterile distilled water and mixed together at a <sup>3</sup>H/<sup>14</sup>C ratio of approximately 3. After clarification by centrifugation at 1000 *g* for 5 min, the solution was applied to the calibrated G-75 Sephadex column and eluted in 0.01 M phosphate buffer, pH 7.5, in 1-ml fractions. Radioactivity was counted in a Beckman scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.) with the following characteristics: <sup>3</sup>H efficiency, 38%; <sup>14</sup>C efficiency, 60%. For establishing the double-labeling patterns, 0.2 ml of each fraction was mixed with 1 ml of water and 6 ml of scintillation fluid and counted under the following conditions: windows adjusted for 10% <sup>14</sup>C spillover into the tritium channel, and less than 0.1% tritium spillover into the <sup>14</sup>C channel. Under these conditions, the overall efficiency of tritium counting was 15% and that of <sup>14</sup>C counting was 30%. The counts per minute were corrected for spillover, and either was plotted directly or converted to disintegrations per minute.

*Discontinuous Acrylamide Gel Electrophoresis.*—Narrow pools of Sephadex G-75 fractions were subjected to analytical acrylamide gel electrophoresis under conditions described by Remold et al. (1). Lyophilized Sephadex fractions in the region of  $K_d = 0.1$ – $0.2$  (6–10 tubes) were dissolved in 1 ml of distilled water and dialyzed against 0.01 M glycine buffer, pH 6.8, for 5 h. A 200  $\mu$ l aliquot was electrophoresed for 4 h on a  $0.6 \times 10$  cm column of 7.5% acrylamide, pH 9.1, at 200 V. After separation, the gel was removed, frozen, and cut into 2-mm slices. Each slice was put into a scintillation vial and eluted in 1 ml of water overnight, 6 ml of scintillation fluid was added, and the vials were counted.

*Isoelectric Focusing.*—

*Analytical acrylamide gels:* Narrow pools (6–10 tubes) of Sephadex G-75 fractions in the region of MIF ( $K_d = 0.1$ – $0.2$ ) were dissolved in 2 ml of distilled water and dialyzed against distilled water for 5 h. To 2 ml of sample was mixed 1 ml of the following solution: 15 ml of acrylamide (30 g/100 ml + bis-acrylamide (0.8 g/100 ml)); 4 ml of tetramethylethylenediamine (1:100), all from Eastman Kodak Co., Rochester, N.Y.; 1.5 ml of ampholine solution (40% LKB no. 8141 ampholine, pH 3–10, LKB Instruments, Inc., Rockville, Md.). The solution was degassed on a vacuum pump for 5 min and 0.1 ml of a 2% ammonium persulfate solution (Fisher Scientific, Pittsburgh, Pa.) was added and the solution poured into a  $0.6 \times 10.5$  cm glass tube. After polymerization was complete (approximately 1 h at room temperature), 0.2% sulfuric acid was added to the upper reservoir and 0.4% triethanolamine to the bottom reservoir, and the gels were subjected to electrofocusing at constant voltage initially at 2 mA/gel. In each experiment, marker sperm whale myoglobin (Schwarz/Mann) was electrofocused in a parallel gel to indicate the completeness of the focusing process. The usual period for focusing was 3–4 h. The gels were frozen and cut into 2-mm slices, and counted. The indicator gel was cut out as well, extracted with 1.5 ml of boiled distilled water for 3 h, and the pH of each fraction measured.

*Preparative electrofocusing:* A simple piece of apparatus for preparative isoelectric focusing of small samples, shown in Fig. 1, was designed by one of us, C.S. It consisted of a Pyrex U

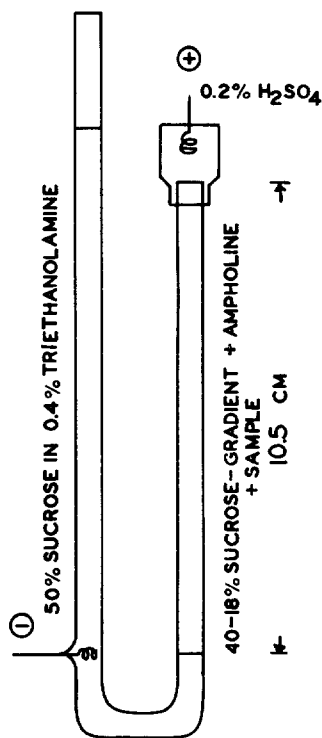


FIG. 1. Micropreparative isoelectric focusing apparatus. Pyrex glass tubing (OD 0.8 cm, ID 0.6 cm) was shaped as shown. The anode well was a rubber ampule stopper with a hole in its center.

tube (OD 0.8 cm; ID 0.6 cm) in the descending end of which a platinum electrode was melted and affixed. For highest recovery, the electrofocusing was carried out without a solid matrix, using a sucrose gradient to stabilize against convection and diffusion. For filling, this long side received 50% sucrose in 0.4% triethanolamine up to the mark. Then the right arm was stoppered in order to fill the left arm to the top with the same solution. The left arm was then stoppered with an ampule cap and the stopper on the right-hand arm was removed. The right-hand arm was then filled with a 40–18% sucrose gradient containing ampholytes and 2 ml of sample. The sucrose gradient was prepared by mixing 1.65 ml of a denser solution containing 0.85 ml of 80% sucrose, 0.75 ml of sample, and 0.05 ml of ampholine solution, pH 3–10, with a lighter solution containing 1.25 ml of sample, 0.35 ml of 80% sucrose, and 0.05 ml of ampholine with constant stirring in a gradient mixer (see Fig. 1). The sample consisted of a narrow pool of lyophilized Sephadex G-75 fractions dissolved in distilled water and dialyzed for 5 h against distilled water. On top of the right arm, a reservoir made from an ampule stopper with a hole in the center was attached, filled with 0.2% sulfuric acid, and the platinum anode was placed in contact with it. The left arm stopper was then removed and the sample was focused for approx. 16 h at 500 V constant voltage, 1 mA initial current. At the completion of the separation, the cathodal end was connected to a peristaltic pump infusing 50% sucrose at the bottom at a rate of 10 ml/h. A total of 20 fractions each containing 0.15 ml were collected from the top of the right-hand column in an LKB fraction collector. They were diluted with 1.5 ml of boiled distilled water and the pH was determined on each sample in a Radiometer pH meter (Radiometer Co., Copenhagen, Denmark). To remove the ampholytes the fractions were dialyzed extensively at 4°C, 24 h against 0.1 M glycine buffer pH 6.0, 24 h against 0.1 M NaCl, and 24 h against distilled water. In later experiments, gelatin (300 µg; Difco Laboratories, Inc., Detroit, Mich.) was added to each fraction to minimize adsorption of active material to the dialysis bag. The dialyzed samples were freeze dried, and later dissolved in MEM plus 15% NGPS for assay of MIF activity. Alternatively, each fraction was passed over a 0.5 × 20 cm Sephadex G-25 column in distilled water to remove ampholytes and sucrose, and then freeze dried directly.

#### RESULTS

*Cumulative Protein Synthesis in PPD-Treated Normal and Tuberculin-Sensitive Lymph Node Cells.*—The first question to be faced in developing an isotopic-labeling technique for mediators of hypersensitivity is whether it is possible to see gross differences in incorporation of labeled amino acids into proteins in the supernatants of antigen-stimulated cultures. For such experiments lymph node cells from normal and tuberculin-sensitive guinea pigs were cultured in leucine-free medium in the presence or absence of PPD (25 µg/ml). [<sup>14</sup>C]leucine was added at the beginning of culture and samples were removed at various times, cells and supernatants were separated by centrifugation, and cell-associated and supernatant proteins were separately precipitated by addition of 5% cold TCA. The results of such an experiment are shown in Fig. 2. The amount of newly synthesized cellular protein generally exceeded that of released protein by 5–10-fold. Tuberculin PPD had neither nonspecific stimulatory nor toxic effects on protein synthesis or release from normal, non-sensitized guinea pig lymph node cells. In contrast, there was a clearly detectable stimulation in leucine incorporation into both cell-associated and released protein in the cultures of tuberculin-sensitive cells stimulated by tuberculin. The increase in synthesis in antigen-stimulated cells was first discernible at

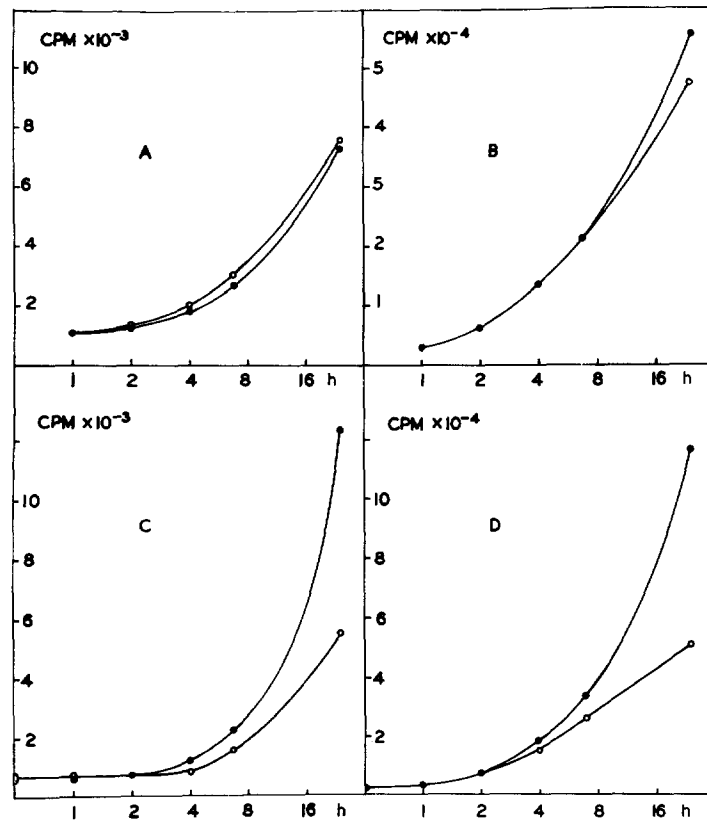


FIG. 2. Cumulative incorporation of [<sup>14</sup>C]leucine into TCA-precipitable protein. The open circles represent cultures in medium alone; the closed circles indicate cultures in medium + PPD (25 µg/ml). (A) Normal lymph node cells: supernatant protein; (B) Normal lymph node cells: cell-associated protein; (C) Tuberculin-sensitive lymph node cells: supernatant protein; (D) Tuberculin-sensitive lymph node cells: cell-associated protein.

approx. 4 h and continued to increase such that by 20 h there was a reproducible difference of twofold or greater in leucine incorporation in both supernatant and cell-associated protein of stimulated cultures. These results follow closely the labeling kinetics described previously by Levy and Rosenberg (10), and correlate with the appearance of MIF activity at about 6 h after stimulation (11).

*Relationship between Labeling Profile on Sephadex G-75 Gel Filtration and Migration Inhibitory Activity.*—Because there was a significant increase in released labeled proteins, it was our expectation that we might see a unique labeled peak associated with MIF activity after gel filtration on Sephadex. In order even to be able to associate a labeled protein fraction with MIF activity, it was necessary to establish precisely on our calibrated columns the

elution volume for MIF. Published results on the molecular size of MIF could not be relied upon because there were considerable differences in conditions for gel filtration and because large pools of fractions were invariably used. In order to provide an internal marker for this fractionation and testing of biological activity, a  $^{14}\text{C}$ -labeled nonstimulated marker supernatant was admixed with each concentrated supernatant (eq 40 ml) to be chromatographed on Sephadex G-75. From the breakthrough peak onward, 100 Sephadex fractions were collected from each column, and a sample from each tube was counted for radioactivity to establish the marker profile (Fig. 3 A) which was remarkably constant in all experiments. The marker profile indicated the breakthrough volume and a second constant peak at  $K_d$  0.6. Three types of samples were fractionated: control supernatants, tuberculin-sensitive cells stimulated with PPD, and tuberculin-sensitive cells stimulated with concanavalin A. In these experiments, each 40 ml of supernatant fractionated represented  $6 \times 10^8$  lymph node cells, i.e., less than one guinea pig equivalent of lymphocytes.

For determination of MIF activity every two tubes were pooled, freeze dried, and reconstituted in MEM plus 15% normal guinea pig serum for testing migration inhibitory activity on normal peritoneal exudate cells. While in previous work it has been common practice to calculate a migration index comparing the migration of cells in antigen-stimulated fractions relative to that in corresponding control fractions, for chemical fractionation purposes it is necessary to have a more absolute standard for 100% migration. It seemed important to be able to discriminate all migration inhibitory or toxic fractions of the column, and to determine later whether they were present only in antigen-stimulated supernatants, rather than calculating only the relative percent inhibition.

The elution pattern on Sephadex G-75 columns of migration inhibitory activity is shown for control supernatant reconstituted with PPD (Fig. 3 B), PPD supernatant (Fig. 3 C), and concanavalin A supernatant (Fig. 3 D). When migration inhibitory activity of every two fractions was analyzed, it became clear that there were many peaks of inhibitory activity associated with the supernatants. In four experiments using control supernatants, it was possible to define prominent inhibitory peaks at  $K_d$  0.0, 0.09, and 0.26 which recurred in all supernatants, control or stimulated. We have chosen to present this particular experiment to indicate the maximal degree of nonspecific inhibitory activity which can be observed from lymphocyte culture supernatants. Nevertheless, in five experiments in which PPD- or con A-stimulated supernatants were examined in detail, it became clear that there was an additional new peak of migration inhibitory activity at  $K_d = 0.15$ , not present in controls, which was associated only with stimulated supernatants. Increased migration inhibitory activities at  $K_d$  0.3-0.4 were seen in Con A supernatants. It was the peak at  $K_d$  0.15 which was presumed to contain the antigen- or mitogen-induced MIF which was studied further.



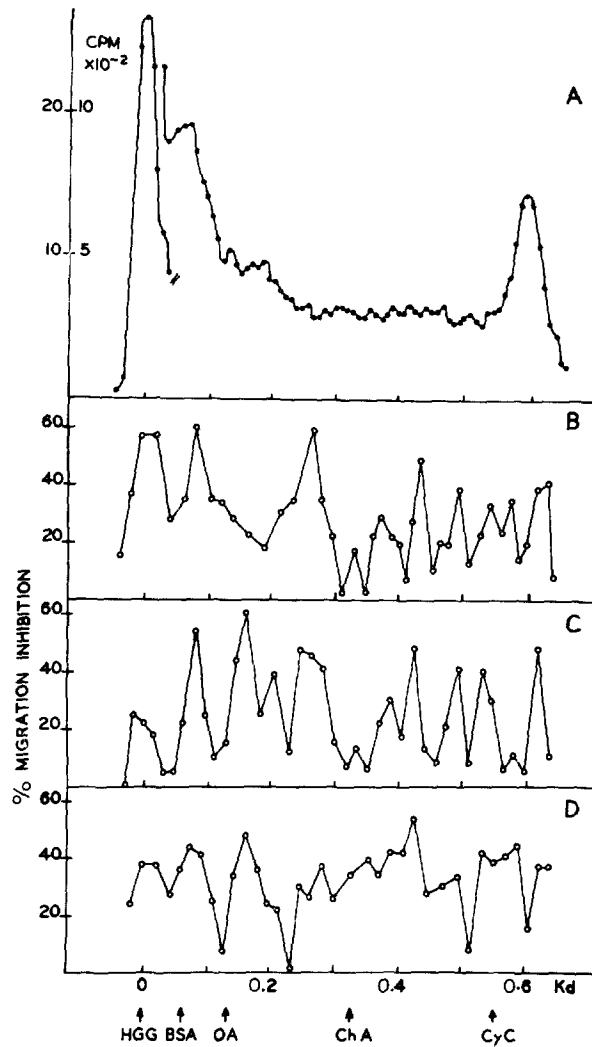


FIG. 3. Migration inhibitory activity of Sephadex G-75 eluates. (A) Marker profile of labeled protein (control supernatant labeled with [ $^{14}$ C]leucine). Concentrated marker (eq 1.5 ml of supernatant) was added to each concentrated supernatant (eq 40 ml), fractionated, and tested for biological activity. (B) Migration inhibition of control supernatant (eq 20 ml). Average percent inhibition of fractions reconstituted in medium and medium + PPD. (C) Migration inhibition of PPD supernatant (eq 20 ml). Average percent inhibition of fractions reconstituted in medium and medium + PPD. (D) Migration inhibition of Con A supernatant (eq 20 cc). Fractions reconstituted in medium alone.

To test a possible antigen dependence or specificity of the biological activity in these fractions, in some experiments every two fractions were pooled, half was reconstituted in medium alone and half in medium containing PPD (25  $\mu\text{g}/\text{ml}$ ). Thus the effect of antigen on migration inhibitory activity was studied. In no case was the pattern of inhibitory activity altered by the addition of PPD, although the addition of PPD consistently enhanced already existing inhibitory activities by approximately 10–15%. This slight nonspecific enhancing property of PPD may account in some measure for earlier reports of antigen specificity of MIF.

*Use of Double-Labeling Technique for Analysis of Products of Activated Lymphocytes.*—When elution profiles of PPD-stimulated and control supernatants labeled with [ $^{14}\text{C}$ ]leucine were compared, normalizing the curves to equal base-line counts, the patterns were essentially indistinguishable and resembled Fig. 3 A. Thus it was virtually impossible to distinguish any single peak increased in the PPD-stimulated cultures at the level of gross labeling. However, even under circumstances in which the bulk of total synthesized proteins produced may be similar, the double-labeling technique permits one to distinguish even small areas in which there is greater incorporation in the stimulated culture relative to the control. Accordingly, PPD-stimulated lymph node cells were cultured in the presence of [ $^{14}\text{C}$ ]leucine and control supernatants in the presence of [ $^3\text{H}$ ]leucine. At 20 h the supernatants were processed, mixed together, and fractionated on the same Sephadex G-75 calibrated column. As can be seen in Fig. 4 B, a broad increased radiolabeled peak was seen at  $K_d$  0.05–0.2. The magnitude of the ratio difference of this peak was invariably small, generally 15% above the base-line ratio. It therefore did appear that there was an increase in some synthesized proteins in the PPD-stimulated culture, relative to control, which eluted in fractions known to contain MIF.

Because the mitogen concanavalin A has been found to induce MIF formation and stimulate lymphocytes to a greater degree than antigens (12, 16), the effect of Con A on the double-labeling pattern was studied. The total labeling profile of a Con A and unstimulated control supernatant are shown in Fig. 4 A. After normalizing the raw data to equal base-line counts, a clear difference in gross counts between the stimulated and control cultures is seen. When Con A-stimulated cultures were labeled with [ $^3\text{H}$ ]leucine and the control with [ $^{14}\text{C}$ ]leucine, the double-label profile indicated up to three radiolabeled peaks, a major one maximal at  $K_d$  0.1 and two smaller at  $K_d$  0.3 and 0.5 (Fig. 4 C). The first peak eluted in the same position as that in which MIF and the radiolabeled peak of PPD-stimulated supernatants were seen, although the increase in ratio with Con A was generally about twofold greater than with PPD. The broad area of this peak indicates that it must be comprised of several labeled proteins. Reversal of labels in this experiment (Fig. 4 B, C) indicated that the increase in ratio is independent of the isotope used and related solely to whether the cultures were stimulated or not. In general we chose to label the stimulated

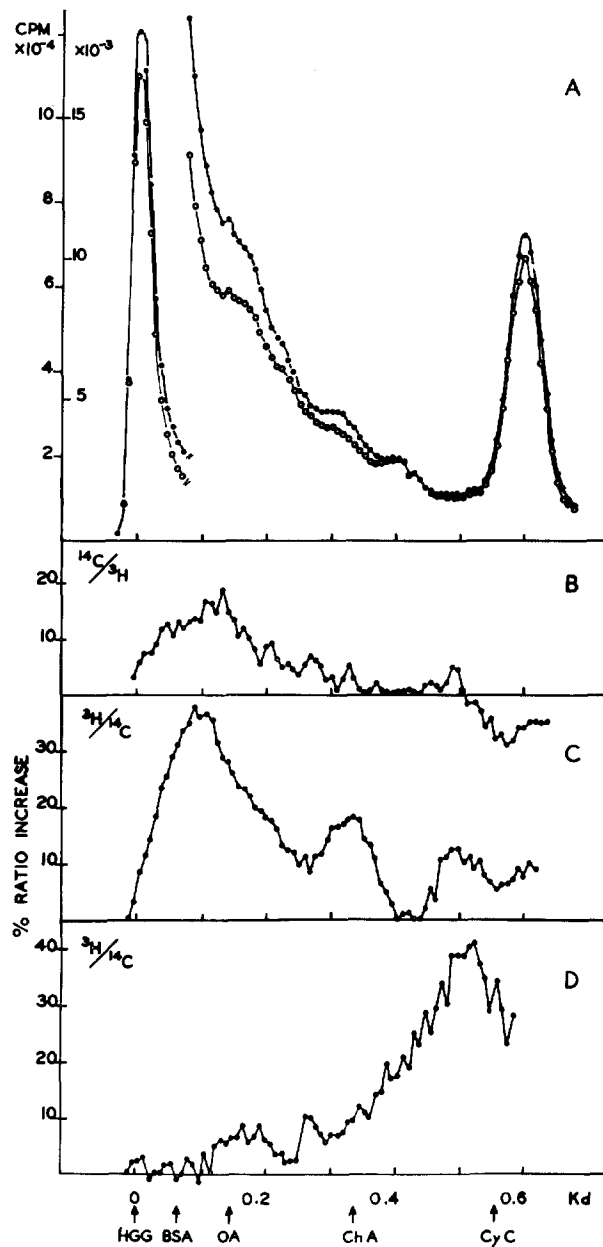


FIG. 4. Double-labeled profile and ratio patterns. (A) Counts per minute (normalized to equal base-line counts) of admixed control supernatant (open circles) labeled with [<sup>14</sup>C]leucine and Con A supernatant (closed circles) labeled with [<sup>3</sup>H]leucine. (B) Ratio of protein counts in PPD supernatant relative to control. In this case, the stimulated culture was labeled with [<sup>14</sup>C]leucine and the controls with [<sup>3</sup>H]leucine. The percent increase in ratio is plotted: the base-line ratio of <sup>14</sup>C/<sup>3</sup>H count was 0.63. (C) Ratio of protein counts in Con A supernatant relative to control. The base-line ratio of <sup>3</sup>H/<sup>14</sup>C count was 3.41. (D) Ratio of pre-labeled protein counts released after Con A stimulation relative to control. Two unstimulated cultures were labeled for 24 h, one with [<sup>14</sup>C]leucine the other with [<sup>3</sup>H]leucine. After washing and chasing with cold leucine, the [<sup>3</sup>H]leucine labeled culture was stimulated for 20 h with Con A. The percent increase in pre-labeled proteins released into the supernatants is shown. Base-line ratio 3.1.

cultures with the weaker isotope ( $^3\text{H}$ ), so that differences would be underestimated if anything. In nine experiments with Con A-stimulated supernatants, the average ratio increase at the  $K_d$  0.1 peak was  $28.2 \pm 3.0\%$ . Thus the ratio-labeling patterns for mitogen- and antigen-stimulated cells both showed an area of increased labeling in the region associated with biological activity. It cannot be emphasized too strongly that the degree of isotope incorporation and the reproducibility of the radiolabeled patterns depends critically on the culture conditions, especially pH, cell density, concentration of stimulant, and amino acid-deficient medium.

*Release of Prelabeled Protein.*—Production of MIF has been found to be inhibited by agents which block RNA or protein synthesis (13, 14, 23), and it has been inferred that MIF represents a protein synthesized *de novo*. To ascertain whether any significant amount of preformed material was released from antigen- or mitogen-stimulated cells with a  $K_d$  similar to MIF, cultures were labeled either with [ $^{14}\text{C}$ ]leucine or [ $^3\text{H}$ ]leucine for 24 h in the absence of stimulation. After that prelabeling period, the cells were washed and incubated in the presence of cold leucine for a further 24 h, during which the [ $^3\text{H}$ ]culture was stimulated with Con A. When the 24–48-h supernatants were filtered on Sephadex G-75 columns, although the total count profiles were similar to Fig. 4 A, a very different ratio pattern of prelabeled materials is seen (Fig. 4 D). It is clear that the large ratio peak of  $K_d$  0.1 present in Fig. 4 C is absent, and that an increased ratio peak at  $K_d$  0.5 is seen. This result suggests that the labeled peak associated with MIF is the result of newly synthesized species of proteins in the size range  $K_d$  0.1, and is not significantly contributed to by preformed materials. The smaller size of prelabeled materials released after antigen stimulation may represent either different species of smaller molecules secreted by the cells, or degradation products of larger molecules. Similarly, the peak width or the “heterogeneity” of the principal peak at  $K_d$  0.1 may indicate either that there are a number of proteins of slightly differing size in that region synthesized by stimulated cultures or that during the 20 h culture period there is partial degradation of a lesser number of synthesized molecules. Further analytical separation of the major radiolabeled peak was necessary to clarify its degree of polydispersity or heterogeneity.

*Analysis of Sephadex G-75 Radiolabeled Peak at  $K_d$  0.13–0.20 by Acrylamide Gel Electrophoresis.*—It had previously been reported that MIF could be separated from a variety of proteins on acrylamide gel electrophoresis and that the peak migration inhibitory activity was obtained in fractions migrating anodally to albumin (1, 16). Consequently, narrow pools of the radiolabeled peak at  $K_d$  0.13–2.0 from Sephadex G-25 columns were subjected to electrophoresis in 7.5% acrylamide gels at pH 9.1 as described by Remold et al. (1). The total labeling pattern for the control and Con A gels is shown in Fig. 5 A, normalized to the same base line of counts, using the same supernatant pools shown in Fig. 4 A, C. Differences in labeling profiles between the stimulated and control

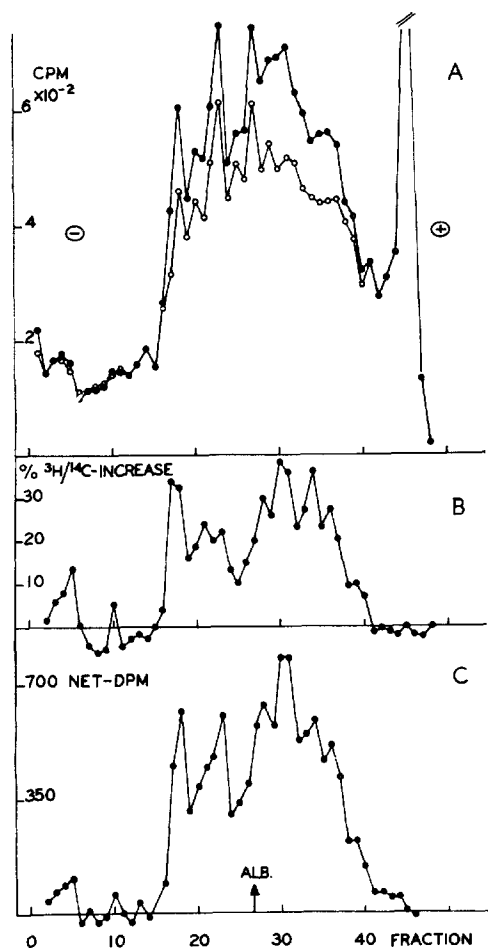


FIG. 5. Labeling pattern of G-75 Peak,  $K_d$  0.13-2.0, on disc. Acrylamide gel electrophoresis (same supernatant pool shown in Fig. 4 A). Total labeling pattern. (A) Counts per minute (normalized to equal base-line counts) of [ $^{14}\text{C}$ ]leucine labeled control and [ $^3\text{H}$ ]leucine labeled Con A fraction. (B) Radiolabeling pattern. The percent increase in stimulated sample is plotted; the base-line ratio of  $^3\text{H}/^{14}\text{C}$  counts was 3.41. (C) Difference curve. Differences in counts between Con A and control curves (A) were corrected and plotted as net disintegrations per minute of stimulated culture above control.

samples were seen more clearly in the ratio curve shown in Fig. 5 B. Even with the narrow Sephadex pool of  $K_d$  0.13-2.0, an increase in labeling of five to six distinct protein species was observed. Clearly the area of greatest increase in labeling in the stimulated fraction was found to migrate anodally to albumin, in the region described by Remold and David (16) to contain MIF.

The double-label technique permits one easily to study those fractions in

which there is an increased ratio of components in stimulated relative to control fractions. However, it is also possible to express the same data as an absolute difference in the incorporation of leucine into the same proteins under standard conditions. The counts are corrected for efficiency into disintegrations per minute and the difference curve represents the absolute increase of labeled leucine incorporated (Fig. 4 C). By correcting further for specific activities the data could be expressed in micromoles of incorporated leucine. Thus different curves could prove useful for quantitating the absolute increase in specific proteins synthesized by activated lymphocytes.

*Analysis of the Ratiolabeled Peak at  $K_a$  0.13–0.20 by Isoelectric Focusing.*—The increase in labeling patterns of proteins migrating faster than albumin could reflect molecules of greater net negative charge than albumin or molecules of smaller size, since acrylamide gels are known also to separate by size (15). Therefore, a more absolute index of the charge characteristics of MIF was sought. Since the isoionic point,  $pI$ , is a characteristic of proteins and independent of the medium in which the isoelectric focusing is carried out, small pools of the ratiolabeled peak ( $K_a$  0.13–0.2) were subjected to ampholine carrier electrophoresis in 7.5% acrylamide gels over a pH gradient of 3–10. The data in Fig. 6 represent results for isoelectric focusing of the same pools of material shown previously (Figs. 4 A, C, 5). A reproducible linear gradient was established from pH 3–8.6.

Two major labeled peaks can be recognized, one at  $pI$  3.5–5, the other  $pI$  5–6. However, the material at  $pI$  3.5–5.0 shows no differences in the ratio of labeling (Fig. 6 B). The difference curve (Fig. 6 C) shows that the greatest difference in synthesis of protein in the Con A-stimulated culture relative to control is found in the area of  $pI$  5–6 averaging  $pI$  5.2 in five experiments. Thus, the ratiolabeled material has a higher  $pI$  than albumin and probably migrates faster than albumin on acrylamide charge gels by virtue of its smaller size.

The size fractionation and isoelectric focusing of the double-labeled supernatants indicated a markedly increased labeling pattern of materials with  $K_a$  value of approximately 0.15, and a  $pI$  value of 5.2. It remained to be demonstrated that there was biological activity associated with this labeled peak. Therefore, a simple micropreparative isoelectric focusing column was developed, shown in Fig. 1. In order to minimize the problems of adsorption to a solid matrix, the preparative ampholine gradient was stabilized with 18–40% sucrose gradient. Again, 40-ml supernatants of control, PPD-stimulated or Con A-stimulated cultures were processed, mixed with [ $^{14}$ C]marker supernatant, and filtered on Sephadex G-75. A pool of 10 tubes of  $K_a$  0.12–0.18 was made and an aliquot of one-third was tested for migration inhibitory activity. An example of the inhibitory activity of this material relative to medium alone is given as follows: control 5%, PPD-stimulated 51%, Con A-stimulated 65% inhibition. The pooled fractions were focused in the microcolumn, and after

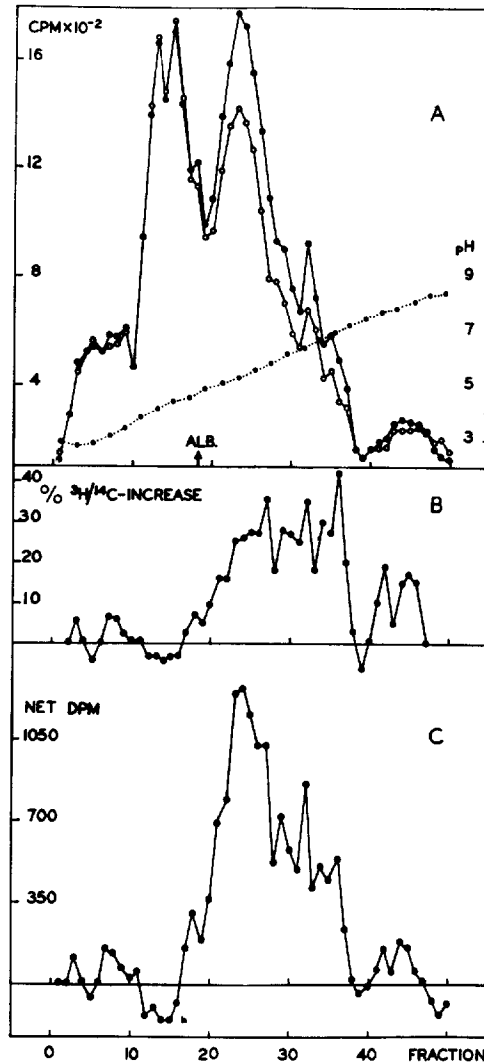


FIG. 6. Labeling pattern of G-75 Peak,  $K_a$  0.13–2.0, on isoelectric focusing gels (see Fig. 5 for details). (A) Total labeling pattern. Dotted line indicates pH gradient. (B) Radiolabeling pattern. (C) Difference curve.

reaching equilibrium, 20 fractions were collected (0.15 ml/fraction). After measuring the pH of each fraction, it was lyophilized and tested for MIF activity. The migration inhibitory patterns of two separate experiments are shown in Fig. 7. It immediately became apparent that the ampholines were not dialyzed away completely, and that some toxicity or spurious migration inhibition was produced, generally at a low level, even in control, non-MIF-

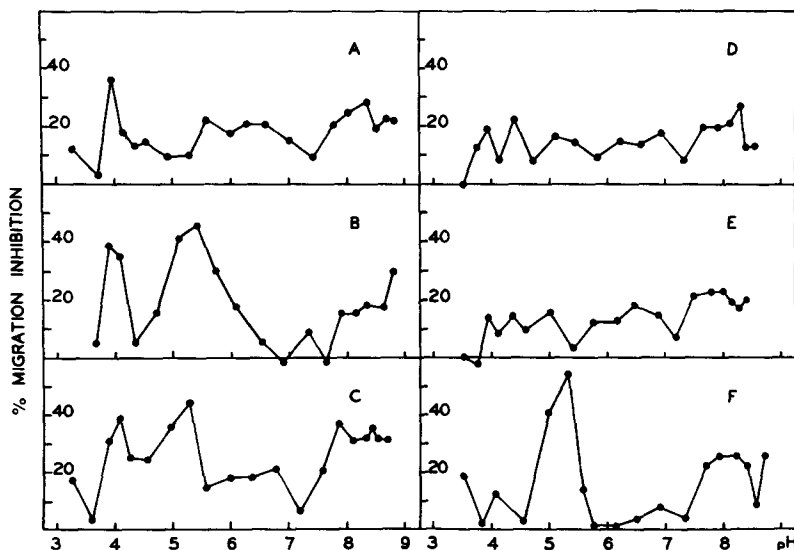


FIG. 7. Migration inhibitory activity of fractions obtained by micropreparative isoelectric focusing. Pool of  $K_d$  0.12–0.18 peak of G-75 columns from 40 ml eq of supernatant was sample. Results of two experiments are shown. (A–C) Migration inhibition of control, PPD and Con A supernatants, respectively. (D) Migration inhibitory activity of ampholine solution alone. (E and F) Migration inhibition of control and Con A supernatants, respectively.

containing Sephadex pools. Figs. 7 A–C were chosen to illustrate a high level of nonspecific inhibition encountered. Even after 3 days of dialysis, marked toxic peaks were present at  $pI$  4.0 and 7.5–8.5. Even so, it was possible to see a markedly increased peak of migration inhibitory activity in the PPD (Fig. 7 B) and Con A (Fig. 7 C) fractions at  $pI$  5–5.5. Addition of gelatin to the fractions diminished losses of activity. The nonspecific inhibitory activity of control supernatants (Fig. 7 E) is essentially accounted for entirely, simply by ampholine toxicity and is no greater than that produced by ampholine solution alone (7 D). In this experiment, there was again a marked increase in MIF activity associated with the fraction of  $pI$   $5.2 \pm 0.3$  pH units, an activity which is found essentially only in stimulated supernatants. Upon preparative isoelectric focusing, significant MIF activity from stimulated supernatants representing less than one guinea pig equivalent of lymph node cells was obtained in five of seven experiments. The average peak inhibition in that fraction in those experiments was  $42.9 \pm 2.7\%$ .

*Composite Picture of Synthesized Products of Activated Lymphocytes.*—By use of techniques described above it is possible to characterize any radiolabeled peak by three parameters: increase in label incorporation above control, as determined by the double-label difference curve;  $K_d$  on Sephadex G-75; and  $pI$  by isoelectric focusing. Individual double-labeled Sephadex fractions be-



tween  $K_d$  0.0 and  $K_d$  0.2 were subjected to analytical isoelectric focusing in gels. The difference curves for the labeling pattern of each species of molecule with distinct  $pI$  was plotted against elution volume on Sephadex G-75. The composite picture so obtained (Fig. 8) indicates the minimum number of different proteins synthesized *de novo* or in increased amounts in Con A- or PPD-stimulated supernatants. A large number of distinct radiolabeled proteins eluted in the void volume, and only the more prominent ones have been plotted. Considering proteins eluting in the included volume of Sephadex G-75, three main peaks could be identified at  $K_d$  0.07, 0.15, and 0.19, which all had very

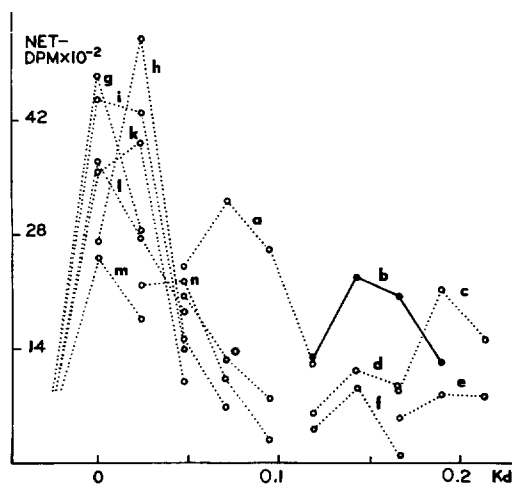


FIG. 8. Composite picture of distinct radiolabeled peaks in Con A-stimulated supernatants derived from two-dimensional analysis of size ( $K_d$  values on G-75 columns) and charge. Each curve represents a protein entity synthesized *de novo* or in increased quantity in stimulated cultures. Solid line indicates radiolabeled peak associated with MIF activity.  $pI$ 's: a, 5.0; b, 5.2; c, 5.1; d, 5.9; e, 6.3; f, 3.3; g, 5.2; h, 6.4; i, 6.1; k, 5.7; l, 5.5; m, 4.9; n, 7.2; o, 7.0.

similar  $pI$  values. This result reinforces the importance of selecting narrow pools of the appropriate  $K_d$  fraction to achieve good resolution in isoelectric focusing. It indicates that the main broad radiolabeling area seen on G-75 columns (Figs. 4 B, C) is undoubtedly polydisperse. Of the fourteen radiolabeled bands plotted, that with a  $K_d$  of 0.15 and  $pI$  of 5.2 is most closely associated with the biological MIF activity.

#### DISCUSSION

It is likely that some products of activated lymphocytes (PALs) will be biochemical mediators of delayed hypersensitivity reactions, and it is clear that they can exert profound biological effects *in vivo* and *in vitro* in minute quantities. For example, Remold et al. (16) and Dumonde et al. (3) found that significant inhibition of macrophage migration in the guinea pig could be pro-

duced by as little as 400 ng of protein. While traditional methods of protein chemistry have been useful in providing us with our present information about the chemical nature of these substances, they have required large amounts of cells and given only small yields. More importantly, it is difficult to see how they can be pressed further into the fine resolution required to characterize the various discrete factors. Further, the quantities of lymphocytes available from hypersensitive human donors are much too small, in general, to be amenable to analysis by these methods.

Our objectives were basically twofold. First, we wished to develop methods for following the PALs in small samples, our upper boundary condition being lymph node cells from one guinea pig. This necessitated the choice of isotopic labeling techniques for marking synthesized proteins, since it had been demonstrated previously that production of MIF was abolished by inhibitors of RNA and protein synthesis (13, 14, 23). Secondly, it was necessary to choose methods in which results could be expressed in terms which were intrinsic chemical parameters of the various molecules. Once these qualitative characteristics are established for each factor, more quantitative radioassays could be applied to their study, e.g., possibly for diagnostic assay. In this paper, we have dealt exclusively with the qualitative problem of developing isotopic labeling methods applicable to purification of MIF, and the relationship between isotopic labeling patterns and biological activity at each step.

The first studies were simply to indicate whether in antigen- or mitogen-stimulated cells an increase in incorporation of labeled leucine into macromolecular protein could be established, and what the kinetics of this response were. The results (Fig. 1) confirm those previously of Levy and Rosenberg (10) on PHA-stimulated cells that a significant increase in total macromolecular protein incorporated into stimulated cultures was discernible as early as 4 h after stimulation. Further, the results showed that although the majority of the incorporation was into cell-associated protein, significant increases in synthesized proteins released into the medium occurred in stimulated cultures.

There has been much previous work on gel filtration patterns of MIF activity using relatively large pools of eluate, and unfortunately many of the estimates for the size range of MIF are discordant (1, 3, 9, 17, 18). For detailed fractionation studies, it is obvious that the smaller the pool from Sephadex, the less the polydispersity and the higher the probability of good resolution by one more fractionation step based on charge. Therefore it was decided to test every two tubes for MIF activity past the void volume of our G-75 columns. When 40-cc supernatants were concentrated and used as sample, it soon became apparent that there were multiple peaks of migration inhibitory activity, even in control samples. The degree of inhibition varied with the preparative procedures and the macrophage populations tested, but the problem of distinguishing a MIF induced by stimulation from a variety of non-specific inhibitory proteins became critical. Because of the nonspecific inhibitory

activities, presentation of results in terms of migration inhibition by stimulated fractions relative to control was not really meaningful in chemical terms. For this reason, we chose to accept the medium control as the standard of 100% migration, which permitted us to define the  $K_d$ 's for the nonspecific inhibitory proteins which may have confused the result. By testing 100 tubes, migration inhibitory activity could be localized within 10 tubes, and the elution volume for the peak activity associated with the unique migration inhibitory peak present only in stimulated supernatants was approximately  $K_d = 0.15$  (Fig. 3). The size marker used to calibrate these columns closest to this peak was ovalbumin, with a  $K_d = 0.13$  and mol wt of 45,000.

While there have been some reports indicating that MIF activity in whole supernatants (11, 19) or Sephadex fractions (20) may have antigen dependence or specificity for antigen, no evidence for specificity could be found in several recent and very thorough studies (1, 3). In the present work we observed that the  $K_d$  0.15 material did not lose migration inhibitory activity or radioactive counts by passage through PPD-immunoadsorbant columns, and thus no evidence for specificity of this MIF was seen here. PPD did, however, non-specifically increase the degree of inhibition slightly in some experiments.

Having established the characteristics for elution of biological activity, attempts were made to introduce isotopic labels into proteins in that peak. The double-label technique, in which a stimulated sample is labeled with an amino acid of one isotope and the control with another, has proved very useful elsewhere for distinguishing small differences in synthesis in proteins over a background of a large amount of labeled and nonlabeled protein. While this study was in progress, Rosenberg and Levy (6) reported that this technique indicated increases in amino acid incorporation into cell-associated proteins in PHA-stimulated lymphocytes, and that the cell fraction showing the greatest increase early after stimulation was the nuclear fraction. In the present experiments when double-labeled supernatants, e.g. control labeled with [ $^{14}\text{C}$ ]leucine stimulated with [ $^3\text{H}$ ]leucine, were admixed and subjected to gel filtration on the same columns in which MIF activity was studied, at least one and often multiple broad peaks of increased labeling were associated with the stimulated fraction, defined here as radiolabeled peaks. The increase associated with the PPD-stimulated supernatants was small, but the peak was always in the region of  $K_d$  approx. 0.1. Because the degree of stimulation was low, we turned to Con A-stimulated culture supernatants. Con A has been shown to affect T cells exclusively (21) in the mouse, and to induce formation of MIF and skin reactive factor in the guinea pig (12). When Con A and control double-labeled supernatant mixtures were fractionated on G-75 columns, a significant broad peak emerged at  $K_d$  approx. 0.1, as well as smaller peaks at  $K_d$  0.35 and 0.5 (Fig. 4). The magnitude of the increase of the  $K_d$  0.1 peak in nine experiments was  $28.2\% \pm 3\%$ . For further fractionation a narrow pool was selected from this broad double-labeled peak in the region containing most MIF activity (ca.  $K_d$  0.15).

Since it had been shown by Remold et al. (1, 16) that MIF could be purified after Sephadex fractionation from other serum proteins by disc acrylamide gel electrophoresis and that it migrated anodally to albumin, the electrophoretic pattern of our narrow radiolabeled pool was examined under their conditions. We were able to resolve five to six peaks, in which the majority of label migrated anodally to albumin (Fig. 5). Thus, the radiolabeled pattern indicated a heterogeneity of the Sephadex material, but confirmed that there was a significant amount of radioactivity in precisely the region identified by Remold et al. as containing MIF activity. However, this migration pattern could have been due either to a higher net charge, or to molecular sieving effects of polyacrylamide. Because relative migration is an ambiguous, parameter we chose isoelectric focusing as our method for charge separation, since at equilibrium proteins migrate to their isoelectric points,  $pI$ , which are intrinsic characteristics of the molecules. The analytical isoelectric focusing results indicated the narrow Sephadex  $K_d$  0.15 pool contained two major labeled areas, one of which was the same in control supernatants, and the other was found to be significantly elevated only in stimulated supernatants (Fig. 6). This area centered at 5.3 indicating a lower net charge than guinea pig albumin ( $pI = 4.8$ ). With a micropreparative isoelectric focusing procedure, it was possible to establish a unique migration inhibitory peak in the region of  $pI$   $5.2 \pm 0.5$  associated with the radiolabeled area (Fig. 7). Because recovery of biological activity from acrylamide gels is low, we chose to stabilize the ampholine gradients with a sucrose gradient to obviate adsorption to a solid matrix. Our results indicate that this activity has a lower net negative charge than serum albumin and that the greater mobility on disc acrylamide gels is probably due to sieving effects since MIF is smaller than albumin. They are also consistent with earlier observations using diethylaminoethyl fractionation in which MIF activity was eluted in the alpha globulin region at lower ionic strength than albumin and prealbumin. (3, 22, 23). Analysis of the radiolabeled profiles on isoelectric focusing gels of individual fractions eluting from G-75 columns between the void volume and  $K_d$  0.2 disclosed the presence of at least 14 radiolabeled peaks synthesized either *de novo* or in increased amounts in the stimulated cultures. Several had similar  $K_d$  values but could be distinguished by  $pI$  values, and vice versa, and the need for using narrow Sephadex pools became readily apparent. It has thus been possible to characterize a variety of products of activated lymphocytes by the increase in isotope incorporation, by  $K_d$  on Sephadex, and by  $pI$  on isoelectric focusing.

This represents a first attempt using new methods and many additional experiments will be required to achieve full purification and characterization of MIF, and to establish whether the specifically radiolabeled peak here associated with MIF activity is indeed the active component. We recognize limitations in the present approach. For example, even though one labeled peak may ultimately be isolated, it may be contaminated with a number of nonlabeled proteins which will not be detectable by our method. Of course the methods

need to be extended to other antigens than PPD, although PPD and Con A did induce the same radiolabeled peak and MIF activity. The quantitative aspects of this method require further study. Dumonde et al. (3) have pointed out the importance of quantitation in terms of protein weight required to give certain percent migration inhibition in dilution experiments, e.g.,  $MI_{30}$ . However, there are difficulties in reporting specific activities on the basis of protein weight, since most of the protein in the supernatants is contributed by contaminating serum from dying cells, conditions that vary from experiment to experiment. The potential of the radiolabeling methods is ultimately to permit quantitation in absolute terms of the increase in isotope, or micromoles of leucine, incorporated into a specific protein in antigen-stimulated cultures. At this time we can only state that the net amount of label found in the radiolabeled peak associated with MIF activity represents 0.1–0.5% of the total macromolecular counts, i.e. synthesized protein, in the supernatants. It is our expectation that the methods would simplify the prevailing complex situation by identifying the 15–20 biological activities already reported with a smaller number of chemical species.

We believe that the radioisotopic methods may prove helpful for permitting the isolation and characterization of many of the molecules in antigen-stimulated lymphocytes synthesized either *de novo* or in increased amounts after antigen stimulation. The methods are sufficiently general in their application to be useful in studying synthesis of cellular components in the various lymphocyte subpopulations, and in macrophages affected by antigen. With further study they may be adapted to useful clinical assays in situations where the availability of lymphocytes is limited.

#### SUMMARY

General methods were developed and applied to the biosynthesis and purification of products of activated lymphocytes available in minute quantities. The activity studied here was the migration inhibitory factor (MIF) produced by purified protein derivative (PPD)- or concanavalin A (Con A)-stimulated lymphocytes obtained from one guinea pig or less. The methods selected yielded results in terms of two chemical parameters characteristic of the molecules involved, namely  $K_a$  on Sephadex G-75 and isoionic point,  $pI$ , on isoelectric focusing.

When supernatants were fractionated on G-75 columns, there were several areas even in control supernatants which produced migration inhibition relative to medium controls. However, in PPD- and Con A-stimulated supernatants, at least one peak of MIF activity was found solely in the stimulated cultures, with a  $K_a$  of 0.15. A double-labeling technique was used to characterize the proteins of this peak. Control, unstimulated cultures were labeled with [ $^{14}C$ ]leucine and stimulated cultures were labeled with [ $^3H$ ]leucine. After mixing the supernatants and G-75 filtration, a major "radiolabeled" broad peak, i.e.

one with increased  $^3\text{H}/^{14}\text{C}$  ratio, was found. When a narrow portion of this peak about  $K_d$  0.15, containing most of the MIF activity, was subjected to analytical isoelectric focusing, all of the label was associated with proteins of lower net charge than albumin. A unique radiolabeled peak was found in PPD- and Con A-stimulated fractions with a  $pI$  of approx. 5.3. A micropreparative isoelectric focusing technique was developed and yielded MIF activity in the same region as the major radiolabeled peak. Further study will be required to ascertain whether the radiolabeled protein is MIF. By following the  $K_d$ ,  $pI$ , and  $^3\text{H}/^{14}\text{C}$  labeling ratio, at least 14 products of activated lymphocytes, synthesized either *de novo* or in increased amounts, could be distinguished.

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