*REACTIONS IN VIVO AND IN VITRO PRODUCED BY A SOLUBLE SUBSTANCE ASSOCIATED WITH DELAYED-TYPE HYPERSENSITIVITY**

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The cellular basis for delayed-type hypersensitivity was established by the passive transfer of delayed-type reactions to normal guinea pigs by means of lymphoid cells obtained from hypersensitized animals.[1] Attempts to demonstrate a mediator of delayed-type hypersensitivity in sera or lymphoid cell extracts from sensitized individuals have been unsuccessful in experimental animals.[2] Recently a technique [3, 4] for studying this immunological response *in vitro* has been developed which is based on the observation[5] that migration of peritoneal exudate cells from sensitized guinea pigs is inhibited by the presence of specific antigen. In this system, it has been found that the immunological information for this response is possessed by the lymphocytes, while the macrophages serve as indicator cells which migrate.[6] When such sensitized lymphocytes are cultured in the presence of specific antigen, they elaborate into the medium a substance, probably a protein, capable of inhibiting the migration *in vitro* of normal macrophages.[6–10] This substance, termed migration inhibitory factor (MIF), is produced by lymphocytes from guinea pigs exhibiting delayed-type hypersensitivity but not from animals producing only circulating antibodies,[3, 6] and is produced only after the sensitized lymphocytes interact with the specific antigen.[11]

This report will describe (1) the production of MIF by sensitized lymphocytes cultured in serum-free media, (2) partial purification of the active material, and (3) the production of skin reactions in normal guinea pigs by such purified preparations.

Materials and Methods.—Animals: Random-bred Hartley guinea pigs were purchased from commercial breeders, and inbred strain XIII guinea pigs were obtained from our own breeding colony.

Sensitization: Guinea pigs were sensitized to tuberculin by the injection of complete Freund's adjuvant (3.3 mg/ml H37Ra mycobacteria, Difco) on one occasion as follows: 0.1 ml into each footpad and 0.6 ml into the nuchal muscle. Three to 6 weeks later, each animal was tested intradermally with 10 μg of tuberculin purified protein derivative (PPD, obtained from the Ministry of Agriculture, Fisheries and Food, Central Veterinary Laboratory, Weybridge, England). Animals showing reactions of 15 mm or greater were used as cell donors.

Tissue cultures of lymphocytes: Lymphocytes were obtained from brachial, axillary, cervical, and inguinal lymph nodes, which were teased apart with stainless-steel rakes[2] in Hank's solution containing 10% normal guinea pig serum (NGPS, prepared from blood obtained by cardiac puncture). The cells were washed twice in Hank's solution by centrifugation at 200 $\times$ g and finally suspended in Eagle's minimum essential medium (MEM) containing 100 U penicillin/ml, and 100 μg streptomycin/ml. Finally, the cell density was adjusted to 1.2×10^7 cells/ml, and the cells were cultured in plastic T-flasks (Falcon Plastic Co.) for 24 hr with PPD, 25 μg/ml. No serum was added. Afterward,

the suspension was centrifuged at 1000 × *g* for 20 min and the supernatant fluid was lyophilized and stored at −70°C. Control preparations consisted of sensitized cells cultured without PPD; PPD (25 μg/ml) was added to the cell-free supernatant before lyophilization. For use, the lyophilized residue was reconstituted to 1/8 the original volume in distilled water, neutralized with 0.1 *N* HCl, and clarified by centrifugation.

Gel filtration: Glass columns (1.5-cm diameter by 125-cm length) were poured with Sephadex G-100 (Pharmacia) suspended in 0.02 *M* Tris HCl–0.1 *M* NaCl buffer at pH 7.2. The columns were calibrated with protein standards of known molecular weight (Mann Research Labs.). The reconstituted supernatants were applied to the columns in sample volumes of 1.5–2.5 ml, the effluent being collected in consecutive 3.0-ml fractions at a rate of 7.5 ml/hr. The optical density of the eluates was measured at 280 and 215 mμ on a Beckman DU spectrophotometer using quartz cells (80% transmission at 215 mμ, Precision Glass Co., Cell 5Q). The effluent was pooled as indicated in Table 1 and reduced in volume by Diaflo membrane dialysis (Amicon Corp., Cambridge, Mass.). Protein was determined by a micromodification of the Lowry method[12] using bovine plasma albumin as standard. In some experiments, 0.1 ml NGPS was added to each fraction prior to concentration. The final volume of the dialysate was adjusted to 1/10 the volume of the original supernatant, and the preparations were sterilized by Millipore filtration.

Cell migration chambers: The reconstituted supernatants or the concentrated G-100 Sephadex fractions were dialyzed against 100-fold vol of saline followed by MEM (each containing antibiotics). The contents of the bags, to which 10% by vol NGPS was then added, were used as the test media in cell migration chambers.

The preparation of these chambers has been described in detail elsewhere.[3, 6] Briefly, peritoneal cells were collected from guinea pigs given 30 ml Bayol 55 intraperitoneally 2 days previously. The cells were washed twice by centrifugation (200 × *g*) in Hank's solution containing heparin 0.5 units/ml. Finally, 5 × 10^6 cells were suspended in 0.2 ml of test medium. The cell suspension was drawn into duplicate capillary tubes (1.5-mm diameter × 75-mm length) which were then plugged with paraffin. A cell button was prepared by centrifuging the tubes for exactly 2 min at 125 × *g*. The capillary tubes were then cut at the cell-medium interface and placed in Mackaness-type tissue culture chambers of a design previously described.[6] The duplicate tubes were placed in each chamber, which was immediately filled with test medium, and the chambers were incubated at 37°C. After 24 hr, the degree of cell migration was observed and recorded by photomicrography.

Reactions in skin sites: The G-100 Sephadex fractions were dialyzed in the cold against a 100-fold vol of Hank's solution or saline. The contents of the bags were drawn into tuberculin syringes and injected intradermally into three to six normal guinea pigs, and the reactions were observed over a 48-hr period. Animals were sacrificed at various intervals, the skin sites were fixed in buffered formalin, and hematoxylin- and eosin-stained sections were prepared.

Results.—In previously reported experiments[6, 13] the supernatants of sensitized lymphocytes cultured with PPD were found to inhibit the migration *in vitro* of normal macrophages; such supernatants will be referred to as inhibitory supernatants. Control supernatants were obtained from (*a*) lymphocytes from tuberculin-sensitive guinea pigs cultured with heterologous antigens, (*b*) lymphocytes from nonsensitive guinea pigs cultured with PPD, and (*c*) lymphocytes from sensitive guinea pigs cultured without PPD, but with PPD added after removal of cells. Such control supernatants consistently failed to inhibit migration. For the studies presented here, we have routinely used the last-mentioned control.

Preliminary studies on the fractionation of supernatants containing 15 per cent NGPS suggested that the active material was eluted from G-200 or G-100

Sephadex in the same fraction as serum albumin. However, a similar fraction from control supernatants was also occasionally found to inhibit macrophage migration *in vitro* and to provoke significant inflammatory reactions when injected intradermally into guinea pigs. One possible cause for this was the presence of relatively large amounts of serum proteins in concentrated supernatants containing serum. To eliminate these extraneous proteins, therefore, we simply omitted the serum from the culture media. The results (Table 1) indicate that, in the absence of serum, MIF is produced by the sensitized cells cultured with PPD. The omission of NGPS from the original cultures allowed the fractionation and concentration of the resultant supernatants without yielding excessive amounts of serum proteins. (Cell viability, as measured by trypan blue exclusion, was found to be comparable in cultures containing 15% NGPS and those free of serum.)

Fractionation: Supernatants of lymphocyte cultures, after lyophilization, reconstitution, and clarification, were applied to calibrated Sepahadex G-100 columns. In general, there was too little protein in the fractions from serum-free cultures to be detected by ultraviolet absorption at 280 mμ, but an elution pattern could be discerned by scanning at 215 mμ. Two peaks were found (Fig. 1), the

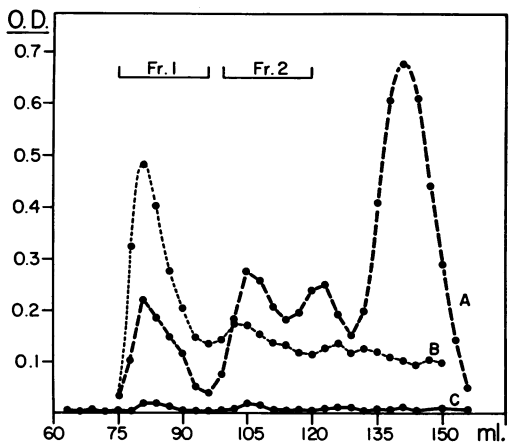

FIG. 1.—Gel filtration of lymphocyte culture supernatants on calibrated Sephadex G-100 column. (*A*) Elution pattern of marker proteins: *first peak*, apo-ferritin, mol wt 980,000; *second peak*, bovine plasma albumin, mol wt 67,000; *third peak*, ovalbumin, mol wt 45,000; *fourth peak*, chymotrypsinogen A, mol wt 25,000. (*B*) Lymphocyte culture supernatant, OD at 215 mμ. (*C*) Lymphocyte culture supernatant, OD at 280 mμ.

first appearing in the void volume, representing material of molecular weight 100,000 or greater, and the second corresponding to an average molecular weight of approximately 67,000 as estimated from the calibration curve.

Macrophage migration in vitro: Preparations from fraction 1 and 2 both from inhibitory and control supernatants were tested for their ability to inhibit the migration of normal macrophages. In six experiments, marked inhibition of migration was produced only by fraction 2 from inhibitory supernatants (Table 1). The total protein in this fraction was 100–500 μg and aliquots diluted 1:5 or 1:10 were effective in inhibiting migration.

Skin reactions: Intradermal injection of 10-times concentrated inhibitory supernatants into normal Hartley guinea pigs caused skin reactions which became manifest in 3–5 hours, were maximal at 8–12 hours, and disappeared by 30 hours.

TABLE 1. *Migration of normal peritoneal exudate cells in sensitized lymphocyte culture supernatants and Sephadex G-100 fractions.*

Material tested	No. of expts.	Per cent migration* (Av. ± SE)
Supernatants	6	32 ± 7.6
G-100 fraction 1	6	122 ± 17.2
G-100 fraction 2	6	35 ± 6.5

* Calculated as follows: 100 × [migration in preparations from sensitized cells cultured with PPD ÷ migration in preparations from sensitized cells cultured without PPD (PPD added after removal of cells)].

TABLE 2. *Skin reactions in normal guinea pigs produced by intradermal injections of supernatants and G-100 fractions.*

Material injected*	No. of guinea pigs	Strain	Diameter (mm.) of erythema ± SE	Induration
Control supernatant	10	XIII	6.9 ± 0.98	0
Inhib. supernatant	10	XIII	11.8 ± 0.63	Mod.
Control fraction 1	15	Hartley	2.4 ± 0.66	0
" "	10	XIII	4.2 ± 0.64	0
Inhib. fraction 1	15	Hartley	3.7 ± 0.82	0
" "	10	XIII	4.7 ± 0.77	0
Control fraction 2	18	Hartley	5.7 ± 0.63	0
" "	10	XIII	5.2 ± 0.95	0
Inhib. fraction 2	18	Hartley	12.5 ± 0.55	Mod.
" "	10	XIII	11.2 ± 1.03	Mod.

* In each experiment, 0.1 ml of each preparation was injected into three to six normal guinea pigs.

These reactions consisted of induration and erythema (Table 2). In contrast control supernatants produced considerably smaller erythematous reactions and no induration. When G-100 fractions were tested, marked induration and erythema was produced only by fraction 2 material from inhibitory supernatants; this was the same fraction responsible for inhibition of macrophage migration *in vitro*. Control fraction 2 material or fraction 1 material from either control or inhibitory supernatants produced slight erythema and no induration. The amount of injected protein in inhibitory fraction 2 which gave positive reactions was 10–50 μg.

To rule out the possibility that these reactions were the result of foreign histocompatibility antigens in the preparations from random-bred guinea pigs, similar experiments were performed with inbred strain XIII animals. These experiments gave identical results (Table 2).

Histological examination of reactions produced by inhibitory supernatants or fraction 2 material derived from them showed, at four hours, an exudate consisting nearly entirely of mononuclear cells. Later neutrophils appeared so that by 14–16 hours an exudate of approximately equal numbers of mononuclear cells and neutrophils was present in the dermis and subcutaneous tissue. In addition, sites injected with fraction 2 from inhibitory supernatants showed focal epidermal necrosis (Fig. 2).

Discussion.—In the present experiments, it has been possible to demonstrate production of the migration inhibitory factor (MIF) associated with delayed-type

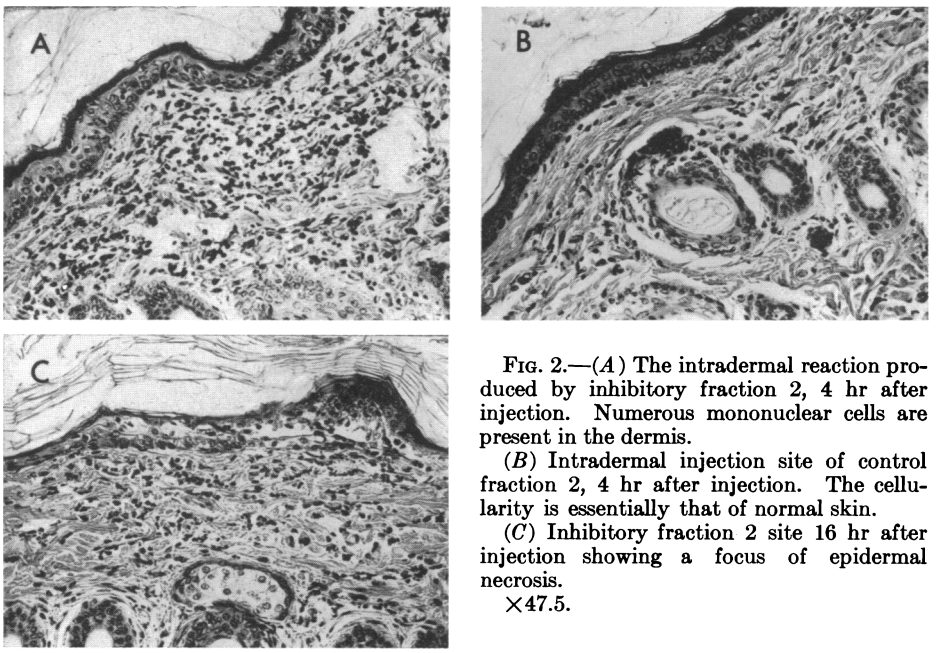

FIG. 2.—(*A*) The intradermal reaction produced by inhibitory fraction 2, 4 hr after injection. Numerous mononuclear cells are present in the dermis.

(*B*) Intradermal injection site of control fraction 2, 4 hr after injection. The cellularity is essentially that of normal skin.

(*C*) Inhibitory fraction 2 site 16 hr after injection showing a focus of epidermal necrosis.

×47.5.

hypersensitivity reactions *in vitro* by lymphocytes cultured in serum-free media. Fractionation of the supernatants of these cultures on Sephadex G-100 columns yielded two protein peaks as detected by ultraviolet absorption at 215 mμ. The first, fraction 1, obtained from either control or inhibitory supernatants, was found to be inactive in producing inhibition of migration of normal peritoneal exudate cells *in vitro*. It may be noted that this fraction, containing molecules of molecular weight greater than 100,000, would be expected to contain immunoglobulins of known immunological classes as well as antigen-antibody complexes. Only fraction 2 prepared from inhibitory supernatants was found to contain MIF. This fraction has an average molecular weight estimated to be 67,000 and, on the basis of size, the possibility that MIF is an immunoglobulin appears remote. The results regarding the size of MIF in these and previous experiments[13] are in agreement with those from other laboratories studying hypersensitivity to protein antigens and conjugates.[9, 14]

While the capillary tube migration system had shown considerable correlation with delayed-type hypersensitivity reactions *in vivo*, it remained to be demonstrated that the material active *in vitro* was capable of provoking positive reactions *in vivo*. Dumonde[9] recently reported a greater reactivity in intradermal skin sites injected with inhibitory supernatants than with control supernatants, both containing serum. However, we had difficulties interpreting reactions produced by supernatants from random bred animals prepared in the presence of 15 per cent serum; these were resolved by utilizing inbred strain XIII guinea pigs for preparing and testing the supernatants and by using serum-free culture media. In these experiments, the fraction which was active *in vitro* has been found to produce dermal reactions in normal guinea pigs which resemble active delayed-

type hypersensitivity reactions in that induration and erythema are produced; histologically the infiltrate consists primarily of mononuclear cells, and epidermal necrosis is present. The time course of these reactions, however, is somewhat accelerated. Whereas active delayed-type reactions are detected at 12 hours and generally reach maximum intensity at 20–30 hours, those produced by inhibitory fraction 2 are manifest at 3–4 hours and appear to be maximal at 10–14 hours. Because the sensitized lymphocytes have been cultured *in vitro* in the presence of antigen for 24 hours in order to obtain the active material, it might be expected that once produced it would effect reactions in a shorter time. In addition, the possibility must be considered that the reactions result in part from molecules other than MIF, not all of which necessarily are implicated in naturally occurring hypersensitivity reactions. In this respect, it must be pointed out that positive reactions were produced by preparations containing as little as 10 μg protein.

These results and those from other laboratories[9, 14] suggest a simple model for delayed-type hypersensitivity reactions. Sensitized lymphocytes, possessing the information for recognizing the specific antigen, would remain inactive until contact with antigen was made in the tissues. Consequently, MIF would be elaborated and would serve to immobilize mononuclear cells, particularly histiocytes, at the local site. This model would account for a number of previous observations on delayed-type hypersensitivity: (*a*) the characteristic histological pattern;[15] (*b*) the failure to obtain a mediator substance in the serum of hypersensitive individuals; (*c*) the failure of cells whose RNA and protein synthetic capacities have been blocked with antimetabolites to effect passive transfer,[16] and (*d*) the delayed-time course of the reaction. That additional factors may be involved in delayed-type reactions is suggested by recent findings[17, 18] that sensitized lymphocytes in the presence of specific antigen can lyse nonspecific target cells in tissue culture monolayers.

This model presents a number of problems for experimentation. For example: What is the mechanism by which sensitized lymphocytes recognize specific antigen and how does this lead to the production of MIF? What is the chemical nature of MIF and is it related to known immunoglobulins? How are macrophages sequestered and immobilized? Do such macrophages become activated to contain or process antigen and to produce tissue damage?

Summary.—Lymphocytes from guinea pigs with delayed-type hypersensitivity to tuberculin, upon contact with PPD *in vitro*, produce a substance which inhibits the migration of normal peritoneal macrophages. In this report, it has been possible to obtain this migration inhibitory factor in serum-free medium. When such culture supernatants were fractionated on Sephadex G-100 columns, the migration inhibitory factor was found in the second fraction, estimated to have an average molecular weight of 67,000. This same fraction, upon intradermal injection into normal guinea pigs, produced reactions characterized by induration, erythema, and mononuclear cell infiltration.

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[1] Landsteiner, K., and M. W. Chase, *Proc. Soc. Exptl. Biol. Med.*, **49**, 688 (1942).
[2] Bloom, B. R., and M. W. Chase, *Progr. Allergy*, **10**, 151 (1967).
[3] George, M., and J. H. Vaughan, *Proc. Soc. Exptl. Biol. Med.*, **111**, 514 (1962).
[4] David, J. R., S. Al-Askari, H. S. Lawrence, and L. Thomas, *J. Immunol.*, **93**, 264 (1964).
[5] Rich, A. R., and M. R. Lewis, *Bull. Johns Hopkins Hosp.*, **50**, 115 (1932).
[6] Bloom, B. R., and B. Bennett, *Science*, **153**, 80 (1966).
[7] David, J. R., these PROCEEDINGS, **56**, 72 (1966).
[8] David, J. R., *J. Exptl. Med.*, **122**, 1125 (1965).
[9] Dumonde, D. C., *Immunopathol., Intern. Symp., 5th*, in press.
[10] Svejcar, J., J. Johanovsky, and J. Pekarek, *Z. Immunitaetsforsch.*, **132**, 182 (1967).
[11] Bennett, B., and B. R. Bloom, *Transplantation*, **5**, 996 (1967).
[12] Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
[13] Bloom, B. R., and B. Bennett, *Federation Proc.*, **27**, 13 (1968).
[14] David, J. R., *Federation Proc.*, **27**, 6 (1968).
[15] Dienes, L., and J. B. Mallory, *Am. J. Pathol.*, **8**, 689 (1932).
[16] Bloom, B. R., L. D. Hamilton, and M. W. Chase, *Nature*, **201**, 681 (1964).
[17] Holm, G., and P. Perlmann, *J. Exptl. Med.*, **125**, 721 (1967).
[18] Ruddle, N. H., and B. H. Waksman, *Science*, **157**, 1060 (1967).