Lymphokine-Induced Phagocytosis in Angiocentric Immunoproliferative Lesions (AIL) and Malignant Lymphoma Arising in AIL

By Charles R. Simrell, Joseph B. Margolick, Gerald R. Crabtree, Jeffrey Cossman, Anthony S. Fauci, and Elaine S. Jaffe

A factor that augmented the phagocytosis of IgG-coated red blood cells by the human monocyte/macrophage line U937 was identified in cell culture supernatants from two of two patients with angiocentric peripheral T cell lymphomas, three of three patients with angiocentric immunoproliferative lesions that were not frankly malignant, and one of two patients with T lymphoblastic malignancies. The factor was not present in supernatants derived from 14 nonangiocentric peripheral T cell lymphomas of other histologic types nor in ten cases of B cell lymphoma and two cases of Hodgkin’s disease. A similar factor was present in the supernatants of concanavalin A (Con A)-stimulated normal peripheral blood mononuclear cells and in the supernatants of IL-2–dependent T cell lines derived from normal peripheral blood. The factor had an apparent mol wt of greater than 50,000 daltons, was heat labile (100°C for two minutes), and stable at pH 2.0. Its stimulation of phagocytosis was independent of any increase in number of Fc receptors. Thus, this factor is probably not γ-interferon. This factor may play a pathogenetic role in the hemophagocytic syndromes associated with certain T cell malignancies and immunodeficient states.

Certain patients with peripheral T cell lymphoma (PTL) develop an erythrophagocytic syndrome that mimics malignant histiocytosis both clinically and pathologically. In those cases studied, the neoplastic cells expressed the phenotypic markers of T cells, whereas the phagocytic cells were cytologically benign and bore markers of the monocyte/macrophage line. One possible pathogenetic mechanism for this phenomenon may involve a lymphokine produced by the neoplastic T cells, which could stimulate the phagocytic cells of the reticuloendothelial system. In order to test this hypothesis, cell suspensions were prepared from peripheral blood mononuclear cells (PBMCs) and tissues involved by lymphoid malignancies of T cell origin and cultured in vitro for 24 hours. The culture supernatants were then tested for their capacity to stimulate the phagocytic activity and the Fc receptor expression of the human monocyte/macrophage cell line U937. This cell line has proved to be a convenient system for the investigation of factors involved in monocyte/macrophage differentiation and function. In addition to supernatants derived from T cell malignancies, culture supernatants derived from cells of patients with non-Hodgkin’s lymphomas of B cell origin, Hodgkin’s disease, as well as a variety of other conditions were also tested. The results indicate that a phagocytosis-inducing factor (PIF), acting independently of any increase in Fc receptor number, is produced by cells from patients with certain T cell lymphoproliferative disorders, particularly angiocentric immunoproliferative lesions such as lymphomatoid granulomatosis or angiocentric T cell lymphoma. This factor may play a role in the pathogenesis of certain hemophagocytic syndromes.

Materials and Methods

Patient Selection and Histopathology

The patients in this study had been referred to the Warren Grant Magnuson Clinical Center at the National Institutes of Health for evaluation by either the Medicine Branch, Clinical Oncology Program, Division of Cancer Therapy, National Cancer Institute, or the Laboratory of Immunoregulation, National Institute of Allergy and Infectious Disease. All surgical pathologic material was reviewed. Malignant lymphomas were classified according to the modified Rappaport Classification for non-Hodgkin’s lymphomas. Hodgkin’s disease was classified according to the Rye modification of the Lukes-Butler scheme. Also studied were specimens from patients with angiocentric immunoproliferative lesions (AIL), a category that includes lymphomatoid granulomatosis and atypical lymphocytic vasculitis. These lesions are characterized by an angiocentric and angiodestructive chronic inflammatory infiltrate involving skin, lung, or other usually extranodal sites. The lymphocytic cells in these cases lacked marked cytologic atypia and were admixed with immunoblasts, plasma cells, histiocytes, eosinophils, and neutrophils. Control specimens included lymph nodes and spleens demonstrating either normal features or reactive lymphoid hyperplasia and peripheral blood mononuclear cells from normal volunteers.

Patient and Normal Donor Mononuclear Cell Culture Supernatants

Fresh biopsy tissue or heparinized peripheral blood were obtained aseptically with informed consent. Solid tissue was minced in RPMI 1640 (GIBCO, Grand Island, NY) and filtered through a wire mesh. Purified mononuclear cells were obtained by density gradient centrifugation through Ficoll-Hypaque (Lymphoprep; Nygaard & Co, Oslo). Mononuclear cell suspensions were incubated at 10^6 cells per milliliter in RPMI 1640 with 10% fetal calf serum (FCS; GIBCO) at 37°C in a 5% CO₂ atmosphere. Following a 24-hour incubation, the supernatant was harvested and stored at 4°C until assayed (generally in less than 24 hours) for the ability to induce phagocytosis and

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Fc receptors. In parallel, a portion of the cells were taken for assay as previously described for E rosette, complement, and Fc receptors, surface immunoglobulin heavy and light chains, and terminal deoxynucleotidyl transferase, as well as cytochemical analysis of acid phosphatase, \( \beta \)-glucuronidase, \( \alpha \)-naphthyl butyrate esterase, acid \( \alpha \)-naphthyl acetate esterase, naphthol ASD-acetate esterase, and alkaline phosphatase. In addition, further phenotypic analysis using monoclonal antibodies directed against other T and B surface antigens was also performed: OKT3, OKT4, OKT6, OKT8 (Ortho Diagnostic, Raritan, NJ); Leu-1, Leu-2a, Leu-3a, Leu-4, and HLA-DR (Becton Dickinson, Sunnyvale, Calif); Lyt3 (New England Nuclear Corp, Boston); Bl J5 (Coulter Monoclonal Antibodies, Hialeah, Fla); and 3A1 (ATCC, Rockville, Md). These studies allowed characterization of the patients’ neoplastic cells within the T, B, or monocyte/macrophage lineages.

### Derivation of T Cell Lines

T cells were purified from normal PBMC by rosetting with sheep red blood cells coated with 2-aminoethylisothiouronium as previously described. Oligoconal T cell lines were then established by plating one to ten T cells per round-bottomed microtiter well in RPMI supplemented with glutamine, antibodies, FCS, 10% to 20% IL-2-containing supernatant derived from the cell line M1A, and phytohemagglutinin (Burroughs-Wellcome, Research Triangle Park, NC) at a final concentration of 2 \( \mu \)g/mL. As feeder cells, 50 \( \times \) 10^6 allogeneic irradiated (4,500 rads) PBMCs were added to each well. After culture for 14 to 17 days, supernatants from wells containing visible colonies were obtained and stored at 4 °C until testing for PIF activity.

### Stimulation of U937 Cells

U937, a human monocyte/macrophage cell line, was obtained from Dr Hillel Koren, Duke University Medical Center. The cells were maintained at 0.4 to 1.6 \( \times \) 10^6 cells per milliliter in RPMI 1640 and adjusted to 2.5 \( \times \) 10^6 cells per milliliter. Rabbit IgG-coated ox red blood cells (IgG-OxRBCs) were prepared by incubation of equal volumes of 5% OxRBCs and the IgG fraction of rabbit anti-bovine RBCs (Cappel, West Chester, Pa) at one-half the hemagglutinating concentration. IgM-OxRBCs were prepared similarly using the IgM fraction of rabbit-anti-bovine RBCs (Cappel). After a 30-minute incubation at 37 °C, the coated RBCs were washed extensively in cold PBS and resuspended to a final concentration of 1% vol/vol.

Equal volumes (100 \( \mu \)L) of U937 cells and IgG-OxRBCs were mixed, pelleted in a table-top centrifuge, and incubated at 37 °C for one hour in a 5% CO\(_2\) atmosphere. In some experiments, uncoated or IgM-coated OxRBCs were also used as targets. The cell pellet was then resuspended, cytocentrifuged, and stained with a modified Wright’s stain. The phagocytic index (PI) was defined as the mean number of ingested red blood cells per 100 U937 cells, as microscopically determined by counting a minimum of 300 U937 cells in each cytocentrifuge smear. The ability of normal rabbit IgM and IgG to inhibit the stimulated phagocytosis of IgG-OxRBCs was tested by the addition of serial dilutions of the antibody directly to the phagocytic assay mixture.

### Fc Receptor Assay

Cell surface Fc receptors were assayed by measuring the Fc receptor specific binding of human myeloma IgG as previously described. Briefly, stimulated or unstimulated U937 cells were harvested, washed free of any contaminating serum-derived IgG, and incubated at 37 °C for 30 minutes with a receptor-saturating concentration of purified monomeric human myeloma IgG generally 5 \( \times \) 10^5 mol/L. Following this incubation, the cells were quickly washed free of unbound immunoglobulin, and bound radioactivity in the cell pellet was determined in a gamma counter. To estimate nonsaturably (ie, nonspecifically) bound IgG, cells were incubated with [125I]-IgG, in the presence of a concentration of unlabeled IgG sufficient to bind 99% of the U937 Fc receptors. Specific binding was determined by subtracting nonspecific binding from total binding, and the results were expressed as molecules of IgG bound, ie, number of Fc receptors per cell. Iodination was performed using 1,3,4,6-tetrachloro-3a,6a-diphenylglycouril (Iodo- gent; Pierce Chemical Co, Rockford, Ill.). Generally, 100 to 250 mg of IgG was iodinated using 0.5 to 1.0 \( \mu \)Ci of carrier-free Na [125I] (New England Nuclear, Boston) and 10 to 25 \( \mu \)g Iodo- gent. The reaction was carried out at room temperature over a five- to ten-minute period. Remaining free iodine was separated from the labeled protein by gel filtration over a small Bio-Gel P-6 column (Bio-Rad, Richmond, Calif). Specific activities of the labeled immunoglobulin were on the order of 100 \( \mu \)Ci/mol. Protein concentration was determined by absorption at 280 nm.
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Normal Media
Normal or Reactive MNC
MLC
Con A-Stimulated PBMNC
Angiocentric T Cell Proliferations

ALL

Non Angiocentric T Cell Lymphomas
B Cell Lymphomas
Hodgkin's Disease

DAUDI

Fig 1. Effect of culture supernatants on U937 phagocytosis of IgG-OxRBCs. Con A- and MIC-derived supernatants increase phagocytosis of IgG-OxRBCs by U937 cells (P ≤ .001). Supernatants derived from two angiocentric T cell malignancies and three angiocentric immunoproliferative lesions have a similar effect (P ≤ .001).

Ultrafiltration

Preliminary mol wt analysis was performed by ultrafiltration of culture supernatants through semipermeable polymer membranes with nominal mol wt limits of 300,000, 100,000, 50,000, 30,000, 10,000, and 1,000 daltons (Amicon, Danvers, Mass). The ultrafiltration system was also used for testing the acid lability of PIF-containing supernatants. The buffered medium of the PIF-containing supernatant was exchanged by diafiltration first with a 0.2 mol/L KCl/HCl, pH 2.0 buffer, and then, 24 hours later, reequilibrated with phosphate-buffered saline, pH 7.4, prior to assay in a 48-hour U937-stimulation culture.

RESULTS

Effect of 24-Hour Culture Supernatants on U937 Phagocytosis of IgG-OxRBCs

Control studies showed that unstimulated U937 cells have modest phagocytic activity, PI = 8.2 ± 3.0 (mean ± SE). In contrast, both MLC supernatants and Con A-stimulated PBMNC supernatants induced marked increases in phagocytic activity, PI = 42.1 ± 3.9 and 38.4 ± 4.2, respectively (P ≤ .001) (Figs 1 and 2). The increase in PI reflected an increase in both the number of phagocytophylactic cells and the number of RBCs ingested by each phagocytic cell. Dose-response experiments demonstrated a rapid rise in phagocytic activity with increasing concentrations of Con A-stimulated PBMNC supernatants from 0.1% to 10.0% (vol/vol), and relatively little further increase in activity with greater concentrations (10% to 50%) (Fig 3). Time course experiments using MLC or Con A to stimulate PIF production showed significant PIF activity in 24-hour supernatants, and maximal activity in 48-hour supernatants (Fig 4).

PIF was identified in PBMNC supernatants from three of three patients with AIL (PI = 136, 87, and 38). PIF was also found in cell culture supernatants derived from two peripheral T cell malignancies (PI = 43.9, 109). Histologic review performed independently of the functional studies revealed that both peripheral T cell lymphomas manifesting PIF activity arose in...
patients with previous diagnoses of AIL, and both lymphomas retained an angiocentric and angiodestructive character. They were further classified in the modified Rappaport scheme as diffuse large cell “histiocytic” type and diffuse poorly differentiated lymphocytic type. Thus, five of the six cases in this study which demonstrated significantly increased PIF activity were either AIL or malignant lymphomas arising in cases of AIL. (PI = 82.8 ± 18.7, P ≤ .001). The sixth positive case was a case of T cell acute lymphoblastic leukemia/lymphoma, (PI = 72).

Phenotypically, the lymphomas demonstrating PIF activity were heterogenous. Of the PTL, one had a mature helper phenotype, and one reacted with pan T cell monoclonal antibodies (Leu-1-positive) but lacked helper- and/or suppressor-associated antigens. The T cell lymphoblastic lymphoma was TdT-positive and had a suppressor cell phenotype. All three malignancies expressed low levels of TAC (1% to 3%) and Ia (HLA-DR) (1% to 18%). Serum, as well as cells, was available in two PIF-secreting patients (one peripheral T cell lymphoma and one AIL). The patients’ cells secreted PIF when cultured in the presence of either FCS or autologous serum. Autologous serum alone had no effect on U937 cells.

Malignant lymphoid proliferations that failed to demonstrate PIF activity included 14 nonangiocentric peripheral T cell neoplasms, further classified as diffuse large cell “histiocytic” type (seven cases), diffuse mixed cell type (five cases), mycosis fungoides (one case), and Sezary cell leukemia (one case). Two of these peripheral T cell lymphomas were from patients who were sero-positive for antibodies to human T cell lymphoma/leukemia virus (HTLV-I) antigens.15 Other negative lymphoid malignancies included ten non-Hodgkin’s lymphomas of B cell origin, further classified as diffuse well-differentiated lymphocytic type (two cases), nodular (follicular) lymphoma (five cases), diffuse large cell type (one case), diffuse mixed-cell type (one case), and diffuse undifferentiated Burkitt’s type (one case). Finally, supernatants from two cases of Hodgkin’s disease, one of lymphocyte-depleted type, and one of nodular-sclerosing type were negative for PIF activity, as was the supernatant from one case of pre-B acute lymphoblastic leukemia.

Culture supernatants of unstimulated cells from normal or reactive lymph nodes, spleen, or normal PBMCNs had minimal PIF activity (PI = 12.3 ± 2.7), not significantly different from unstimulated controls, (P = .404). A culture supernatant derived from mononuclear cells isolated from a reactive pleural fluid did demonstrate phagocytosis-inducing activity (PI = 12.4). Fifty percent of the pleural fluid mononuclear cells were shown to be T cells, 30% were polyclonal B cells, and the remainder were mesothelial cells and macrophages.

**Effects of 24-Hour Culture Supernatants on U937 Fc Receptors**

Unstimulated U937 cells had approximately 30,000 (30,700 ± 1,900) Fc receptors per cell. U937 cells that had been stimulated by an MLC culture supernatant demonstrated an approximate threefold increase in the number of cell surface Fc receptors (86,000 ± 8,000), (P ≤ .001). U937 cells exposed to Con A-stimulated PBMCNC supernatants consistently failed to show any increase in Fc receptors (21,000 ± 1,700), despite the marked augmentation of phagocytic activity. Similarly, no other supernatants, including those of four patient-derived supernatants that contained significant PIF activity, showed any Fc receptor-inducing activity (Fig 5).

Although U937 cells incubated with supernatant derived from Con A-stimulated PBMCNs showed enhanced phagocytosis of IgG-OxRBC, they did not ingest IgM-OxRBC (data not shown). The addition of
Fig 5. Effect of culture supernatants on U937 IgG-FcR. MLC supernatants stimulate an increase in IgG-Fc receptor number on U937 cells (P < 0.001). Supernatants from Con A-stimulated normal MNC or patient-derived supernatants do not stimulate an increase in Fc receptor number.

Fig 6. PIF production by T cell lines. Supernatants from four of 21 IL-2-dependent T cell lines contain PIF activity comparable to that of Con A-stimulated normal PBMNC supernatants.

Fig 7. Preliminary estimate of PIF mol wt by ultrafiltration. Note marked loss of PIF activity in ultrafiltrate containing molecules >50,000 daltons. Some loss of activity in higher mol wt ultrafiltrates may represent filtration of high mol wt complexes.

Production of PIF by Long-term T Cell Lines

Supernatants from four of 21 T cell lines derived from normal PBMNC contained PIF activity comparable to that of Con A-stimulated normal PBMNC supernatants (Fig 6). Thus, a highly purified T cell population is capable of generating PIF. This does not preclude the possibility, however, that some non-T cells may also produce the same or a related activity.

Preliminary Characterization of PIF in Con A-Stimulated PBMNC Supernatant

Ultrafiltration of PIF-containing Con A-stimulated PBMNC supernatants, as well as one patient-derived supernatant, indicated a mol wt of greater than 50,000 daltons (Fig 7). The activity was sensitive to tempera-
two patients with T cell lymphoblastic malignancies. The activity was not seen in supernatants derived from 14 other peripheral T cell lymphomas of other histologic types nor in ten cases of B cell lymphoma and two cases of Hodgkin's disease. These findings strongly suggest that secretion of this factor may be a characteristic of ALL and the malignant lymphomas that supervene in this disease. The ALL are a related group of lymphoproliferative disorders for which several diagnostic terms have been employed: lymphomatoid granulomatosis, polymorphic reticulosis, and atypical lymphocytic vasculitis. These lesions are angiocentric and angiodestructive lymphoid proliferations comprised of an admixture of lymphocytes and other inflammatory cells. Despite the polymorphic character of the infiltrate, the lymphocytes may show some cytologic atypia, and approximately 30% of patients develop frank malignant lymphoma. Therefore, it has been argued that in at least some patients the disease may be neoplastic at the outset.16

We hypothesize that such a phagocytosis-inducing factor may play a role in the pathogenesis of an erythrophagocytic syndrome seen in association with certain T cell malignancies and, in fact, this syndrome appears with greater prevalence in angiocentric lymphoma than in other peripheral T cell lymphomas.1 Indeed, one of the patients in the present study whose cell culture supernatants contained PIF was one of six recently reported cases who died with this syndrome.5

The secretion of lymphokines by neoplastic T cells is a well-known phenomenon, and certain syndromes and clinical stigmata are recognized to occur as a consequence of lymphokine production by neoplastic cells in vivo. Neoplastic cells from patients with Sezary syndrome often secrete migration inhibition factors (MIF) in vitro and in vivo,19 and the polyclonal hypergammaglobulinemia seen in Sezary syndrome has been related to the function of the malignant cells as helper cells in vivo.20 A case of cutaneous lymphoma secreting an immunosuppressive lymphokine has been

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The identity of the PIF activity described in this study is as yet unknown. It may be a newly described lymphokine or may represent another functionally defined activity of some other previously known lymphokine. A number of T cell-derived lymphokines are known to have a stimulatory effect on cells of the monocyte/macrophage line,26 including MAF, colony-stimulating factor (CSF), and γ-interferon.27,33 Guyre et al,30 as well as Ralph et al,33 have recently reported that γ-interferon induces an increase in Fc receptors on U937 and that the Fc receptor-inducing factor in mitogen-stimulated PBMNC supernatants may be γ-interferon. It is unlikely that the PIF activity described here is due to γ-interferon, since the activity is not associated with an increase in Fc receptors, has an apparent mol wt of more than 50,000 daltons, is stable at pH 2.0, and is only slightly inhibited by very high concentrations of monoclonal anti-γ-interferon. Moreover, high concentrations of purified human γ-interferon induced only a comparatively minimal increase in phagocytosis in this system. PIF activity is sensitive to temperature conditions that do not affect human MAF.34 However, we cannot, at this point, rule out the possible role of CSF in this phenomenon. Recently, Olsson et al35 have described a 46,000 to 55,000 dalton differentiation-inducing factor (DIF) in culture supernatants of mitogen-stimulated T lymphocytes or in the supernatants of the HTLV-infected T cell line HUT-102. DIF can induce differentiation of the human promyelocytic cell line HL-60 and can augment the degree of retinoic acid-induced differentiation of U937 cells, as measured by nitro blue tetrazolium reduction. DIF alone, however, did not induce differentiation of U937 cells. The relationship between DIF and the PIF activity identified in our experiments needs further study.

This study describes a potential mechanism for the production of a hemophagocytic syndrome in patients with benign or malignant T cell proliferations. In T cell malignancies, the syndrome could occur as a result of a function of the abnormal T cells that mimics a physiologic function of normal activated T cells.1 Hemophagocytic syndromes also have been described in patients with endogenous and/or iatrogenic immunodeficiency and viral or other infections.36,37 In these cases, the hemophagocytic syndrome might occur as a consequence of excessive lymphokine produced by activated T cells in a misregulated immune state.

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