Lymphokine Regulation of Macrophage-Derived Growth Factor Secretion Following Pulmonary Injury

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Rat alveolar macrophages secrete enhanced levels of a growth factor for fibroblasts following acute lung injury. The authors have previously identified this anionic, heat-labile substance as macrophage-derived growth factor (MDGF) and have now focused on how its release by alveolar macrophages is controlled following lung damage. In response to pulmonary injury induced with a single intratracheal instillation of bleomycin sulfate, the lymphocyte count rose from undetectable to 23% of the cells retrieved in lavage fluid. Spontaneous MDGF secretion by macrophages cultured from the same lungs generally paralleled the changes in lymphocyte counts over time. To test whether lymphokine(s) secreted by alveolar lymphocytes regulated MDGF secretion by macrophages in this model, the authors exposed normal macrophages harvested from control rat lungs to lymphokine preparations *in vitro*. Lymphokines provided a powerful stimulus to normal macrophages, inducing MDGF secretion at dilutions as low as 10^{-6}. Fractionating T-lymphocyte subsets by adherence to monoclonal antibodies indicated that the W3/25$^+$ cells elaborated the macrophage-stimulating lymphokine, but that OX8$^+$ cells did not. Furthermore, recombinant murine gamma interferon was capable of substituting for native lymphokine in activating MDGF secretion by normal macrophages. It is concluded that immune stimulation of fibroblast proliferation requires cooperative interaction of both lymphocytes and macrophages in this model of acute lung injury. (Am J Pathol 1985, 121:261–268)

ALVEOLAR macrophages harvested from the lungs of humans[1] and rats[2] secrete a growth factor for fibroblasts (macrophage-derived growth factor, MDGF). Normal alveolar macrophages, like blood monocytes,[3] can be induced to secrete MDGF after *in vitro* stimulation.[2] "Spontaneous" secretion of MDGF appears to occur following acute lung injury in humans[1] as well as in experimental models. We have previously shown in the rat[2] that this substance has physical and biologic properties similar to those of MDGF released by human and murine macrophagelike cell lines.[4,5] At the same time, several laboratories have demonstrated that MDGF from rodent cell lines is distinct biochemically and biologically from interleukin-1.[4,6]

Rat alveolar macrophages release MDGF following acute lung injury induced by intratracheal instillation of bleomycin; however, bleomycin added *in vitro* has no direct effect on MDGF release by cultured normal macrophages.[2] Thrall and colleagues have carefully analyzed the cells obtained by bronchoalveolar lavage at various stages during the development of bleomycin-induced fibrosis in the rat.[7] They found lymphocytes to be present among lavage cells within 3 days of lung injury. They further used the mouse monoclonal antibodies W3/25 and OX8 to identify nonoverlapping T-cell subsets in lavage fluid. W3/25$^+$ T cells exhibit helper functions, mixed lymphocyte responsiveness, and graft-versus-host functions; OX8$^+$ T cells exhibit allogeneic suppression of the induction of antibody-forming cells and cytotoxicity functions in the rat.[8] The T-cell population retrieved by lavage contained equal portions of W3/25-positive and OX8-positive cells at 3, 7, and 14 days after lung injury, a ratio distinct from the 2:1 ratio found in peripheral blood and lymph nodes. Thrall and colleagues speculated that this changing pattern of T lymphocytes in the air spaces must be an important step in the evolution of fibrotic lesions.

We have speculated that release of MDGF by alveolar macrophages, rather than being a spontaneous

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event, might be triggered indirectly by other limbs of the immune system acting on naive alveolar macrophages. Lymphokines have been shown to influence a number of macrophage functions, including induction of endocytosis,[9] production of hydrogen peroxide,[10] expression of Fc receptors,[11] and potentiation of cytolytic activity.[12-14] This known diversity of effects of lymphocyte secretory products on macrophage functions prompted us to examine their possible role in stimulating MDGF secretion by pulmonary macrophages following acute lung injury.

Materials and Methods

Isolation and Culture of Cells

Early passages of fibroblasts derived from lungs of male Fischer 344 rats (Harlan Sprague Dawley, Walkersville, Md) were cultured in 25-sq cm flasks (Corning, Corning, NY). The fibroblasts were fed twice weekly with minimum essential medium (MEM) (GIBCO Laboratories, Grand Island, NY) containing 10% (vol/vol) fetal bovine serum (GIBCO), penicillin (100 U/ml, Sigma Chemical Co., St. Louis, Mo), streptomycin (100 μg/ml, Sigma) and fresh glutamine (2 mM, GIBCO), incubated at 37 C in an atmosphere of 5% CO_2/95% air, and subcultured weekly. Fibroblasts used in all experiments were below population doubling level 25.

Plasma-derived serum, serum depleted of platelet-derived growth factor, was prepared from a single lot of pooled human serum as described by Glenn and Ross.[3]

We collected alveolar cells using standard lavage procedures as previously described.[2] Briefly, rats were anesthetized with sodium pentobarbital (10 mg/100 g body weight) and killed by exsanguination. The lungs were removed with the airway intact and lavaged repeatedly with 10 boluses of calcium- and magnesium-free phosphate-buffered saline (PBS). The volume instilled each time was equal to one-thirtieth of the rat's body weight. Cells were collected from lavage fluid by centrifugation at 500 rpm in a clinical centrifuge for 10 minutes and washed two times in serum-free MEM containing glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and Fungizone (100 μg/ml). The cells were dispersed in a minimal volume of the same medium; aliquots were removed for cell count in a hemacytometer and cell differential count by cytocentrifuge (Shandon Southern, Sewickley, Pa). We stained the slides with May-Grünwald-Giemsa stain and counted a minimum of 300 cells per slide. Cell viability was greater than 96% as determined by trypan blue exclusion.

Pulmonary lavage cells were fractionated into adherent and nonadherent cells by incubating the mixed population of cells at 1.2×10^6/ml in serum-free MEM in 24-mm multiwell plates (Costar, Cambridge, Mass). After a 2-hour incubation at 37 C in 5% CO_2/95% air the medium was removed and centrifuged for collection of the nonadherent cells. Nonadherent cells were suspended in ammonium chloride (0.85%) for 10 minutes at 4 C for lysing red blood cells.

Preparation of Alveolar Lymphocyte Supernatants

Alveolar cells (from bleomycin-injured lungs) not adhering to tissue culture plates (nonadherent cells) were either cultured as a whole, lymphocyte-rich population (40–55% lymphocytes) or fractionated by one of the following two methods: 1) Ficoll-Hypaque (Ficoll, Sigma; Hypaque, Winthrop Laboratories, New York, NY) density gradient centrifugation, or 2) "panning" for subsets of T lymphocytes.[15] Cells were cultured at 1.2×10^6 cells/ml in Newman-Tytell medium. Supernatants collected after 24 hours were diluted in PBS and tested both for their ability to stimulate fibroblast proliferation directly and for their ability to stimulate MDGF secretion by macrophages.

Ficoll-Hypaque density gradient centrifugation was performed by the method of Boyum.[16] Nonadherent cells were layered over Ficoll-Hypaque and centrifuged at 600*g* for 30 minutes at 4 C. The mononuclear cell fraction and the granulocyte fraction were washed and cultured separately as described above.

Adherence to immobilized monoclonal anti-lymphocyte antibodies ("panning") was used to separate W3/25-positive and OX8-positive T-lymphocyte subsets (Accurate, Westbury, NY).[15] For this procedure plates were initially incubated with rabbit anti-mouse IgG (20 μg/ml, Accurate) in PBS (16 hours, 4 C), after which the plates were washed with cold PBS. Nonadherent cells (10^6/ml) were incubated in separate 35-mm Petri plates with monoclonal antibody W3/25 or OX8 (1:100 dilution in PBS with 2% plasma-derived serum) for 60 minutes at 4 C with occasional swirling. The nonadherent cell monoclonal antibody complex was centrifuged for 10 minutes in a clinical centrifuge, the supernatants were removed, and the cells were washed in 3 ml of PBS containing 2% plasma-derived serum twice for removal of unbound antibody. The cell pellet was resuspended in 500 μl of the same buffer and incubated in the plates coated with rabbit anti-mouse IgG for 60 minutes on ice with occasional swirling. Suspended and loosely attached cells were removed by gentle aspiration and discarded. The bound cells were then harvested by vigorous, repeated aspiration. Cells were washed and cultured as described above.

Preparation of Macrophage Supernatants

Total lavage cells were plated in Neuman-Tytell medium (GIBCO), and nonadherent cells were removed after 2 hours. Monolayers of adherent cells (>96% macrophages) were washed vigorously with PBS and cultured in Neuman-Tytell medium (1.2×10^6 cells/ml) containing fresh glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). For *in vitro* stimulation studies, duplicate cultures received the same medium with or without phenol-extracted lipopolysaccharide (30 μg/ml, Sigma). The lipopolysaccharide was added *in vitro* to assure that each lot of macrophages was capable of responding maximally to a standard stimulant. After 24 hours the medium was decanted, filtered through 0.2-μ filters, and stored at −20 C. Macrophage supernatants were tested for growth factor activity based on their ability to induce proliferation of quiescent fibroblasts (described below). Adherent lavage cells from bleomycin-treated rats were handled similarly.

Lymphokine Stimulation of Control Alveolar Macrophages

Macrophages from control animals were used to test the ability of lymphokine preparations to induce MDGF secretion. Supernatant fluids from cultured nonadherent cells or from populations of lymphocytes purified by Ficoll-Hypaque or "panning" were tested for their ability to stimulate MDGF secretion. Macrophages were cultured in the presence of lymphocyte supernatants for 24 hours, after which the macrophage media were collected for MDGF assay. Lymphocyte supernatants were routinely tested at a dilution of 1:10 unless otherwise noted. At this dilution any contaminating residual MDGF in crude lymphocyte supernatants was not detected.

We used recombinant murine gamma interferon (IFN-γ) (Genentech, Inc., South San Francisco, Calif) to test a purified lymphokine for its ability to stimulate normal alveolar macrophages to release MDGF as well as its ability to directly modulate fibroblast proliferation.

Fibroblast Proliferation Assay

Growth factor activity in culture medium from macrophages and lymphocytes was determined by monitoring the entry of quiescent adult rat lung fibroblasts to the cell cycle using ^{3}H-thymidine incorporation as an index of DNA synthesis.[2,3] Fibroblasts were seeded at a density of 4200 cells in 200 μl of MEM containing 10% fetal bovine serum (see above) in flat bottom 96-well microtiter plates (CoStar, Cambridge, Mass) and incubated for 4 days, at which time they were visually confluent (approximately 1.5×10^5 cells/well). After 4 days, 100 μl of test substance (macrophage or lymphocyte supernatants or recombinant IFN-γ or control medium) and 30 μl of fresh medium (MEM containing 10% plasma derived serum) was added to each well. Fibroblasts were incubated with test medium for 18 hours, after which the medium was replaced with 200 μl of labeling medium containing 0.5 or 1.0 μCi/ml of ^{3}H-methyl thymidine (2 Ci/mM, Amersham, Arlington Heights, Ill) in MEM with 10% fetal bovine serum. After 2 hours, labeled fibroblasts were harvested by trypsinization and collected on glass fiber filters with the use of a cell harvester (Cambridge Technology, Cambridge, Mass), and isotope incorporation was determined by liquid scintillation spectrometry. Counting efficiency for tritium was routinely 45%. Fresh medium containing 10% fetal bovine serum served as a positive control and typically caused a 6–10-fold increase in ^{3}H-Tdr incorporation (as measured at 18 hours) and a 65–78% increase in fibroblasts number (at 48 hours). The baseline level of ^{3}H-Tdr incorporation by fibroblasts receiving fresh medium alone (800–1100 dpm/well) was subtracted from all values for test medium. Lipopolysaccharide had no direct effect on fibroblasts in this assay.

Induction of Lung Injury

Lung injury was induced in adult rats by a single direct peroral intratracheal administration of bleomycin sulfate (0.6 mg) as previously described.[17] Barrier-maintained male Fischer 344 rats (Harlan Sprague Dawley, Walkersville, Md) weighing 180–200 g were used in all studies. Animals were sacrificed 7 days after bleomycin instillation unless otherwise noted.

DEAE-Sephacel Chromatography of MDGF

To determine whether the growth factor released by alveolar macrophages in response to *in vitro* exposure to lymphokine was similar to standard MDGF produced by macrophages treated with lipopolysaccharide and the factor released by macrophages collected from bleomycin-treated lungs, we compared their elution profiles from DEAE-Sephacel.[4] Supernatants from macrophages cultured in the presence of lymphokine for 24 hours were pooled and dialyzed exhaustively against phosphate buffer (0.1 M, pH 7.2). The material was chromatographed on a DEAE-Sephacel column at room temperature as previously described.[2] The column bed volume was 5 ml, and 2-ml fractions were collected.

After loading the sample, the column was washed with 10 bed volumes of 0.1 M phosphate buffer, and growth factor was eluted with a linear salt gradient increasing from 0 to 1.0 M NaCl in 60 ml of phosphate buffer at the same pH. Column fractions were dialyzed against phosphate buffer, and 100 μl aliquots of each fraction were tested for growth factor activity in triplicate.

Data Analysis

Data are presented as means ± SEM of triplicate samples. Statistical significance was determined with the Student *t* test. MDGF released by cultures of macrophages stimulated with test substances was expressed as percentage of the maximal growth factor released by parallel cultures exposed to lipopolysaccharide (30 μg/ml) *in vitro*. Thymidine incorporation in response to lipopolysaccharide-treated macrophage supernatants was comparable to positive control values produced by addition of 10% fetal bovine serum to quiescent fibroblasts.

Results

To examine the relationship between lymphocytes (or their secretory products) and MDGF secretion following acute lung injury, we examined the temporal relationship between the appearance of lymphocytes in alveolar lavage fluid and the spontaneous release of MDGF from macrophages. Figure 1 shows the differential count of lavage cells during the 35 days following intratracheal administration of bleomycin sulfate. Lavage fluid from respiratory pathogen-free control animals consistently contained virtually all macrophages (>98%), with only rare granulocytes and lymphocytes. Following acute lung injury, the proportions of lymphocytes, polymorphonuclear leukocytes, and eosinophils in lavage fluid were elevated. All three cell types were increased in quantity within 1 day of lung injury. Subsequently, the population of lymphocytes continued to rise to a peak level of 23% (Day 7). Granulocytic cells rose briefly but returned to normal levels within 7–12 days. The number of total lung cells retrieved by lavage did not vary during the course of injury (10 ± 1 × 10^6), and therefore these percentages represent an absolute rise in the number of lymphocytes in the alveolar spaces. Cultures of alveolar macrophages retrieved from lungs of normal rats secreted only trace levels of MDGF, as assessed by tritiated thymidine uptake by confluent adult rat lung fibroblast cultures. In contrast, macrophages retrieved from injured lungs "spontaneously" secreted progressively increased amounts of MDGF (Figure 1). Maximal secretion occurred with cells retrieved by lavage 5–7 days after lung injury. This

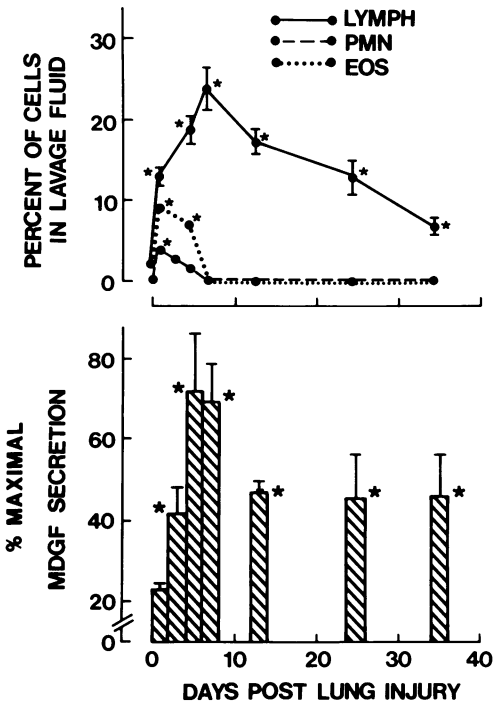

Figure 1—Differential cell count and "spontaneous" secretion of MDGF from adherent macrophages following acute lung injury. **Upper panel**—Although the total number of lavage cells retrieved from control and bleomycin-injured animals was constant (10 ± 1 × 10^6), the proportion of cell types varied with time after lung injury. The remainder of the cells collected by lavage were macrophages. Data are expressed as mean ± SEM. The number of rats sacrificed at a given time varied from 3 to 10. **Lower panel**—Growth factor secreted by cultured macrophages was monitored by fibroblast proliferation (see Materials and Methods). Data are expressed in relation to the (maximal) amount secreted by macrophages in response to lipopolysaccharide *in vitro*.

time course generally paralleled the proportion of lymphocytes found in lavage fluid.

Initial determination of the cellular source of growth factor activity was made by fractionating lavage cells harvested 5 days after lung injury into adherent and nonadherent populations. The adherent cells were 97% macrophages, as judged by light microscopy (viability 92% by trypan blue exclusion). Adherent macrophages spontaneously produced high levels of MDGF, as assayed by increased fibroblast tritiated thymidine incorporation (three to four times above control). However, supernatants of nonadherent cells (viability, 85%) consisting of a mixed population of cells (48% ± 2% lymphocytes, 19% ± 1% polymorphonuclear leukocytes, 18% ± 1% eosinophils and 14% ± 2% macrophages on Day 5) failed to elevate thymidine incorporation by fibroblasts consistently above control levels.

To determine whether lymphokines released by the newly recruited nonadherent alveolar cell population might modulate MDGF secretion, we tested secretory products of these cells for their ability to induce MDGF secretion by normal macrophages. We cultured the

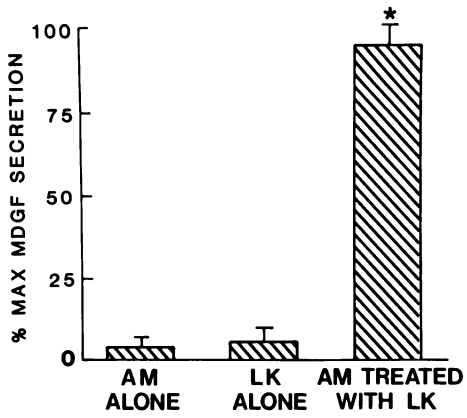

Figure 2—Induction of MDGF secretion by lymphokine. Conditioned media from crude, nonadherent alveolar lymphocyte cultures were tested at 1:100 dilution for their ability to stimulate secretion of MDGF by macrophages. Normal alveolar macrophages release little or no MDGF (*AM alone*) unless exposed *in vitro* to crude lymphokine (*AM treated with LK*). Crude lymphokine (*LK alone*) has no direct effect on fibroblast cultures at this dilution. The *bars* represent the mean ± SEM of triplicate samples. The *Asterisk* indicates $P < 0.05$, compared with control (fibroblast monolayers treated with fresh medium alone).

lymphocyte-rich fraction of nonadherent cells obtained from rats 7 days after bleomycin-induced lung injury. Dilutions of culture medium supernatants derived from these nonadherent cells were then applied to cultures of normal rat lung macrophages for 24 hours, and the macrophage culture medium was assayed for MDGF activity (Figure 2). Fibroblast proliferation occurred only in the presence of secretory products of macrophages which had been stimulated with nonadherent cell supernatants. The maximal level of MDGF release following lymphokine stimulation was comparable to levels induced by direct *in vitro* macrophage stimulation with bacterial lipopolysaccharide. To control for the possibility that nonadherent cell supernatants might directly stimulate fibroblast proliferation, we also added them directly to microwell cultures of confluent fibroblasts. Figure 2 shows that this supernatant fluid ("LK alone") had no such direct effect on fibroblasts. Moreover, supernatants of nonadherent cells were potent inducers of MDGF secretion by normal alveolar macrophages: half maximal response was elicited at dilution of 10^{-6} (Figure 3).

The crude nonadherent cell population contained variable numbers of lymphocytes, polymorphonuclear leukocytes, eosinophils, and macrophages at early time points (Figure 1). To further pinpoint the cellular source of the macrophage-stimulating activity, we passed the nonadherent cells collected at Day 5 through a Ficoll-Hypaque gradient to separate mononuclear from granulocytic cells. The fractionated cell populations were cultured for 24 hours, and their supernatants were tested for their ability to induce MDGF release by control macrophages. Supernatants from the resulting lymphocyte-rich fraction (77% ± 8% lymphocytes) were twice as effective on a per cell basis at inducing MDGF secretion as was the mixed population of crude nonadherent cells (Figure 4), which indicates that a lymphokine modulates MDGF release. The granulocyte-rich fraction, on the other hand, did not induce MDGF release from control macrophages.

To be sure that the fibroblast growth factor activity released by control macrophages following *in vitro* exposure to lymphokine was the same factor released from normal macrophages in response to lipopolysaccharide

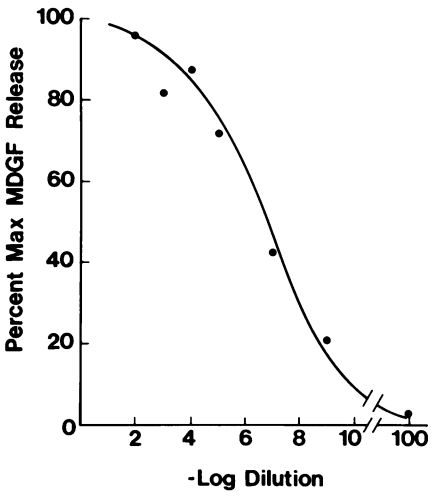

Figure 3—Macrophages respond to lymphokine in a dose-dependent manner. Normal alveolar macrophages were plated at 1.2×10^6 cells/ml in Neuman–Tytell medium and cultured in varying dilutions of lymphokine for 24 hours. Unconcentrated conditioned medium from these adherent macrophages was then assayed for MDGF activity. Data represent the mean ± SEM of triplicate determinations.

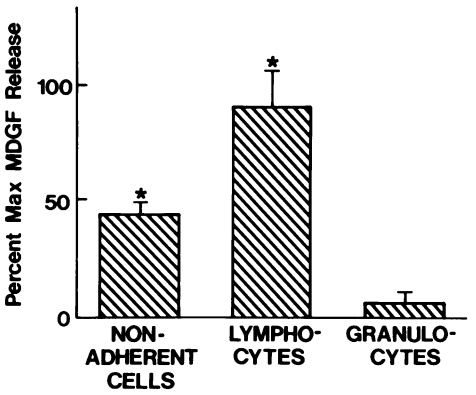

Figure 4—Cellular source of macrophage stimulating activity. Control macrophages were exposed to culture supernatants of total unfractionated nonadherent lavage cells (*bar on left*) or from nonadherent cells fractionated by Ficoll-Hypaque density gradient centrifugation into mononuclear cell and granulocyte fractions. Macrophage supernatant fluids were then collected and assayed for growth factor release. None of the nonadherent cell supernatant fluids were capable of causing fibroblast proliferation directly (data not shown). Data represent the mean ± SEM of quadruplicate wells. *Asterisks* indicate $P < 0.05$, compared with fibroblast monolayers treated with fresh medium alone.

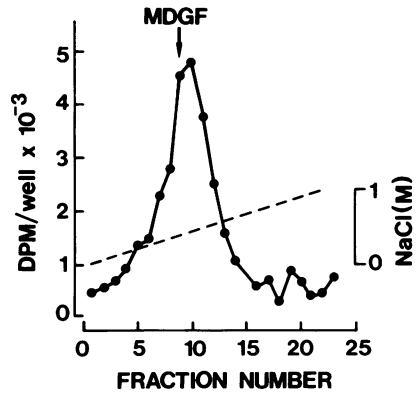

Figure 5—DEAE-Sephacel chromatography of growth factor secreted by control macrophages treated with lymphokine *in vitro*. Supernatants from macrophages treated *in vitro* with lympholine were pooled and chromatographed on DEAE-Sephacel as described in Materials and Methods. MDGF was eluted with the use of a linear salt gradient from 0 to 1 M NaCl (*dashed line*), and 100 μl aliquots of 2-ml fractions were tested for MDGF activity. The arrow indicates the elution peak of MDGF released by control macrophages stimulated *in vitro* with lipopolysaccharide, which is identical to that of MDGF released spontaneously by macrophages collected from bleomycin-exposed rats. Data represent the mean ± SEM of quadruplicate assays.

as well as the factor released spontaneously by alveolar macrophages collected from bleomycin-treated rats, we chromatographed lymphokine-stimulated macrophage supernatants on a DEAE-Sephacel column. The growth factor in lavage fluid was eluted at the same salt concentration as MDGF released by control macrophages stimulated *in vitro* with lipopolysaccharide and by macrophages from bleomycin-treated rats (Figure 5). The bulk of the protein content was eluted from the column before initiating the salt gradient (>94%).

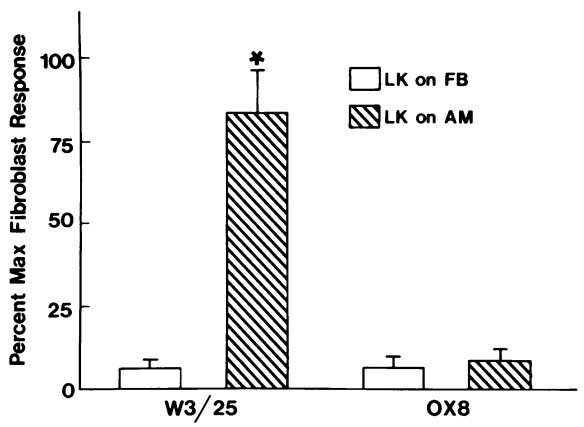

Figure 6—W3/25+ cells regulate MDGF secretion. Separation of lavage-derived lymphocytes into subsets of T lymphocytes, W3/25⁺ and OX8⁺, was made by adherence to monoclonal antibodies specific for cell surface antigens. The T-cell supernatants were tested at a dilution of 1:10 for their ability to stimulate MDGF secretion. Supernatants from W3/25⁺ and OX8⁺ cells had no direct effect on ^{3}H-Tdr incorporation by quiescent monolayers of fibroblasts (*open bars*). The level of MDGF secreted by W3/25⁺ stimulated macrophages was similar to the level released by control macrophages stimulated *in vitro* with lipopolysaccharide. Data represent the mean ± SEM of quadruplicate wells. The *asterisk* indicates $P < 0.05$, in comparison with fibroblast monolayers treated with supernatants from unstimulated control macrophages.

To further characterize the cell population responsible for elaborating lymphokine we used "panning," a technique which sorts cells by adherence to monoclonal antibodies prepared against cell surface antigens. Lymphocytes were separated into W3/25⁺ and OX8⁺ T-cell subsets. The cells were cultured as described in Materials and Methods, and supernatant fluids were collected after 24 hours. Figure 6 shows that only W3/25⁺ cells elaborated lymphokine capable of stimulating normal macrophages to release MDGF. The induction of MDGF secretion elicited by lymphokine produced by W3/25⁺ cells was similar to the maximal level of MDGF released by control macrophages treated with lipopolysaccharide *in vitro*. In contrast, supernatants from OX8⁺ cells failed to induce significant MDGF release.

Recombinant murine IFN-γ was tested for its ability to substitute for lymphocyte culture supernatant fluid in inducing MDGF secretion (Figure 7). IFN-γ (1 unit/ml) induced a sixfold increase in the release of MDGF, as compared with untreated control macrophages. The level of MDGF released by macrophages treated with IFN-γ was not different from the maximal amount secreted after stimulation with lipopolysaccharide *in vitro*. At this concentration, IFN-γ itself had no direct effect on fibroblast proliferation.

Discussion

Increases in the number of lymphocytes in lavage fluid have been reported in a variety of inflammatory pulmonary disorders, including sarcoidosis,[18] hypersensitivity pneumonitis,[19] and interstitial pulmonary fibrosis.[20,21] Similar changes have been reported in animal models of interstitial lung disease: Thrall and colleagues demonstrated a transient influx into the alveolar space

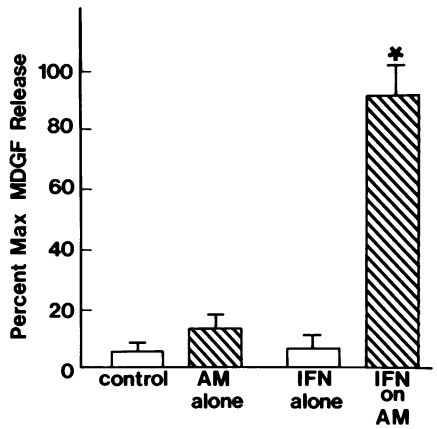

Figure 7—Effect of interferon on MDGF release. Recombinant murine interferon-gamma (*IFN*) substitutes for crude lymphokine in stimulating MDGF release when added directly to normal alveolar macrophages at a concentration of 1 U/ml. This concentration of interferon has no direct effect on fibroblast proliferation (*open bars*).

of both helper and suppressor T cells, as defined by W3/25 and OX8 monoclonal antibodies, with an excess of helper cells as compared with the proportions of these two cell types in blood.[7] The studies reported here describe a similar increase in the proportion of lymphocytes retrieved by lavage after lung injury in the rat. Our results show further that these infiltrating cells are capable of influencing the behavior of effector cells resident in the alveolus.

A number of lymphokines have been reported to affect macrophage function (for review, see Rosenstreich[14]). These include, among others, migration inhibition factor, macrophage activating factor, colony stimulating factor, macrophage cytotoxic factor, and the interferons. The changes in macrophage function observed after stimulation by these mediators include increased killing of tumor cells[22] and bacteria,[23] endocytosis,[9] production of hydrolytic enzymes,[24] and secretion of collagenase.[25]

The data shown here provide evidence for an additional regulatory role of macrophages by lymphocytes within the injured lung, by which T cells release a lymphokine, which controls the function of alveolar macrophages. Figure 2 demonstrates that nonadherent cell supernatant fluid can induce resting alveolar macrophages to secrete MDGF, an anionic peptide capable of inducing fibroblast proliferation. As with other functions stimulated by lymphokines, control macrophages respond to very low levels (10^{-6} dilution) of the material (Figure 3).

Parallel studies to probe the immunoregulatory potential of alveolar lymphocytes lavaged from the normal lung could not be carried out. The paucity of lymphocytes in pooled samples of lavage fluid from multiple control rats precluded such an approach. However, after lung injury, peripheral blood lymphocytes did not appear to secrete lymphokine that was capable of inducing MDGF (data not shown). This finding may mean that lymphocytes are both recruited to the air space and also sensitized to an undefined antigen during the early days following lung injury.

Studies of subsets of T lymphocytes separated by panning (Figure 6) indicate that W3/25-positive T cells release the lymphokine responsible for triggering the release of MDGF by normal alveolar macrophages. The absence of an effect on MDGF secretion of supernatants of OX8-positive cells is more difficult to interpret. OX8-positive cells are present in almost the same numbers as W3/25$^+$ cells after bleomycin-induced acute lung injury.[7] However, evidence suggests that they represent several functional populations: for instance, a portion of large granular lymphocytes which act as natural killer cells express OX8 surface antigen.[26]

To further confirm that specific lymphocyte secretory products are capable of stimulating MDGF secretion, we demonstrated that a purified lymphokine, recombinant murine IFN-γ, can substitute for the helper T-cell–derived lymphokine. This observation, in conjunction with studies of monoclonal antibody-purified T cells, confirms that lymphocytes alone among the nonadherent cell population are capable of stimulating MDGF secretion. In this regard, studies in other systems have shown that IFN-γ can substitute for lymphocyte-derived mediators.[11,13,27,28]

There is evidence that lymphocytes from a variety of sources (including peripheral blood and cloned cell lines) are capable of directly influencing fibroblast replication.[29] We also found that crude supernatants from unfractionated nonadherent lavage cells had a small direct effect on fibroblast growth. However, in our studies these small direct effects were probably due to residual nonadherent alveolar macrophages in the lymphocyte preparations. Thus, tenfold dilutions of these mixed cell supernatants had no effect on fibroblast proliferation. In contrast, lymphokines continued to induce near-maximal MDGF release by macrophages at dilution of 10^{-4} (Figure 4). These relationships suggest that the major effect of these secretory products of air space lymphocytes on fibroblast growth is mediated indirectly through the alveolar macrophage.

The relationship between the growth factor we have described and the growth factor secreted by human macrophages remains problematic. Studies of cultured lines of macrophagelike cells have yielded markedly different results from studies in freshly harvested alveolar macrophages. Like MDGF from rodent alveolar macrophages, the MDGF secreted by the U937-1 macrophagelike cell line acts synergistically with plasma-derived serum to allow density-arrested BALB/c3T3 cells to initiate DNA synthesis,[5] and it can therefore be defined as a competence factor.[30] A polypeptide proliferation factor is also released by human alveolar cells.[1] It, however, has been characterized as a progression factor with an action which is independent of plasma-derived serum but dependent on the presence of a competence factor such as platelet-derived growth factor in the medium.[1] The apparent discrepancies between the human studies cited above may rest more on methodologic considerations than on real biologic differences. MDGF from U-937-1 cells was tested in a classically defined competence factor assay[30] involving confluent mouse target cells. In contrast, Bitterman and colleagues[1] employed preconfluent human fetal fibroblasts held in low-serum medium in order to limit spontaneous proliferation. These culture conditions provide considerably more sensitive assay conditions but make direct comparison of MDGF as defined in other studies difficult.

In conclusion, our observations indicate that mesenchymal cell amplification during pulmonary inflammation and remodeling is a coordinate function of at least two immune effector cell populations acting in concert. The elaboration of lymphokines by helper T cells in the alveolar space provides a plausible explanation for the "spontaneous" MDGF secretion by alveolar macrophages which follows acute lung injury. Whether it is the dominant or sole determinant of MDGF release after lung injury remains to be determined.

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