

# INTERLEUKIN 4 INDUCES CULTURED MONOCYTES/MACROPHAGES TO FORM GIANT MULTINUCLEATED CELLS

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Giant multinucleated cells (GMCs)<sup>1</sup> were identified long ago as having a possible role in tumor growth (1), removal of foreign particulate matter (2), and sequestration of tubercle bacilli (3). Since that time many others have confirmed that GMCs are associated with granulomatous lesions formed in response to foreign bodies, viruses, and bacteria (4, 5). For example, the immune response to tubercle bacilli results in a classic granulomatous lesion (6), which presents as a large number of macrophages and GMCs surrounding an infectious focus with a layer of lymphocytes aggregating around the circumference. Because these lesions invariably show extensive focal recruitment of macrophages/monocytes, it was proposed that monocytes were the precursors of GMCs. These multinucleated forms are also called Langhans' giant cells, giant foreign body cells, and giant cells of inflammation.

Further studies indicated that GMCs were formed as a result of cell fusion rather than abnormal cell division (6), but the causative agent for this process was unknown. In 1973, Galindo (7) demonstrated that GMCs could develop in vitro from normal rabbit alveolar macrophages treated with supernatants of *Bacillus Calmette-Guerin* (BCG)-sensitized lymph node cells. These workers then postulated that a lymphokine, termed macrophage fusion factor (MFF), was released from sensitized T lymphocytes upon specific antigen stimulation (8). The organization of granulomatous lesions, with their mantle of lymphocytes, also implicates the involvement of T cell-derived lymphokines in vivo.

It is well known that activated T cells produce a diverse array of factors that affect various macrophage activities, in addition to the formation of GMCs (8). The T cell activities described include inhibition of the migration of macrophages (MIF) (9–11), aggregation of macrophages (MAagF) (12, 13), and activation of macrophages to efficiently kill tumor cells (MAF) (14). The relationship between MFF, MAF, MAagF, and MIF has not yet been defined. Recently, it was shown that the mouse T cell product, IL-4, induces tumoricidal activity in cultured macrophages (15) in a way similar to the activity previously described as MAF.

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<sup>1</sup> *Abbreviations used in this paper:* CSF, colony stimulating factor; G-CSF, granulocyte CSF; GMC, giant multinucleated cell; GM-CSF, granulocyte/macrophage CSF; MAF, macrophage activation factor; MAagF, macrophage aggregation factor; MIF, migration inhibition factor; M-CSF, macrophage CSF; MFF, macrophage fusion factor.

In the present communication, we attempt to further clarify the relationship between these various factors and IL-4 using bone marrow and alveolar cell cultures.

### Materials and Methods

**Cytokines and Antibodies.** Purified mouse rIL-3 (16), recombinant granulocyte colony stimulating factor (G-CSF), and recombinant macrophage colony stimulating factor (M-CSF) were provided by colleagues at DNAX Research Institute (Palo Alto, CA). Purified mouse rIL-4 (17) and recombinant granulocyte/macrophage colony stimulating factor (rGM-CSF) (18) were a gift of Schering-Plough Research (Bloomfield, NJ). 1 U of each factor is defined as that amount that stimulates half-maximal [<sup>3</sup>H]thymidine incorporation of an appropriate factor-dependent cell line cultured at  $5 \times 10^4$  cells/ml. The anti-IL-4 mAb 11B11 (19), was kindly provided by R. Coffman (DNAX Research Institute). 11B11 antibodies were used at 25  $\mu$ g/ml, a concentration that completely blocks the biological activity of 500 U/ml of IL-4. Anti-mouse IFN- $\gamma$  mAb, XMG-1 (20), was a gift of T. Mosmann (DNAX Research Institute). XMG-1 antibodies used at 20  $\mu$ g/ml abolish the activity of IFN- $\gamma$  at 78 ng/ml.

**Cell Culture.** Basic growth medium for all of our cell cultures consists of RPMI 1640 medium (M. A. Bioproducts, Walkersville, MD) supplemented with 50  $\mu$ M 2-ME and 5–10% FCS (Irvine Scientific, Santa Ana, CA). For soft agar cultures, varying numbers of cells were plated in 35-mm culture dishes (Falcon; Becton Dickinson & Co., Lincoln Park, NJ) containing 1 ml of basic medium and 0.3% (wt/vol) agar or basic agar medium further supplemented with various purified factors as indicated in individual experiments. For liquid cultures, cells from various organs were suspended at  $5 \times 10^5$ /ml in basic medium containing various combinations of growth factors as indicated. All cultures were incubated at 37°C in 5% CO<sub>2</sub>.

**Assay For The Formation of Multinucleated Cells in Bone Marrow and Peritoneal Cell Cultures.** Bone marrow cells were harvested from the femurs of adult CBA/J mice (The Jackson Laboratory, Bar Harbor, ME) as previously described (21). Cells were plated at  $10^5$ /ml in two-chamber tissue culture slides (Lab-Tek, Naperville, IL) in basic medium containing one of the hemopoietic growth factors with or without IL-4. Cultures were incubated at 37°C in 5% CO<sub>2</sub>. At various times, cultures were terminated and cytopins of the nonadherent cells, as well as the original Lab-Tek slides, were fixed and stained with Wright-Giemsa (Sigma Chemical Co., St. Louis, MO) for microscopic evaluation. Multinucleated cells were enumerated and reported as percent of adherent cells present in an average of 160 fields per slide when viewed at  $\times 400$  magnification. Multinucleated cells are defined as cells with three or more nuclei per cell.

Nonstimulated alveolar macrophages were washed from the lungs of mice using broncho-alveolar lavage (22). Briefly, a blunted 18-gauge needle was inserted into the trachea of a mouse and secured with sterile thread. The lungs were washed 6 times with 1 ml volumes of cold Hank's Balanced Salt Solution (HBSS) (Gibco Laboratories, Grand Island, NY). Cells were then plated at  $1 \times 10^5$  cells/ml and assayed for GMC formation as described for bone marrow.

**Histochemical Stains.** Using established histochemical methods and reagents from Sigma Chemical Co., cells adherent to Lab-Tek chamber slides were stained with nonspecific esterase, peroxidase, alkaline phosphatase, or acid phosphatase.

**Autoradiography.** Bone marrow cells were harvested and cultured overnight at  $5 \times 10^5$  cells/ml in basic medium supplemented with IL-3 (200 U/ml). Half the cells were then pulsed with 0.5  $\mu$ Ci/ml [<sup>3</sup>H]thymidine (Amersham Corp., Arlington Heights, IL) for 4 h. Labeled cells were then mixed with nonlabeled cells in various ratios and plated in tissue culture chamber slides at  $10^5$  cells/ml with or without 250 U/ml IL-4. On day 4 of culture, nonadherent cells were gently removed and slides were fixed in methanol for 5 min. Slides were then dipped in nuclear track emulsion (Kodak No. 165 4433) and kept in the dark at 4°C for 3 d. After warming to room temperature, slides were developed in the dark for 3 min in Kodak D-19 developer (No. 146 4593), rinsed briefly, fixed in

## 600 INTERLEUKIN 4 INDUCES FORMATION OF MULTINUCLEATED CELLS

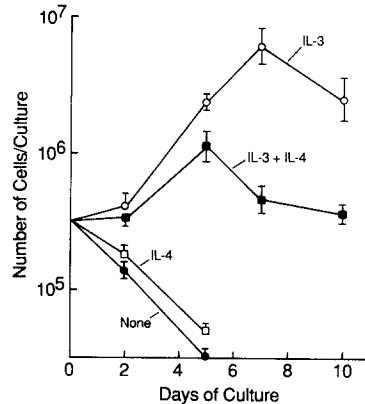


FIGURE 1. Effect of IL-4 on the viability and proliferation of bone marrow suspension cultures. Nonadherent bone marrow cells were suspended at  $5 \times 10^5/\text{ml}$  in basic medium (●) and in basic medium supplemented with IL-4 (200 U/ml) (□), IL-3 (200 U/ml) (○), or IL-3 plus IL-4 (■). At various time points, nonadherent cells were removed and the viable cells counted using trypan blue dye. The values reported are the mean  $\pm$  SD of triplicate cultures.

Kodak fixer (No. 197 1746) for 3 min and rinsed in distilled water before staining with Wright-Giemsa for microscopic examination.

### Results

*Bone Marrow Cells Stimulated with IL-4 in Liquid Suspension Cultures Show Inhibition of IL-3-dependent Growth and Formation of Giant Multinucleated Cells.* Nonadherent bone marrow cells were cultured in unsupplemented liquid medium or in medium containing IL-4, IL-3, and IL-4 plus IL-3. At various time intervals, cell viability, growth, and morphology were assessed. There was a rapid decrease in the number of viable nonadherent cells present in unsupplemented cultures and in IL-4-stimulated cultures (Fig. 1). The number of nonadherent cells increased 15-fold in cultures containing IL-3 but only 3-fold in cultures containing IL-3 plus IL-4. Further, the growth curves were different. Maximum cell numbers were reached by day 6 when stimulated with IL-3 and by day 4 when stimulated with IL-3 and IL-4. Wright-Giemsa staining indicated that ~80% of the cells present in cultures stimulated by IL-3 with or without IL-4 were granulocytes and macrophages at various stages of differentiation. The other 20% consisted of undifferentiated progenitor cells and mature and immature forms of red blood cells, eosinophils and mast cells. Although IL-4 inhibited the IL-3-dependent proliferation of hemopoietic cells, it did not inhibit cell differentiation. This result is consistent with the ability of IL-4 to inhibit IL-3-dependent colony formation in soft agar bone marrow cultures (23).

Next, the bottom of each culture dish was examined by phase microscopy for the generation of adherent cells. After 10 d of culture, there were a few scattered patches of adherent cells in the unsupplemented cultures and in cultures containing only IL-4. However, there was a relatively large adherent population present in cultures stimulated with IL-3 and IL-3 plus IL-4. The adherent layers appeared different in these two latter cultures. In the presence of IL-4, the adherent monolayer was approximately fourfold less dense and it contained many large cells with irregular borders. After staining with Wright-Giemsa, the adherent layer stimulated by IL-3 was found to consist of a uniform population of mononuclear cells typical of macrophages and fibroblasts (Fig. 2A). In contrast, the adherent layer present in cultures stimulated by IL-3 plus IL-4 was composed of both mononuclear and multinucleated cells (Fig. 2B). The multinucleated

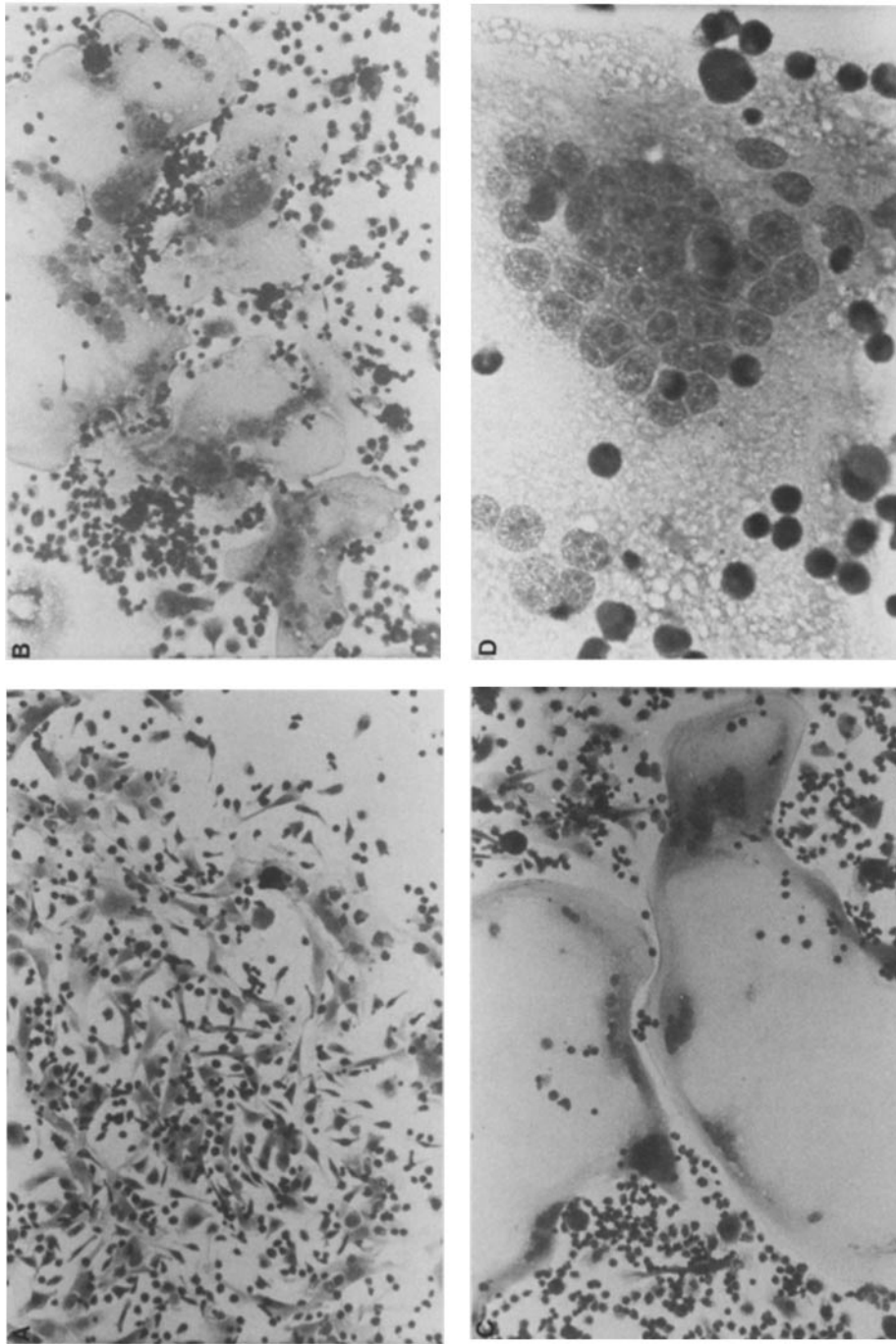


FIGURE 2. Photomicrographs of adherent bone marrow cells stained with Wright-Giemsa. Adherent cell monolayers formed in cultures supplemented with IL-3 in the absence (A) or presence (B-D) of IL-4. (B and C). The size variation of GMCs after 5 d of culture ( $\times 250$ ). The organization of a nuclei cluster is shown in D ( $\times 998$ ).

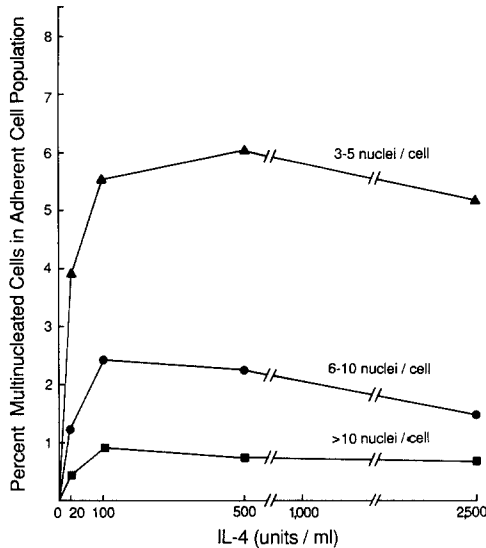


FIGURE 3. Dose-response curve for IL-4 induced multinucleated cell formation. Bone marrow cells were incubated in tissue culture chamber slides with IL-3-supplemented medium and varying concentrations of IL-4. On day 4, duplicate slides were stained with Wright-Giemsa. A total of 160 fields per slide (500–1,000 cells) were examined using a  $\times 40$  objective and a  $\times 10$  eyepiece. The values reported are the average number of multinucleated cells observed per 100 cells counted.

cells showed considerable variation with respect to size and number of nuclei per cell (Fig. 2, B–D). They usually formed in clusters and displayed a strong avidity for plastic since no GMCs were observed in cytopsin preparations of the non-adherent population.

The few patches of adherent cells present in cultures containing no growth factor or IL-4 alone were found to consist primarily of fibroblasts surrounded by several macrophages. In the IL-4-containing cultures, an occasional multinucleated cell was associated with these cell clusters. No multinucleated cells were found in unsupplemented cultures. Thus, it appears that IL-4 is necessary to induce the formation of multinucleated cells. However, the high frequency of GMCs found only in cultures supplemented with both IL-4 and IL-3 suggests that they derive from IL-3-dependent mononuclear cells.

*Quantitation of Multinucleated Cell Formation.* In the following studies, the optimal conditions for the formation of multinuclear cells were determined. Unless otherwise stated, these assays were performed with cell cultures supplemented with IL-3 at 200 U/ml to sustain the viability and proliferation of the target population. This amount of IL-3 was found to support maximum growth of bone marrow cells in liquid medium or soft agar cultures. Multinucleated cells were classified into three categories according to the number of nuclei per cell (i.e., 3–5, 6–10, and >10). We found that the frequency of multinucleated cells of all sizes was optimal using IL-4 at 100 U/ml or more, although a small response was seen at concentrations as low as 20 U/ml (Fig. 3). Control cultures failed to yield >0.8% multinucleated cells of the smallest category (3–5 nuclei/cell), and no giant multinucleated cells (>10 nuclei/cell) were documented.

The time course for the formation of multinucleated cells from bone marrow cultures optimally stimulated with IL-4 (250 U/ml) plus IL-3 (200 U/ml) is shown in Fig. 4. Multinucleated cells in all three categories were first noted on day 2 of culture and reached maximum numbers by day 5. Giant multinucleated cells

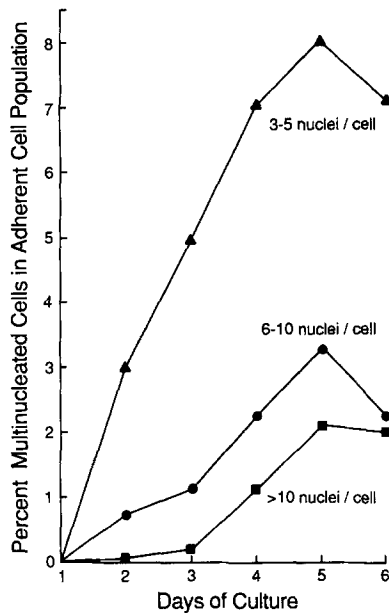


FIGURE 4. Time course of IL-4-induced multinucleated cell formation. Bone marrow cells were cultured in medium containing IL-3 (200 U/ml) and an optimal concentration of IL-4 (250 U/ml). Duplicate cultures were terminated at the time points indicated and stained slides were scored as described in Fig. 2 legend. The values reported are the average number of multinucleated cells observed per 100 cells counted.

with over 50 nuclei (Fig. 2C) were more frequent after prolonged incubation (4–5 d).

*Cell Fusion Is Required for the Formation of Giant Multinucleated Cells.* Other investigators (8, 24) have shown that the formation of multinucleated cells, after stimulation of cell cultures with lymphocyte conditioned medium, is dependent on cell fusion. Our observation that IL-4 has an inhibitory effect on the factor-dependent proliferation of cultured bone marrow cells (Fig. 1) raised the possibility that IL-4 may induce the formation of multinucleated cells by interfering with normal cell division. To discern between asynchronous cell division and cell fusion, we performed the following autoradiographic study. Bone marrow cells were incubated in IL-3 overnight to stimulate proliferation and increase the number of cells that would incorporate radiolabeled nucleotides. Nonadherent cells, which contained the majority of actively dividing cells, were harvested and divided into duplicate cultures. One culture was pulsed with 0.5  $\mu\text{Ci/ml}$  [ $^3\text{H}$ ]-thymidine for 4 h, which resulted in 32% of the cells incorporating label without loss of cell viability or growth. Aliquots of these cells were then mixed with the unlabeled cells in ratios of 3:1, 1:1, and 1:3. Cultures consisting entirely of radiolabeled cells or of various cell mixtures were incubated for 4 d in IL-3 plus IL-4, and then prepared for autoradiography. The highest number of radiolabeled nuclei per GMC was found in cultures consisting entirely of labeled cells. Furthermore, the number of radiolabeled nuclei per GMC consistently decreased as we decreased the ratio of labeled to unlabeled cells from 3:1 (Fig. 5A) to 1:3 (Fig. 5B). This finding is consistent with cell fusion rather than asynchronous division where the nuclei within a given cell would be all unlabeled or all labeled but with a lower specific activity. With asynchronous division, only the ratio of labeled to unlabeled GMCs, not the ratio of radiolabeled nuclei per cell, would change to reflect the portion of labeled precursors used to initiate the cultures.

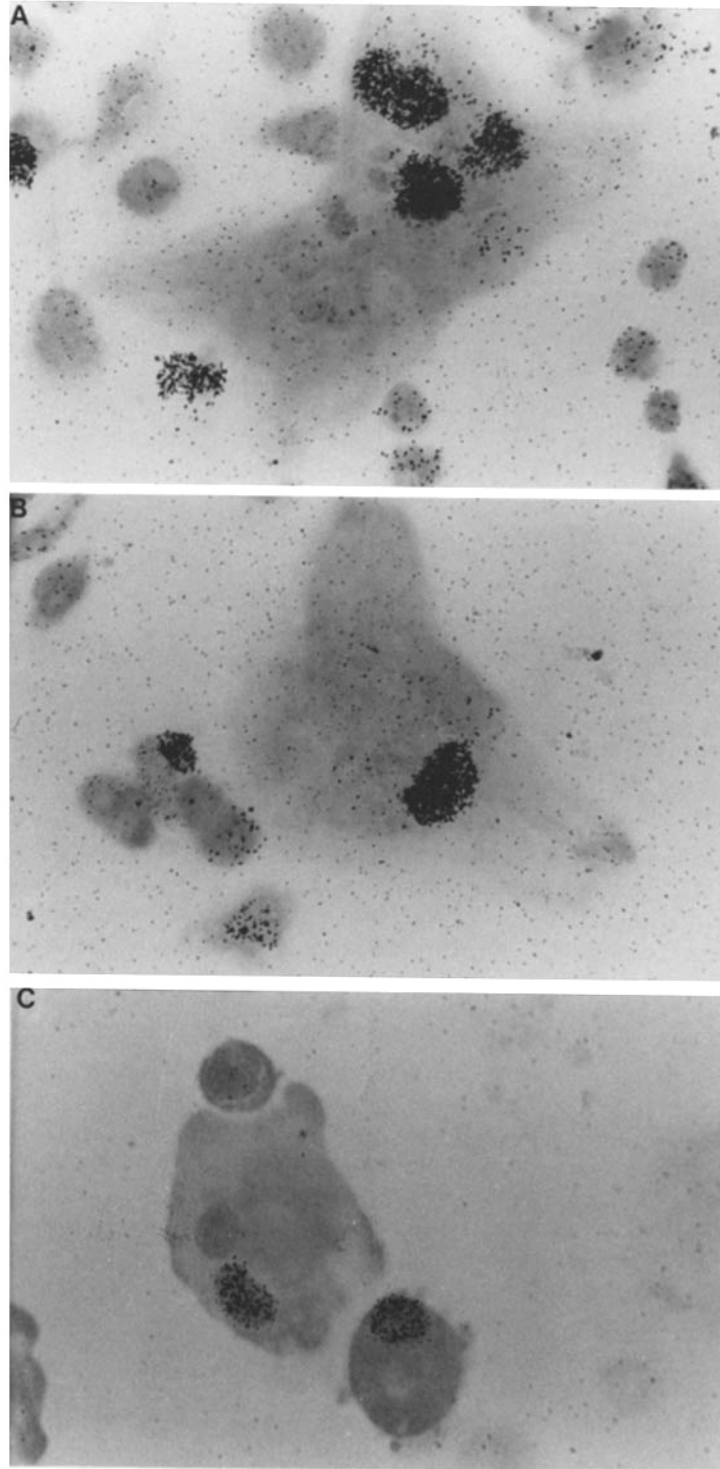


FIGURE 5. Photomicrographs of Wright-Giemsa-stained GMCs after autoradiography. Pictures show the frequency of radiolabeled nuclei per GMCs present in cultures prepared by mixing [ $^3\text{H}$ ]thymidine-labeled and nonlabeled bone marrow cells in a 3:1 ratio (A) and a 1:3 ratio (B). Radiolabeled nuclei are covered by silver grains. Unlabeled nuclei are evident by their weak staining with hematoxylin. Binucleated cell with one labeled and one unlabeled nuclei is shown in C (A, B, and C:  $\times 1088$ ).

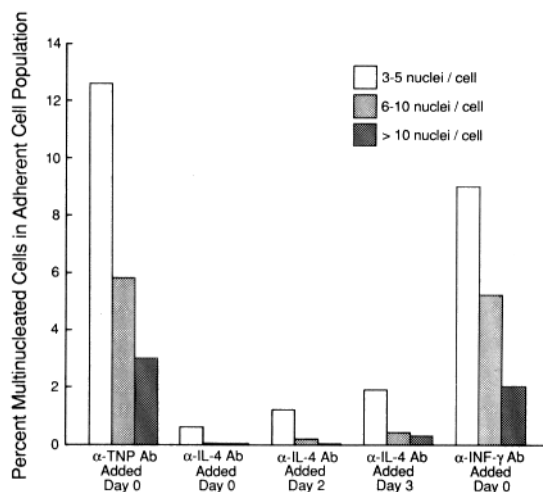


FIGURE 6. The formation of multinucleated cells is completely blocked by anti-IL-4 antibody but not by anti-IFN- $\gamma$  antibody. Bone marrow cells were cultured at  $10^5$  cells/ml in basic medium supplemented with IL-3 (200 U/ml) and IL-4 (200 U/ml). Anti-IL-4 antibody at 25  $\mu$ g/ml was added to IL-4 containing cultures on days 0, 2, and 3. An isotype matched anti-TNP antibody (25  $\mu$ g/ml) and an anti-IFN- $\gamma$  antibody (20  $\mu$ g/ml) were added to separate IL-4-treated cultures on day 0. Duplicate cultures were scored on day 5 and the values reported are the average number of multinucleated cells observed per 100 cells counted.

We also found a substantial number of binucleated cells with one heavily labeled and one unlabeled nucleus (Fig. 5C), an unlikely outcome of nuclear division.

*IL-4 Appears to Directly Mediate the Formation of Multinucleated Cells.* To determine whether the fusion of mononuclear cells was the result of a direct or an indirect effect of IL-4, anti-IL-4 antibodies were added to bone marrow cultures at various times after their initiation in medium containing IL-3 and IL-4. As expected, the formation of multinucleated cells was completely blocked by IL-4-specific antibodies added on day 0 of culture (Fig. 6). An isotype-matched anti-TNP antibody used at the same concentration had no effect. When anti-IL-4 antibodies were added on day 2 or 3, after multinucleated cells begin to appear, the total number of GMCs produced by day 5 was reduced to levels similar to that found on day 2 or 3 in kinetic experiments (Fig. 3). Moreover, there was a complete absence of cells with >10 nuclei, demonstrating that optimal formation of GMCs requires constant stimulation by IL-4. These results indicate that IL-4 directly mediates cell fusion rather than inducing the production of a fusion factor in the first 2–3 d of culture whose activity would be unaffected by the delayed addition of anti-IL-4 antibodies. This conclusion was further supported by the finding that IL-4-specific antibodies completely neutralized the ability of conditioned media from 5-d-old cultures containing GMCs to induce fusion of fresh bone marrow cells (data not shown).

It has been reported that IFN- $\gamma$  can induce the fusion of human peripheral blood monocytes (25). To verify that mouse IFN- $\gamma$  was not involved in the fusion of IL-4-treated cells, anti-IFN- $\gamma$  antibodies were added to bone marrow cells on day 0 of culture. We found that these antibodies had no significant effect on the development of GMCs in the presence of IL-4 (Fig. 6).

*Macrophages Appear to be the Precursors of IL-4-induced GMCs.* Previous work has suggested that macrophages give rise to GMCs (6, 7). Our results agree with these studies. First, GMCs were present in cultures stimulated with IL-4 in combination with M-CSF or GM-CSF but not with IL-4 plus G-CSF (data not shown). Similar to the results obtained with IL-3, none of these hemopoietic growth factors alone induced the formation of GMCs. Thus, IL-4 can elicit the



production of multinucleated cells but only when used as a costimulant with growth factors that efficiently sustain the growth and viability of macrophages. Second, all multinucleated cells were positive for nonspecific esterase and acid phosphatase but were negative for peroxidase and alkaline phosphatase, as is characteristic of macrophages. Finally, we assessed the effects of IL-4 on cells obtained by broncho-alveolar lavage. The majority of these cells (>90%) were macrophages as judged by morphological and staining characteristics. The generation of GMCs with time was evaluated using culture conditions described above. In the presence of IL-4, the kinetics of GMC formation was equivalent to that observed with heterogenous bone marrow cell cultures (data not shown).

*IL-4 Promotes Aggregation and Inhibits Migration of Bone Marrow Macrophages.* Bone marrow cells at  $5 \times 10^4$  were seeded into soft agar cultures containing M-CSF with and without IL-4. After 7–10 d of incubation, macrophage colonies stimulated by M-CSF had a smooth appearance with cells migrating into the surrounding agar (Fig. 7A). In contrast, cells within macrophage colonies stimulated by M-CSF plus IL-4 became clumped and failed to migrate (Fig. 7B).

### Discussion

A granuloma is a localized inflammatory immune response. While the role of the granuloma is to contain and eradicate foreign agents, the physiological relevance of giant multinucleated cells within this lesion is unclear. Early in the immune response, a large number of granulocytes and macrophages appear in the injured tissue. Apparently, most of these cells are recruited from the bone marrow (26, 27), although some cells are derived from the proliferation of local precursors (28). The number of lymphocytes detected in the area is much smaller and the mechanism by which they home to the specific site is unknown. Subsequent immobilization, maturation and activation of the inflammatory cells are essential to the efficient containment and elimination of foreign agents (29, 30). The duration of the inflammatory response may be prolonged as in cases of indigestible foreign bodies and infections with certain parasites or virulent intracellular organisms. GMCs are most often associated with these chronic granulomatous inflammations. In these cases, there is usually extensive tissue damage followed by fibrosis which may exaggerate the pathology of the disease (31, 32).

Using various animal models, *in vivo* and *in vitro* studies have suggested that the initiation, maintenance, and resolution of granulomatous inflammations are dependent on soluble T cell factors. It has been shown that sensitized T cells, as well as granulomatous tissue infiltrated with lymphocytes, produce factors that inhibit the migration and promote the agglutination of normal macrophages *in vitro* (13, 22, 29, 33). These activities would account for the compact appearance of granulomas. Furthermore, T cell-derived factors enhance the phagocytic, bactericidal, and tumoricidal properties of macrophages, activities (34–36), which have been linked to an effective granulomatous response. Another indication for T cell products is the finding that in some granulomas, antibodies are synthesized locally (29). It is envisioned that binding of immune complexes may further activate inflammatory cells with Fc receptors and aid in antigen clearance. Finally,

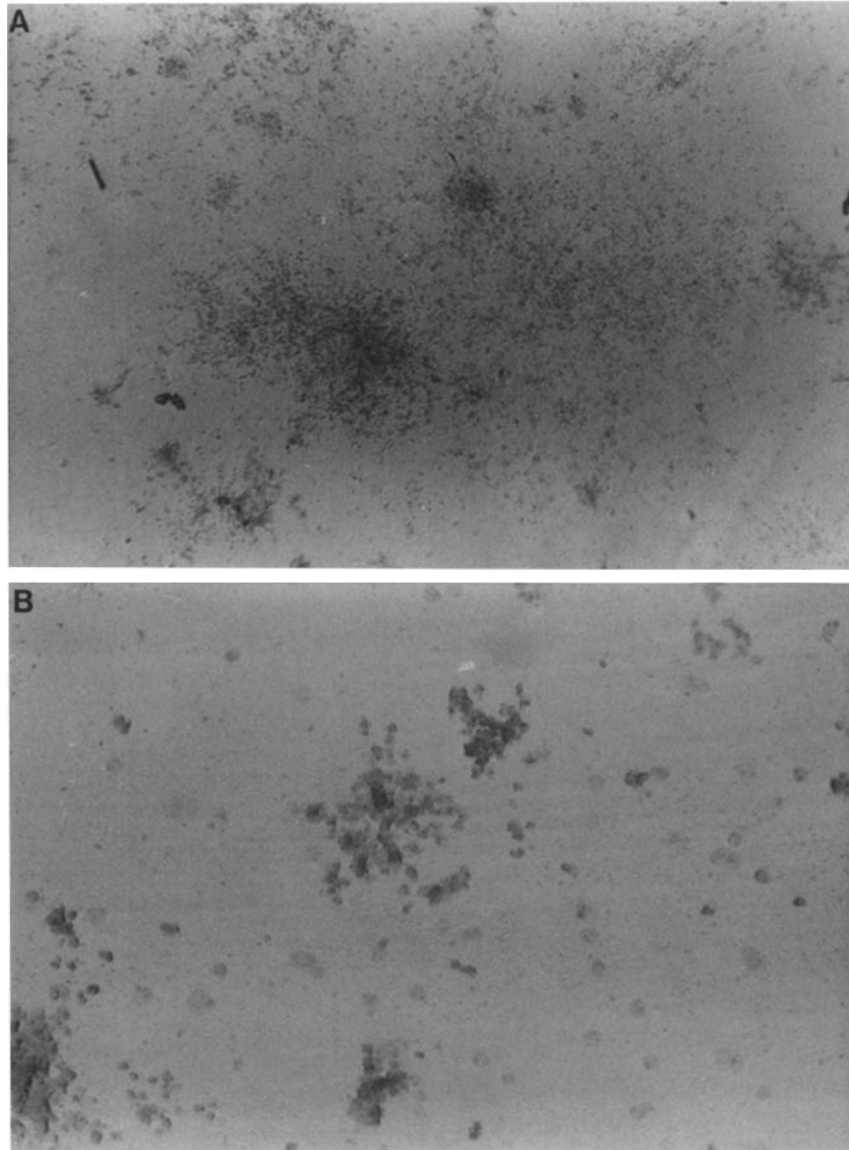


FIGURE 7. Photomicrographs of macrophage colonies formed in the presence and absence of IL-4. Bone marrow cells were cultured at  $10^5$  cells/ml in soft agar cultures supplemented with M-CSF (200 U/ml) without (A) and with (B) IL-4 (100 U/ml). Pictures show the general appearance of the colonies between 7 and 10 d of culture.

it has been shown that T cell derived factor(s) induce the fusion of macrophages to form giant multinucleated cells (8). Because multinucleated macrophages are not functionally superior to mononuclear cells (37-42), their proposed role as a last attempt to sequester persisting agents (38) seems likely but lacks formal documentation.

Evidence for the role of T cell-derived factors in the granulomatous response

has been largely by inference. At present, most lymphokines that mediate certain aspects of the response are chemically undefined. The data presented herein clearly demonstrate that purified rIL-4 directly stimulates multinucleated cell formation via the fusion of CSF-dependent bone marrow and alveolar macrophages. Our results also show that IL-4 is capable of inducing their aggregation and inhibiting their migration. These activities, previously ascribed to separate factors termed MFF, MAagF, and MIF, provide compelling evidence for IL-4 as one of the mediators of a classic granulomatous response. However, other lymphokines may mediate similar biological activities. In human studies, IFN- $\gamma$  has been shown to induce the fusion of blood monocytes (25) and exhibit MIF-like activity (43).

Because IL-4 mediates multiple biological activities, it may account for many of the cellular interactions that typify granulomas. Recently, it has been reported that IL-4 has MAF activity (15). Moreover, it not only functions as a potent costimulator of B cell proliferation and Ig production, but it also affects the factor-dependent growth of other cell lineages (44). In particular, the ability of IL-4 to enhance the growth of granulocyte progenitors in response to G-CSF (23, 45) and macrophage progenitors in response to M-CSF (23) may be relevant to the expansion of inflammatory cells within the granulomatous lesion. However, we have also shown that IL-4 may have antagonistic effects on the proliferation of more immature hemopoietic progenitor cells (23). Under certain assay conditions, IL-4 appears to directly or indirectly inhibit the stromal cell-dependent growth of early B lineage cells and the IL-3-dependent proliferation of early monopotent and multipotent myeloid progenitor cells. This raises the possibility that negative regulation by IL-4 may be instrumental in diminishing the inflammatory response by preventing excessive expansion of the progenitor cell pool, thus minimizing local tissue damage and fibrous repair (29). In this context, the potential use of IL-4 and its antagonists in the clinical management of disseminated granulomatous disease merits further study.

### Summary

Giant multinucleated cells (GMCs) are associated with granulomatous lesions that form in response to various infectious and noninfectious agents. The present study shows that mouse IL-4 induces the *in vitro* formation of GMCs by factor-dependent bone marrow and alveolar monocytes via cell fusion. GMCs appear 2 d after incubation of cell cultures with 20 U/ml or more of IL-4. Anti-IL-4 mAbs block the appearance of GMCs in these cultures, indicating that IL-4 acts directly on monocytes to promote fusion and does not secondarily induce the production of other soluble fusion factors. In soft agar cultures, IL-4 also causes the aggregation of macrophages and diminishes their migration. The role of IL-4 in a granulomatous inflammatory response is discussed.

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