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In Vitro Correlates of Delayed Hypersensitivity in Man: Ambiguity of Polymorphonuclear Neutrophils as Indicator Cells in Leukocyte Migration Test

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Delayed cutaneous hypersensitivity (DCH) of 12 normal adult subjects to purified protein derivative (PPD) of *Mycobacterium tuberculosis*, streptococcal streptokinase-streptodornase (SK-SD), and *Candida albicans* Dermatophytin O (DO) was assayed in vivo by skin testing and compared with such in vitro correlates of cellular immunity as lymphocyte transformation (LT) and inhibition of leukocyte migration (ILM) from microcapillary tubes or in agarose gel. LT was shown to be the best in vitro correlate of specific lymphocyte sensitization with all antigens. In the ILM assays, PPD showed good correlation with in vivo DCH and in vitro LT; SK-SD showed partial correlation; DO showed no correlation, not being active in any of the ILM tests. Cell distribution and morphology of stained migration patterns, ILM tests performed on separated populations of lymphocytes and polymorphonuclear leukocytes (PMN), as well as the ability of test antigens to stimulate PMN cells to reduce nitroblue-tetrazolium dye, indicated that in ILM tests mononuclear cells were not inhibited in their migration, whereas migration of PMN cells appeared to depend on their direct reaction with the test antigens.

The clinical importance of cell-mediated immunity, or delayed-type hypersensitivity, is now widely recognized. The importance of cellular immunity to infection is demonstrated by the apparent association of resistance and delayed hypersensitivity in listeriosis (17), salmonellosis (7), tuberculosis (8), and brucellosis (18). Recently, the specific nature of such an association was shown in tularemia (5). Furthermore, cellular immunity is of great clinical significance in transplantation and tumor immunity and in immune deficiency states. Reliable in vitro methods for the evaluation of cellular immunity in man, where in vivo skin testing often is not possible, not desirable, or insufficiently sensitive, would be very valuable for clinical investigation and diagnosis.

In the present investigation, the cell-mediated immunity of 12 normal human volunteers was assayed in vivo by delayed cutaneous hypersensitivity and in vitro by inhibition of leukocyte migration, both from capillary tubes and in agarose gel, and by stimulation of lymphocytes to proliferate, as measured by ^{14}C-thymidine uptake. Our subjects, chosen from among employees of a bacteriological laboratory, were tested with purified protein derivative (PPD) of *Mycobacterium tuberculosis*, as well as with streptococcal and *Candida albicans* antigenic preparations. Our purpose was to evaluate the usefulness of microtechniques for these in vitro assays (methods requiring a reasonably small sample of peripheral blood) as a reliable correlate of delayed hypersensitivity in man.

MATERIALS AND METHODS

Subjects. The 12 human volunteers studied were members of health professions, the majority of them employees of a hospital bacteriological laboratory.

Antigens. A streptokinase-streptodornase (SK-SD) preparation containing 20,000 and 5,000 units of the enzymes, respectively (Varidase, Lederle Laboratories, American Cyanamid Co., Pearl River, N.Y.), was used as the streptococcal antigen. Dermatophytin O (DO) (Hollister-Stier Laboratories, Spokane, Wash.), undiluted, was used as *C. albicans* antigen.

Both preparations were dialyzed extensively against saline to remove preservatives and then sterilized by filtration and diluted appropriately, when necessary, with sterile saline. PPD of *M. tuberculosis* was a kind gift from the Central Veterinary Laboratory, Weybridge, England. This PPD was dissolved in saline at required concentrations, sterilized by filtration, and used for cell cultures and migration inhibition studies. Tuberculin PPD, Mantoux (Connaught Medical Research Laboratories, Toronto, Canada), intermediate-strength stabilized solution, was used for skin tests. Endotoxin (Bacto-Lipopolysaccharide *Escherichia coli* 026:B6, Difco Laboratories, Detroit, Mich.) was dissolved in saline at required concentrations.

Skin tests. Test antigens, contained in 0.1-ml volumes, were injected intradermally in the forearm with a tuberculin syringe and a 27-gauge, 0.5-in needle. At 24 and 48 h, the reactions were recorded in millimeters of erythema and induration. Reactions consisting of at least 5 mm of erythema and induration were considered positive; those exhibiting only erythema but no induration were recorded as ±. Initially, 1:100 dilution of DO and 40/10 U of SK-SD were used; if negative, tests were repeated with 1:10 dilution of DO and 400/100 U of SK-SD. Tuberculin sensitivity was known in most cases from the records of the Department of Public Health. Where this information was not recorded, skin tests were done with intermediate-strength tuberculin solution containing 5 U.S. units per test.

Preparation of leukocytes. Venous blood (10 ml) was drawn and mixed with phenol-free heparin, 50 U/ml. For the preparation of unseparated leukocytes, the heparinized blood was mixed with 6% dextran in saline (Dextran 200-C, MW 252000, Sigma Chemical Co., St. Louis, Mo.), 0.2 by volume, and allowed to sediment for 1 h at 37 C. The leukocyte-rich plasma then was expelled through the needle into a collection tube. Cells were centrifuged for 10 min at 200 × *g*, the supernatant was decanted, and the cell pellet was washed three times with Hanks balanced salt solution (Grand Island Biological Co., Grand Island, N.Y.). Separation of leukocytes was performed by the method of Böyum (4) on Ficoll-Hypaque gradient, final density 1.077 g/ml (Ficoll, Pharmacia Fine Chemicals, Uppsala, Sweden; Hypaque-M, 75%, Winthrop Laboratories, Sterling Drug, Inc., New York, N.Y.). The lymphocyte fraction consisted of 85 to 90% lymphocytes, 5 to 12% monocytes, and 3 to 5% granulocytes; the granulocyte fraction had 100% granulocytes, almost all of them polymorphonuclear neutrophils (PMN). Both fractions contained at least 95% viable cells, as determined by the trypan blue dye exclusion test.

Lymphocyte cultures. For lymphocyte transformation studies, cells were cultured by a modification of the procedure described by Stites et al. (28). The separated lymphocytes were washed three times with phosphate-buffered saline (Dulbecco, pH 7.2) and resuspended in Eagle minimum essential medium (MEM) (Grand Island Biological Co.) containing 100 U of penicillin and 100 μg of streptomycin per ml, 1% L-glutamine, and 10% human AB serum by volume. Cells (2–3 × 10^5) in 1 ml of medium were placed in plastic tubes (12 by 75 mm; Falcon tube no. 2054, Falcon Plastics, Oxnard, Calif.), and the antigens were added to achieve the following concentrations per milliliter: PPD, 10 to 300 μg; SK, 50 to 300 U; undiluted DO, 0.05 to 0.2 ml. All cultures were done in duplicate or triplicate. Cultures were incubated at 37 C in a moist atmosphere of 5% CO_2 and 95% air for 5 days, at the end of which period 0.1 μCi of ^{14}C-methyl-thymidine (New England Nuclear Corp., Boston, Mass.; specific activity 24.5 mCi/mmol) was added to each culture tube in 0.5 ml of medium. After an additional 24 h of incubation, 2 × 10^6 to 4 × 10^6 mouse spleen "carrier" cells were added to each tube to increase the bulk of the cell pellet, and the cultures were harvested in the cold by centrifugation at 800 × *g* for 15 min by serially suspending them once in saline, twice in 5% trichloroacetic acid, and once in absolute methanol. The dry residue was dissolved by incubation in 0.5 ml of hyamine hydroxide (New England Nuclear Corp.) for 3 h at 56 C and transferred to scintillation counting vials with 10 ml of Omnifluor (New England Nuclear Corp.) in toluene (4 g of Omnifluor in 1 liter of toluene). Radioactivity was measured with a Nuclear-Chicago liquid scintillation counter, 720 series. The variation between replicate cultures seldom exceeded ±5%. The results were expressed as stimulation index (SI), the ratio of ^{14}C-thymidine incorporation in cultures with antigen present relative to cultures without antigen from the same individual. The actual baseline values (cultures without antigen) ranged from 80 to 200 counts/min.

For the production of migration inhibitory factor (MIF), cultures of 5 × 10^6 cells were set up in 3 ml of medium 199 (Grand Island Biological Co.) that contained antibiotics and glutamine but no serum supplement, and either no antigen or PPD (25 μg/ml), SK (50 U/ml), or undiluted DO (0.05 ml/ml), respectively. After 3 days of incubation as described above, culture supernatant fluids were collected by centrifugation, extensively dialyzed in the cold against saline and distilled water, and lyophilized.

Leukocyte migration. Capillary migration was performed by a modification of the method described by Federlin et al. (12). Unseparated or separated leukocytes were washed three times in Hanks balanced salt solution, resuspended in MEM containing antibiotics, glutamine, and 10% by volume tissue culture horse serum (Baltimore Biological Laboratories, Cockeysville, Md.), and were counted. Cells were centrifuged and resuspended in the same medium to a concentration of 70 × 10^6 cells/ml. Sterile 10-μliter microcapillary tubes (Microcaps, Drummond Scientific Co.) were filled with this cell suspension, sealed at one end with clay, and centrifuged for 5 min at 150 × *g*. Tubes were cut at the cell-fluid interface with a steel scorer, and the cell-containing butts were placed, two per chamber, in Mackaness-type microchambers (capacity 0.75 ml) attached to the cover slip with a speck of silicone grease. Microchambers were covered with cover slips, sealed with paraffin, and filled with the same medium containing either no antigen or up to 300 μg of PPD per ml, up to 300 U of SK per ml, and up to 0.15 ml of undiluted DO per ml. At no time was a higher antigen concentration used

than the one which proved to be stimulatory or nontoxic in lymphocyte transformation studies, or both.

After incubation for 24 h at 37 C, migration patterns were projected and traced, and the areas were measured by planimetry. The areas of migration in duplicate test chambers (four tubes) were averaged, and the results were expressed as migration index, the ratio of migration in chambers containing antigen relative to migration in chambers without antigen. In keeping with the established convention, inhibition of migration was arbitrarily considered to be present if the migration index was less than 0.80. To study cell distribution of migration patterns, chambers were carefully drained with a syringe. In some cases, before drainage of chambers, migration patterns were fixed by replacing the medium with 10% Formalin in saline for 5 min. Cover slips with migration patterns were removed, air dried, stained with Wright-Giemsa stain (Harleco, Philadelphia, Pa.), and mounted with permount.

Migration in agarose gel was performed essentially as described by Clausen (6). Washed leukocytes were resuspended in medium 199 containing antibiotics, glutamine, and 10% horse serum. Preincubation with antigens (the same concentrations as used for capillary tube migration) was for 1 h at 37 C. Agarose was from Litex (Glostrup, Denmark) or from L'Industrie Biologique Française (Gennevillers, France). Migrations were carried out in tissue culture dishes (60 by 15 mm; Falcon, no. 3002). Migration patterns were fixed and stained as described by Gaines et al. (13). Migration areas were calculated by measuring the diameter, and the results were expressed as migration index in the same manner as described above for capillary migration.

Capillary migration of peritoneal exudate cells. MIF supernatant fluids were assayed on normal guinea pig peritoneal exudates which had been induced 3 days before sacrifice by the intraperitoneal injection of 20 ml of sterile light mineral oil. The method was that described by David et al. (9). Lyophilized MIF supernatant fluids, prepared as described above, were dissolved in 1.5 ml of MEM containing 15% by volume of pooled normal guinea pig serum, and the duplicate migration chambers were filled. Results were expressed as migration index.

Nitroblue-tetrazolium reduction. The nitroblue-tetrazolium (NBT) reduction test was performed by a modification of the method first described by Park et al. (22). Venous blood (1 ml) was collected in a plastic tube containing 5 to 10 U of preservative-free heparin (Calbiochem, Los Angeles, Calif.). To a small plastic cup with a V-shaped bottom (as used for chemical autoanalyzers) were added 0.05 ml of 0.2% aqueous solution of NBT dye (Grade III, Sigma Chemical Co.), 0.05 ml of pyrogen-free phosphate-buffered double-strength saline, pH 7.2, and 0.1 ml of heparinized blood. For the stimulated NBT test, the appropriate amounts of antigens, contained in a minimal volume (never exceeding 0.05 ml), were also added. Cups were capped, mixed by gentle shaking, and incubated at 37 C for 30 min. At the end of the incubation period, blood was mixed with a microcapillary pipette, and thin cover-slip smears were made, air dried, stained with Wright-Giemsa stain, and mounted with permount. The percentage of PMN cells containing black deposits of reduced NBT dye was determined by counting 100 PMN cells under oil immersion.

RESULTS

Comparison of cell-mediated responses. Cell-mediated responses of 12 normal subjects were studied in vivo by skin testing for delayed hypersensitivity and in vitro by inhibition of migration of peripheral blood leukocytes from microcapillary tubes and in agarose gel, as well as by stimulation of cultures of peripheral blood lymphocytes to proliferate (Table 1). Almost all of the subjects studied exhibited delayed cutaneous hypersensitivity (DCH) to the three test antigens: purified protein derivative of *M. tuberculosis*, streptococcal streptokinase-streptodornase, and Dermatophytin O derived from *C. albicans*. In every case, this hypersensitivity was reflected by lymphocyte transformation (LT) in culture, expressed by elevated SI values (values of 2.0 or greater, the threshold chosen arbitrarily). Lack of delayed hypersensitivity was accompanied by stimulation indexes of less than 2.0.

The correlation of leukocyte migration inhibition with DCH and LT varied with the test antigens. In the case of PPD, there was good agreement between the inhibition of leukocyte migration, both from microcapillary tubes and in agarose gel, and lymphocyte transformation. In the case of SK-SD, the correlation was only partial; only in about one-half of the subjects was the leukocyte migration inhibited, at least by one migration method, in the presence of positive DCH and elevated SI values. Furthermore, the migrations from microcapillary tubes and in agarose gel varied in their response to SK-SD: sometimes they followed the same trend, either that of inhibition or the absence of it, and sometimes they did not. In the case of DO, the most striking feature was the complete lack of inhibition of leukocyte migration by either method in any of the subjects. This antigen exhibited a complete lack of correlation with positive DCH and elevated SI values of most subjects.

Gross examination of migration patterns showed, in most cases, a fairly even cell distribution (Fig. 1A). Only occasionally could a more dense inner zone, surrounded by a lighter "halo" area, be visualized in either uninhibited (Fig. 1B) or inhibited (Fig. 1C) migration. Microscope examination of stained migration patterns showed a gradually decreasing cell

TABLE 1. Cell-mediated responses of 12 normal subjects

Individual subjects	Testing antigens											
	Purified protein derivative				Streptokinase-streptodornase				Dermatophytin O			
	DCH[a]	Migration index[b]		SI[c]	DCH	Migration index		SI	DCH	Migration index		SI
		Capillary tubes	Agarose gel			Capillary tubes	Agarose gel			Capillary tubes	Agarose gel	
1	+	0.41	0.47	54.0	+	0.65	0.33	7.8	+	1.1	0.92	9.7
2	−	0.87	0.73	1.3	+	0.58	0.85	6.0	+	1.0	0.98	2.9
3	±	0.50	0.76	6.1	+	0.90	0.82	2.3	+	0.90	0.92	5.6
4	+	0.37	0.47	48.0	+	0.84	0.76	11.8	+	0.96	0.95	9.4
5	+	0.39	0.19	125.0	+	0.36	0.21	198.0	+	0.98	0.85	30.0
6	+	0.66	0.48	5.6	+	0.62	0.81	105.0	+	0.97	0.88	38.0
7	+	0.62	0.37	86.0	+	0.90	0.30	172.0	+	1.2	0.91	45.0
8	+	0.58	0.62	22.6	+	0.88	0.84	3.8	±	0.94	0.96	3.3
9	+	0.48	0.59	44.0	+	0.84	0.91	12.3	+	0.92	0.95	8.4
10	+	0.51	0.39	32.0	+	0.61	0.72	26.6	+	1.08	0.92	20.2
11	+	0.62	0.51	8.2	±	0.90	0.84	3.1	−	1.05	0.90	1.3
12	−	0.84	0.76	1.6	−	0.85	0.93	1.8	+	1.02	0.96	7.8

[a] DCH, delayed cutaneous hypersensitivity.

[b] Expressed as area of cell migration in chambers containing antigen/area of cell migration in chambers without antigen.

[c] Stimulation index = ratio of ^{14}C-thymidine incorporation in cultures with antigen present relative to cultures without antigen from the same subject.

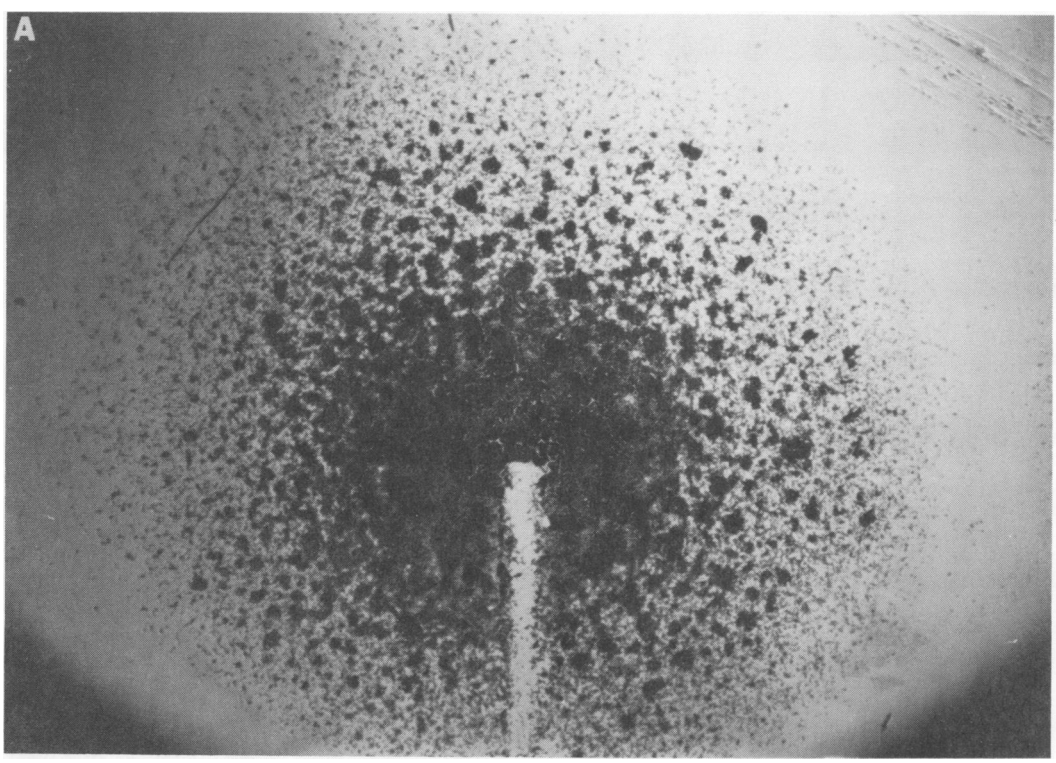

FIG. 1. *Leukocyte migration exhibiting (A) no inhibition and a fairly even pattern of cell distribution; (B) no inhibition and a more dense inner zone surrounded by a lighter "halo" area; (C) inhibition and a more dense inner zone surrounded by a "halo" area. ×16. Formalin fixed, Wright-Giemsa stained.*

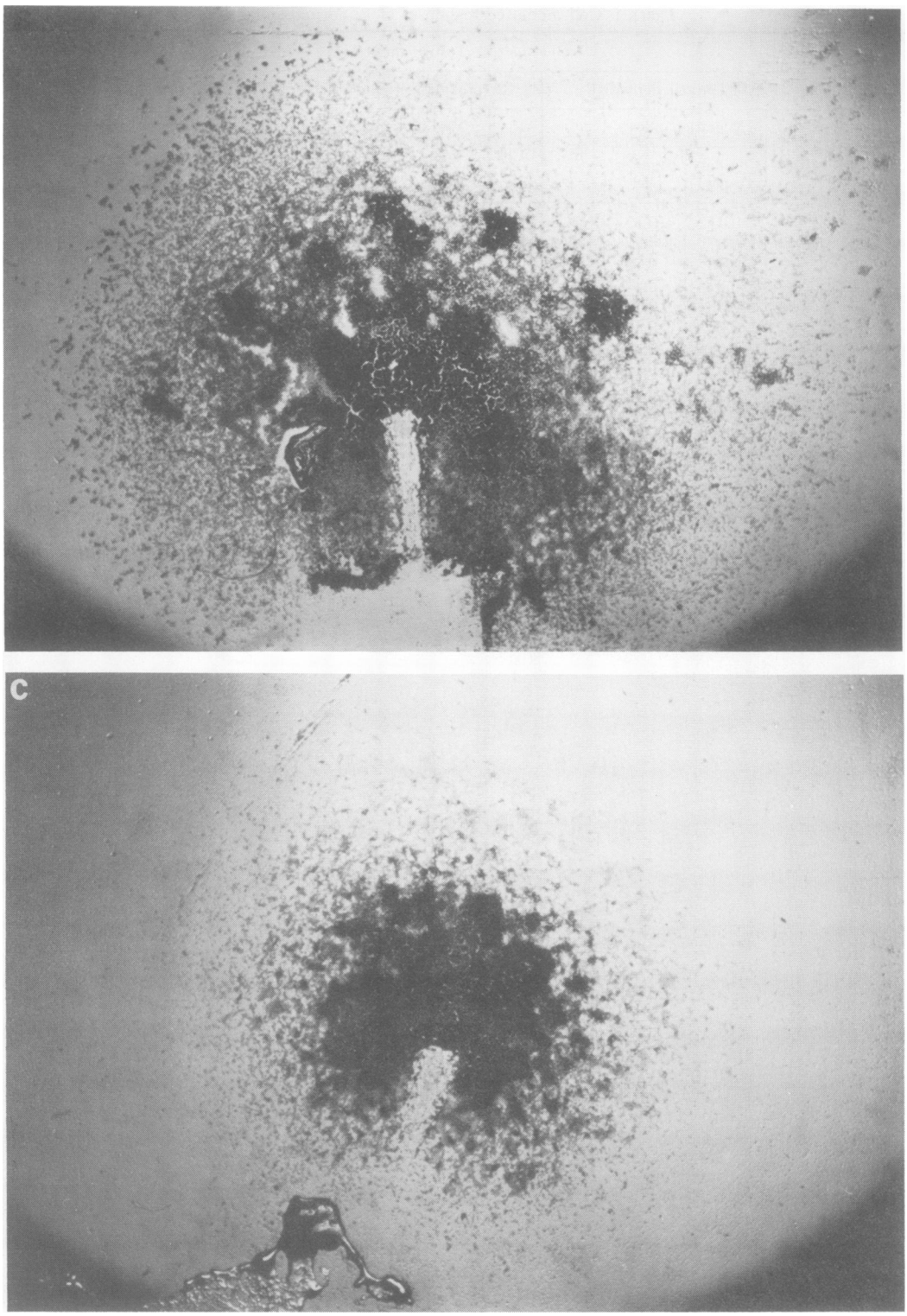

FIG. 1 B–C.

density toward the periphery. The ratio of PMN cells to mononuclear cells changed only slightly, however; mononuclear cells constituted approximately 50% of the cell population in inner areas (Fig. 2A) and 30 to 40% in outer areas (Fig. 2B). In a few cases of clearly defined outer "halo" patterns, more mononuclear cells were present in these areas (Fig. 2C) than in the outer areas of migration patterns that did not show this distinction.

Assay of MIF from supernatant fluids of lymphocyte cultures. In order to investigate the curious dichotomy of the response to DO, it was decided to determine whether MIF was produced by lymphocytes sensitized to this antigen. Lymphocytes from two subjects, who had been shown to be sensitive to all three antigens by DCH and LT, were cultured as described above. Supernatant fluids from these cultures were processed and assayed on peritoneal exudate cells of a normal guinea pig (Table 2). All three antigens proved to be capable of producing MIF from the lymphocytes of sensitized subjects, thus indicating that the failure of DO to inhibit migration of leukocytes might not be caused by the inability of this antigen to generate MIF. At concentrations expected to be present in assayed supernatant fluids, none of the antigens alone showed any inhibition of migration.

Reduction of NTB dye by PMN leukocytes. The effect of DO and other test antigens on the PMN cells, the major indicator cell in leukocyte migration tests, was further investigated by determining the capacity of these antigens to stimulate PMN cells to reduce NBT dye in vitro. The results of such stimulation of PMN cells from all 12 subjects under study are shown in Table 3, with the endotoxin of *Escherichia coli* added as a positive control. It can be seen readily that, in addition to endotoxin, both PPD and SK-SD stimulate PMN cells quite well, whereas DO fails to do so.

Comparison of migration from microcapillary tubes of unseparated and separated leukocytes. The above findings with DO—its ability to produce MIF from sensitized lymphocytes and its apparent inability to stimulate PMN cells to reduce NBT dye—indicated that the effect of this antigen, and perhaps of other test antigens also, on the leukocyte migration might depend upon direct interaction with PMN cells. To investigate this possibility, additional leukocyte migrations

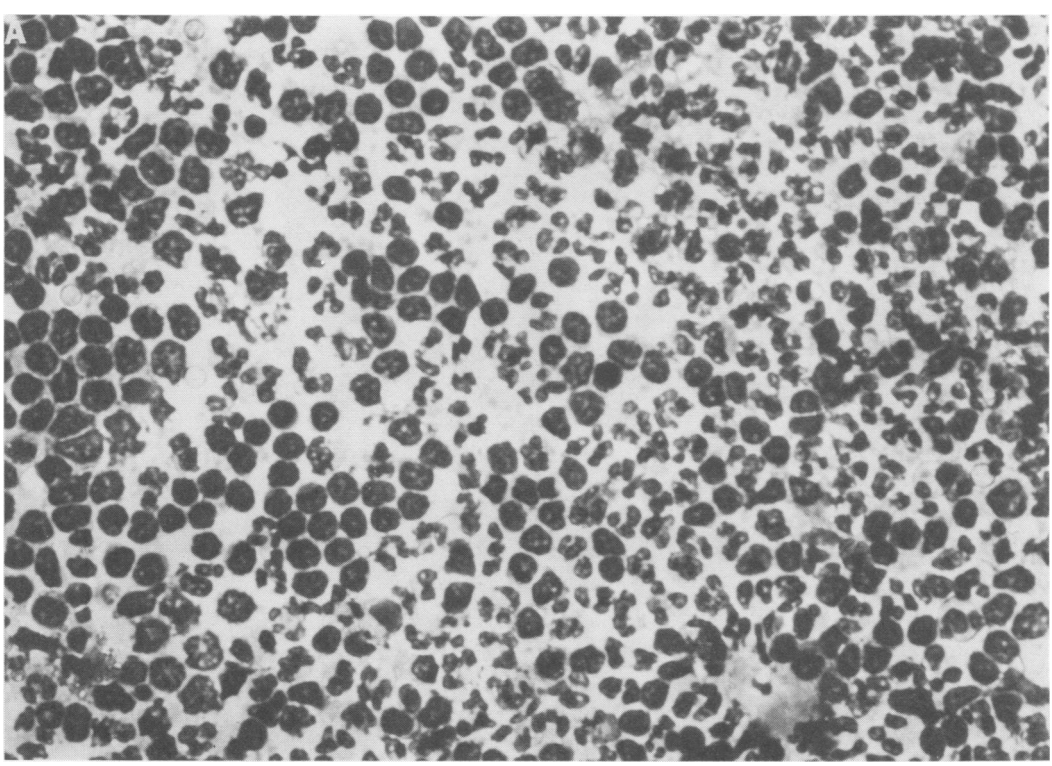

FIG. 2. *Cell distribution of leukocyte migration patterns. (A) Inner areas; (B) outer areas; (C) outer "halo" areas. ×640. Wright-Giemsa stained.*

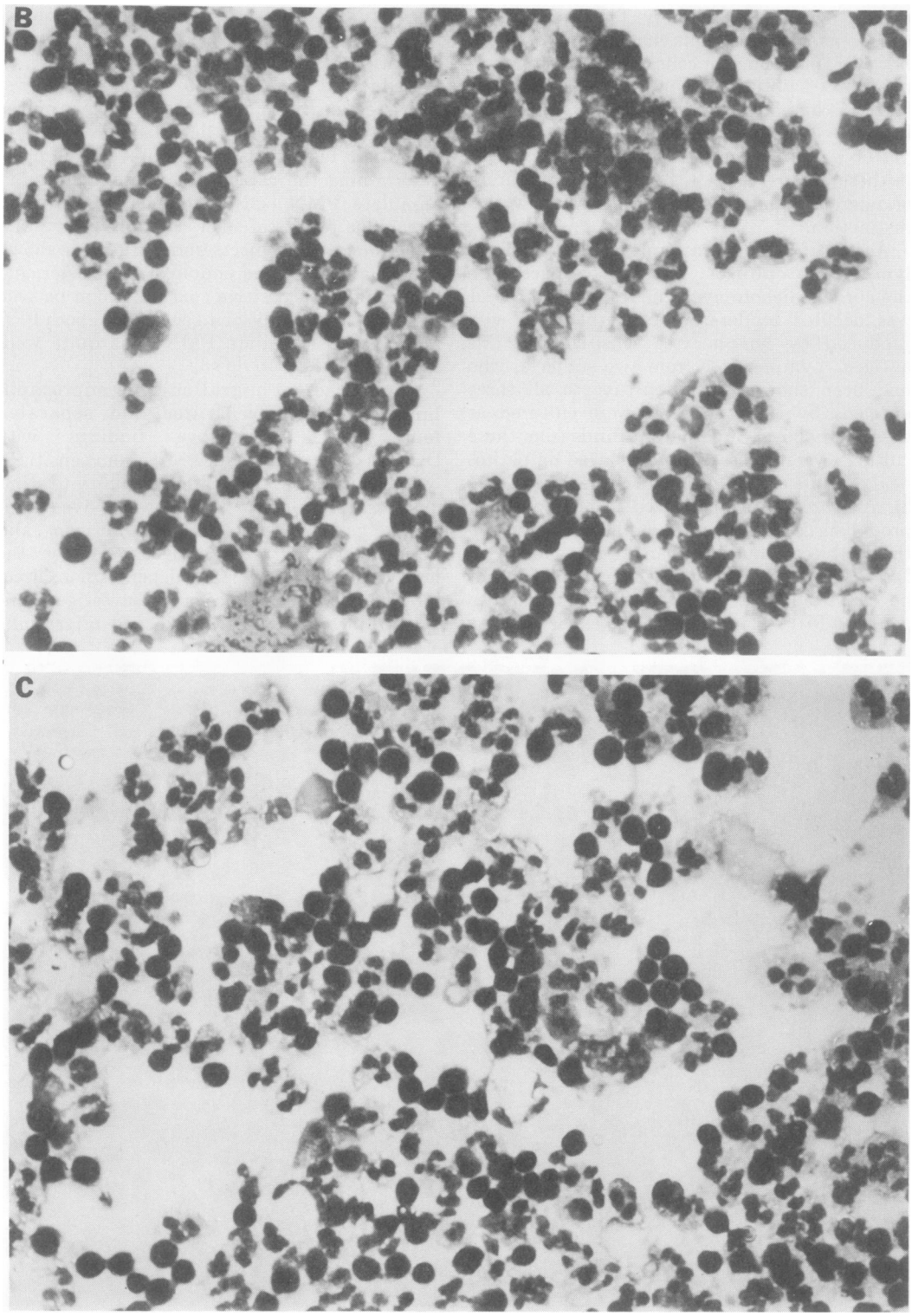

FIG. 2 B–C.

from microcapillary tubes were performed on six of the subjects under study by using separated populations of lymphocytes and PMN cells. The results of these migrations, compared with the migrations of unseparated leukocytes, are shown in Table 4. The striking feature of this comparison is that separated mononuclear cells (the vast proportion of them lymphocytes) are not inhibited at all by any of the antigens. On the other hand, such migration inhibition as is achieved by PPD and SK-SD with unseparated leukocytes is reflected in the corresponding inhibition of separated PMN cells. As predicted, DO failed to inhibit the migration of PMN cells.

TABLE 2. *Effect of supernatant fluids from human lymphocyte cultures on the migration of peritoneal exudate cells of a normal guinea pig*

Lymphocyte cultures of two different subjects	Materials added				
	None (baseline)	Supernatants from lymphocytes cultured with:			
		No antigen	PPD	SK-SD	DO
Expt 1	1.0[a]	1.08	0.52	0.58	0.49
Expt 2	1.0	0.98	0.46	0.60	0.52

[a] Expressed as migration index (see Table 1).

DISCUSSION

It was not our intent to select and compare individuals who were skin test positive or skin test negative for the test antigens but, rather, to compare various techniques in a group sensitized to these antigens. Within this group, we found excellent correlation between delayed cutaneous hypersensitivity and in vitro lym-

TABLE 3. *In vitro reduction of nitroblue-tetrazolium dye by unstimulated and stimulated polymorphonuclear leukocytes of 12 normal subjects*

Individual subjects	Base line[a]	PMN[a] stimulated with			
		Endotoxin (1 μg)	PPD (10 μg)	SK-SD (40/10 U)	DO (0.05 ml)
1	2	42	54	26	3
2	1	36	65	16	2
3	3	22	36	22	2
4	4	51	62	24	4
5	2	48	75	34	4
6	6	95	50	25	8
7	3	39	100	80	6
8	2	26	41	21	1
9	2	90	86	50	3
10	1	43	32	19	2
11	5	52	59	30	6
12	2	31	35	18	4
Mean	3	48	58	30	4
Range	1-6	22-95	32-100	16-80	1-8

[a] Expressed as percentage of NBT-positive PMN cells.

TABLE 4. *Comparison of migration from microcapillary tubes of unseparated and separated leukocytes*

Individual subjects	Testing antigens								
	PPD			SK-SD			DO		
	WBC[a]	L[b]	PMN[c]	WBC	L	PMN	WBC	L	PMN
1	0.41[d]	1.35	0.62	0.65	1.23	0.60	1.1	0.95	1.0
2	0.87	0.98	0.94	0.58	1.04	0.54	1.0	1.09	1.05
3	0.50	1.02	0.75	0.90	1.0	0.80	0.90	1.1	0.92
4	0.37	0.94	0.50	0.84	1.08	0.90	0.96	1.12	0.93
5	0.39	1.08	0.45	0.36	0.96	0.68	0.98	1.06	0.90
6	0.66	1.04	0.51	0.62	1.04	0.56	0.97	1.05	0.93

[a] White blood cells, unseparated leukocytes.
[b] Separated lymphocytes.
[c] Separated polymorphonuclear granulocytes.
[d] Migration index (see Table 1).

phocyte transformation (Table 1), indicating that the ability of lymphocytes to be stimulated by antigens reflects their state of specific sensitization to these antigens. A note of caution is in order, however. PPD tuberculin was shown to act as a mitogen on bone marrow-derived cells of nonimmune mice and guinea pigs (29), triggering DNA synthesis in vitro. In man, lymphocytes from tuberculin skin test-negative individuals can be stimulated by high doses of PPD (21), possibly indicating the presence of low-affinity cells for the antigen in these individuals, whereas in skin test-positive individuals, the best stimulation was achieved with low concentrations of the antigen, consistent with the presence of high-affinity cells. Also, an almost universal occurrence of antibodies to tubercle bacilli in sera from nontuberculous and tuberculous individuals (1) suggests that most persons have been sensitized to the tubercle bacillus to one degree or another, either by direct exposure or by cross-reaction. In view of extensive cross-reactivity among bacterial antigens (19), a considerable degree of cross-sensitization should be expected in the adult human population.

At the same time, it should be stressed that, in immunodeficient or anergic persons, delayed cutaneous hypersensitivity does not always correlate with lymphocyte transformation. As an example, we found good lymphocyte stimulation with PPD in six anergic patients with tuberculosis who had a negative tuberculin skin test (G. Senyk and W. K. Hadley, unpublished observations).

Lymphocytes from animals and man with delayed hypersensitivity produce a number of soluble factors when cultured with the specific antigen. Dumonde et al. called these soluble mediators of cellular immunity "lymphokines" (11) or, more recently, lymphocyte activation products (10). One of these factors, the migration inhibitory factor, assayed by the inhibition of migration of guinea pig peritoneal macrophages, has been a useful tool for the in vitro demonstration of delayed hypersensitivity (14). It has been used to study cell-mediated immunity in a variety of laboratory animals (reviewed in 3) and may play an important role in host defense against infection when released by specifically triggered lymphocytes. Simon and Sheagren (26) demonstrated in guinea pigs that committed lymphocytes, when stimulated with a specific antigen, are able to enhance greatly the ability of normal macrophages to kill antigenically unrelated bacteria. These investigators were unable to identify the MIF directly as an activator of macrophage bactericidal function (27). On the other hand, MIF appears to be the same (20) as a soluble factor elaborated by the antigen-stimulated lymphocytes of guinea pigs, which activates macrophages in the sense of promoting increased adherence, spreading, phagocytosis, and glucose oxidation through the hexose monophosphate pathway.

There have been many efforts to develop a reliable MIF assay in man. Bendixen and Söborg (2) described an MIF assay based on the inhibition of migration of peripheral blood leukocytes by specific antigen (tuberculin). Others (16), however, have found this inhibition difficult to reproduce. The Bendixen-Söborg method also requires 50 to 100 ml of blood, clearly excluding it as a routine diagnostic tool, especially in testing several antigens or in testing children. Thor et al. (30) and Rocklin et al. (23) described an in vitro assay based on the production of a soluble factor by sensitized human blood lymphocytes which had been stimulated with a specific antigen that inhibits the migration of normal guinea pig peritoneal exudate cells from capillary tubes. This indirect method also requires a large blood sample and considerable time to perform because of the need for extensive dialysis of culture supernatant fluids. Attempts to simplify and to miniaturize in vitro assays based on migration of peripheral blood leukocytes have led to the development of a microcapillary method by Federlin et al. (12) and an agarose gel method by Clausen (6), with PPD as the testing antigen in both cases.

By using these latter two methods essentially as described by their authors, we found good correlation of the leukocyte migration methods with delayed cutaneous hypersensitivity and lymphocyte transformation only in the case of PPD. The results with SK-SD varied considerably, both in correlation with DCH and LT and in correlation between the two migration tests (Table 1). DO was completely inactive in migration tests. Assaying the test antigens on separated populations of leukocytes, we found that mononuclear cells were not inhibited in their migration by any of the antigens, whereas PMN cells were inhibited by PPD and SK-SD whenever unseparated leukocytes exhibited such inhibition (Table 4). Thus, we are faced with a definite ambiguity of the PMN cell as an indicator cell of migration of unseparated leukocytes. On one hand, we have found no evidence in the literature that MIF exerts a direct inhibitory effect on the human PMN cells in the same manner that it does on peritoneal macrophages of guinea pigs. We find that MIF is produced in response to all three of our antigens, as shown

by the assay of MIF on guinea pig peritoneal exudate cells (Table 2), yet one of these antigens (DO) has consistently failed to inhibit leukocyte migration. On the other hand, production of soluble, biologically active factors is not confined to the lymphocytes. Recently, a factor, called neutrophil-immobilizing factor, has been described (15) which can be released by PMN leukocytes upon exposure to endotoxin under certain conditions, and which directly and irreversibly inhibits the response of human neutrophils to diverse chemotactic stimuli without impairing their viability. Thus, factors other than MIF can inhibit the migration of human PMN cells in situations involving direct interactions of leukocytes with bacterial antigens. It is apparent, therefore, that what the PMN cell does, or does not do, upon contact with soluble bacterial antigens within the framework of leukocyte migration tests may depend upon the complex interplay of diverse influences and events, the release of MIF by lymphocytes, triggered specifically or otherwise, being but one of them. In this connection, it is interesting to note that DO, which does not inhibit the migration of leukocytes, also fails to stimulate PMN cells to reduce NBT dye (Table 3). This would indicate some basic defect in the capacity of this antigen to interact with PMN cells. It should be emphasized also that the stimulation of PMN cells by PPD and SK-SD to reduce NBT dye does not appear to be immunologically specific, since such stimulation occurred in all subjects studied, including those who were skin test negative to these antigens.

Rosenberg and David (24), using large capillaries and large numbers of human leukocytes, reported that the inhibited leukocyte migration cultures usually exhibit a dense inner area, consisting of mixed leukocytes, and a lighter "halo" area, composed almost entirely of neutrophils. They suggested that only the mononuclear cells were inhibited from migrating, even though, in a few experiments with separated lymphocytes, low levels of specific antigen (SK-SD) failed to inhibit the migration of these cells. Our own experience with the cell distribution and morphology of stained migration patterns from microcapillary tubes was different. The gross pattern of most migrations exhibited a fairly even cell distribution (Fig. 1A). We observed the more dense inner zone and the outer halo area in a few instances of both uninhibited and inhibited migration (Fig. 1B, C). However, stained fields from the halo area clearly show a large number of mononuclear cells in this area (Fig. 2C). Furthermore, using the same high concentrations of antigens which inhibit migration of mixed leukocytes, we failed to observe any migration inhibition of mononuclear cells but did observe the migration inhibition of PMN cells in some cases (Table 4).

The search for a satisfactory and practical MIF assay in man continues. Recently, an MIF assay of human leukocytes was described (25) which used human lymphoblastoid cell line as an indicator cell of migration. Once again, PPD was the only antigen tested. To be universally applicable and practical as a diagnostic tool in determining the status of cellular immunity in man, the MIF assay would have to be reliable and reproducible with a variety of soluble bacterial, fungal, and viral antigens. In addition, it should be possible to run such an assay on a reasonable amount of blood, preferably not exceeding 10 ml. Such a goal remains to be achieved.

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