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1α,25-Dihydroxyvitamin D_3 promotes fusion of mouse alveolar macrophages both by a direct mechanism and by a spleen cell-mediated indirect mechanism

(cell fusion/lymphokine/vitamin D_3/concanavalin A/phytohemagglutinin)

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ABSTRACT **Extensive fusion was induced in mouse alveolar macrophages by treatment with conditioned media obtained from spleen cell cultures treated with 15 μg of phytohemagglutinin or concanavalin A per ml or with 12 nM 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$]. The fusion rate was 80–90% on day 3. In addition, 1α,25$(OH)_2D_3$ added directly to alveolar macrophages induced fusion of about 35% of the cells on day 3, whereas direct addition of phytohemagglutinin and concanavalin A did not enhance fusion at all. When conditioned media from spleen cell or T cell cultures treated with 12 nM 1α,25$(OH)_2D_3$ were applied to a Sephadex G-100 column, a fusion factor (M_r 37,000–70,000) could be separated from 1α,25$(OH)_2D_3$. 1α,25$(OH)_2D_3$ induced fusion at 0.012–120 nM in a dose-dependent manner both by direct action and by spleen cell-mediated indirect action, but the fusion rate was always much greater in the latter than in the former at each concentration of the vitamin. Of the vitamin D_3 derivatives tested, 1α,25$(OH)_2D_3$ was the most potent, followed successively by 1α,24*R*,25-trihydroxyvitamin D_3, 1α-hydroxyvitamin D_3, 25-hydroxyvitamin D_3, and 24*R*,25-dihydroxyvitamin D_3. These results clearly indicate that 1α,25$(OH)_2D_3$ induces fusion of mouse alveolar macrophages by both a direct and an indirect mechanism, the latter mediated by spleen cells, probably by T cells.**

Vitamin D_3 is well known as an antirachitic agent that stimulates bone mineralization. In 1952, Carlsson demonstrated for the first time that vitamin D stimulates not only bone mineralization but also bone mineral mobilization (1). Vitamin D_3 is metabolized first in the liver to 25-hydroxyvitamin D_3 [25-$(OH)D_3$] and then in the kidney mainly to 24*R*,25-dihydroxyvitamin D_3 [24*R*,25$(OH)_2D_3$] and 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] (2). The latter metabolite is thought to be the active form of vitamin D_3 in enhancing bone resorption mediated by osteoclasts (3).

Recent evidence indicates that cells of the macrophage-monocyte series are possible precursors of osteoclasts (4, 5). Osteoclasts are defined as multinucleated giant cells with ruffled borders. The number of these giant cells responsible for bone resorption is known to be very small in the skeletal tissues of vitamin D-deficient animals (6). Administration of vitamin D_3 to rats fed a vitamin D-deficient, low calcium diet increases the number of mononuclear phagocytic and multinucleated giant cells in bone (6). These results suggest that vitamin D plays a critical role in the induction of osteoclasts.

Multinucleated giant cells are also observed in inflamed tissues (7, 8). In chronic inflammation, the release of lymphokines is likely to occur. Inflammatory conditions are known to lead to activation of macrophages. Multinucleated giant cells are thought to represent a specialized form of macrophages activated by lymphokines (9–11). In 1981, Sone *et al.* (12) reported that extensive fusion of rat alveolar macrophages was induced by *in vitro* treatment with cell-free supernatant fluids obtained from concanavalin A (Con A)-stimulated syngeneic lymphocytes. Here we report that the naturally occurring hormone, 1α,25$(OH)_2D_3$, induces fusion of mouse alveolar macrophages at a very high rate by a direct mechanism and also by an indirect mechanism, the latter mediated by spleen cells.

MATERIALS AND METHODS

Animals and Drugs. Six- to 8-wk-old male mice, ddY strain (Shizuoka Laboratory Animal Center, Shizuoka, Japan), were used for all experiments. Phytohemagglutinin (PHA) from kidney beans (type V) and Con A from jack beans (grade IV) were purchased from Sigma. 25$(OH)D_3$ was obtained from Philips-Duphar (Amsterdam). 1α,25$(OH)_2D_3$, 24*R*,25$(OH)_2D_3$, and 1α-hydroxyvitamin D_3 [1α$(OH)D_3$] were the gifts of I. Matsunaga (Chugai Pharmaceutical, Tokyo). 1α,24*R*,25-Trihydroxyvitamin D_3 [1α,24*R*,25$(OH)_3D_3$] was synthesized biologically by incubating kidney homogenates from vitamin D-deficient chickens with 24*R*,25$(OH)_2D_3$ (13). 1α,25$(OH)_2$[23,24-^{3}H]D_3 (specific radioactivity, 81 Ci/mmol; 1 Ci = 3.7×10^{10} Bq) was obtained from Radiochemical Centre (Amersham, England).

Culture Media. Eagle's minimal essential medium (EME medium, Nissui, Tokyo) was supplemented with sodium pyruvate, nonessential amino acids, and 2-fold concentrated vitamin solution [complete EME medium (CEME medium)], as reported (12). Human serum from healthy adult volunteers was heat-inactivated for 30 min at 56°C. Fetal calf serum was obtained from GIBCO. The concentration of 1α,25$(OH)_2D_3$ in human serum used was 0.12 nM.

Conditioned Media from Spleen Cells and T Cells. Spleens collected from mice aseptically were washed with Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (HBSS, pH 7.2), minced, and suspended in HBSS. The resulting suspension was filtered through gauze and centrifuged for 5 min at 1,200 rpm. Contaminating erythrocytes were eliminated from the cell pellet by adding 0.83% NH_4Cl in 10 mM Tris buffer (pH 7.4). The cells

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Abbreviations: PHA, phytohemagglutinin; Con A, concanavalin A; 1α,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; 1α,24*R*,25$(OH)_3D_3$, 1α,24*R*,25-trihydroxyvitamin D_3; 1α$(OH)D_3$, 1α-hydroxyvitamin D_3; 25$(OH)D_3$, 25-hydroxyvitamin D_3; 24*R*,25$(OH)_2D_3$, 24*R*,25-dihydroxyvitamin D_3; HBSS, Hanks' balanced salt solution; EME medium, Eagle's minimal essential medium; CEME medium, complete EME medium; MFF, macrophage fusion factor.

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were washed three times with HBSS and suspended in CEME medium containing 5% human serum or 10% fetal calf serum. T cells were separated from isolated spleen cells by the method of Julius *et al.* (14) by using nylon fibers and CEME medium containing 10% fetal calf serum. T cells were obtained from spleen cells in yields of approximately 20%. Spleen cells or T cells (5×10^6 per ml) were incubated for 1–3 days with PHA, Con A, or vitamin D_3 metabolites in CEME medium containing 5% human serum. After incubation, cell-free conditioned media were stored at −20°C until use.

Purification of Macrophage Fusion Factor (MFF) from Conditioned Media. Conditioned media (20 ml for spleen cell and 35 ml for T cell cultures) were concentrated by ultrafiltration in an Amicon chamber equipped with a UM 10 membrane (Amicon, Lexington, MA) and the insoluble materials were removed by centrifugation. Then, $1\alpha,25(OH)_2[^3H]D_3$ (12,000 cpm) was added to the concentrated media, and the media were applied to a Sephadex G-100 column (1.25 × 45 cm). The column was eluted with 0.14 M NaCl/15 mM glycylglycine buffer, pH 7.2. Protein was determined by the method of Bradford (15), and radioactivity was measured with a liquid scintillation counter (Packard model 3255). Molecular weights were estimated by comparison with protein markers (human serum albumin, M_r 66,000; ovalbumin, M_r 45,000; α-chymotrypsinogen, M_r 27,000; egg white lysozyme, M_r 14,000). Fusion rate was assayed in the presence of 10 or 20% eluates in CEME medium containing 5% human serum.

Fusion Assay of Alveolar Macrophages. Alveolar macrophages were obtained by the tracheobronchial lavage method described previously (12). Mice were anesthetized and exsanguinated by severing both renal arteries. The lungs were lavaged six times with 1.5 ml of HBSS prewarmed at 37°C. Lavaged cells were washed with HBSS and suspended in CEME medium containing 5% human serum at a concentration of 4×10^6 cells per ml. The cells obtained consisted mostly of macrophages; the remaining cells were erythrocytes. The suspended cells (3 μl) were plated in the center of wells and incubated for 30 min at 37°C under 5% CO_2 in air. The resulting monolayers were rinsed thoroughly with CEME medium to eliminate the contaminating erythrocytes. Then, conditioned media from the stimulated spleen cells or T cells or CEME media containing 5% human serum and either Con A, PHA, or vitamin D_3 metabolites were added to the monolayers of alveolar macrophages. After incubation, the wells were rinsed with HBSS and dried immediately. Alveolar macrophages were fixed and stained with May/Grünwald/Giemsa.

The percentage of fusion of alveolar macrophages was determined by counting the total number of nuclei within giant cells (3 or more nuclei per cell) to a total of at least 400 nuclei.

Fusion rate (%) of alveolar macrophages = (total number of nuclei within giant cells/total number of nuclei counted) ×100.

RESULTS

Fusion of Alveolar Macrophages by Conditioned Media Obtained from Mitogen- or $1\alpha,25(OH)_2D_3$-Stimulated Spleen Cells. Fusion of alveolar macrophages was dependent both on the period of stimulation of spleen cells by PHA, Con A, or $1\alpha,25(OH)_2D_3$ and on the period of incubation of alveolar macrophages with conditioned media obtained from stimulated spleen cell cultures. Fig. 1*A* shows the time course of fusion with conditioned media from the stimulated spleen cell cultures. In this experiment, spleen cells were incubated for 1–3 days with 15 μg of PHA or Con A per ml or with 12 nM $1\alpha,25(OH)_2D_3$ and the fusion rate was assayed on day 3. Preliminary experiments have proved that the concentrations of

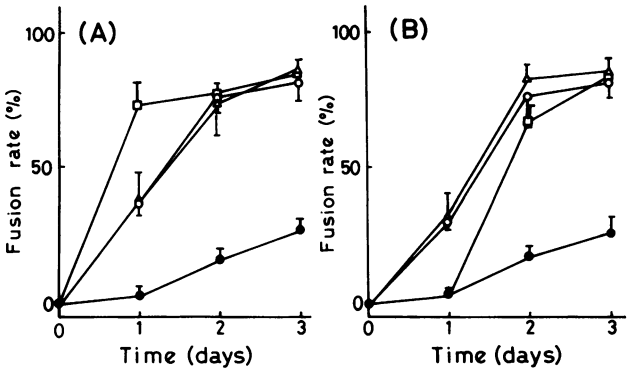

FIG. 1. Time course of change in fusion of alveolar macrophages induced by conditioned media obtained from spleen cell cultures treated with PHA, Con A, or $1\alpha,25(OH)_2D_3$. (*A*) Spleen cells were incubated with PHA, Con A, or $1\alpha,25(OH)_2D_3$ for 1–3 days, and the conditioned media obtained were added to the fusion assay system and incubated for 3 days. (*B*) Spleen cells were incubated with PHA, Con A, or $1\alpha,25(OH)_2D_3$ for 3 days, and the conditioned media obtained were added to the fusion assay system and incubated for 1–3 days. PHA (○), Con A (△), and $1\alpha,25(OH)_2D_3$ (□) were used at concentrations of 15 μg/ml, 15 μg/ml, and 12 nM, respectively. Conditioned medium for the control (●) was obtained from spleen cell cultures incubated with vehicle. Data are means ± SEM of three to eight experiments.

PHA and Con A used are optimal to obtain maximal responses. About 80% of the alveolar macrophages fused to form multinucleated giant cells, similarly with each of the three conditioned media from spleen cell cultures. Stimulation by $1\alpha,25(OH)_2D_3$ caused nearly the maximal fusion rate on day 1 and that by mitogens on day 2 (Fig. 1*A*). Conditioned media from spleen cell cultures treated with PHA or Con A for 3 days appeared to induce cell fusion earlier than those treated with $1\alpha,25(OH)_2D_3$, but by day 3 the maximal fusion rate was almost the same (Fig. 1*B*).

Fig. 2 shows the morphology of multinucleated giant cells formed by conditioned media from spleen cell cultures treated for 3 days with vehicle (*A*), 12 nM $1\alpha,25(OH)_2D_3$ (*B*), and 15 μg of PHA (*C*) or Con A (*D*) per ml. Both the mitogens and $1\alpha,25(OH)_2D_3$ induced large multinucleated cells (both foreign body type and Langhans type) to a similar extent, whereas conditioned medium without inducers increased cells with only a limited number of nuclei.

Direct Effect of Mitogens and $1\alpha,25(OH)_2D_3$ on Fusion. Conditioned media from stimulated spleen cell cultures contain both mitogens or $1\alpha,25(OH)_2D_3$ and a fusion factor produced by spleen cells. Thus, the question remains whether the promoting effect of conditioned media on fusion of alveolar macrophages is due to a direct action of PHA, Con A, and $1\alpha,25(OH)_2D_3$ or to an indirect action involving a fusion factor released from stimulated spleen cell cultures. Fig. 3 shows the time course of fusion when 15 μg of PHA or Con A per ml or 12 nM $1\alpha,25(OH)_2D_3$ was added directly to the fusion assay system of alveolar macrophages. Neither PHA nor Con A induced fusion. But $1\alpha,25(OH)_2D_3$ was the direct cause of about 35% of the alveolar macrophages forming multinucleated giant cells, though the maximal fusion rate was much lower than that induced by conditioned media from $1\alpha,25(OH)_2D_3$-stimulated spleen cell cultures.

Various derivatives of vitamin D_3 added directly to the fusion assay system had dose–response effects on the fusion of alveolar macrophages (Fig. 4). $1\alpha,25(OH)_2D_3$, at 0.12–120 nM, induced fusion in a dose-dependent manner. $1\alpha,24R,25(OH)_3D_3$ was 1/10th as active as $1\alpha,25(OH)_2D_3$. $1\alpha(OH)D_3$, $25(OH)D_3$, and $24R,25(OH)_2D_3$ exhibited similar effects but only at concen-

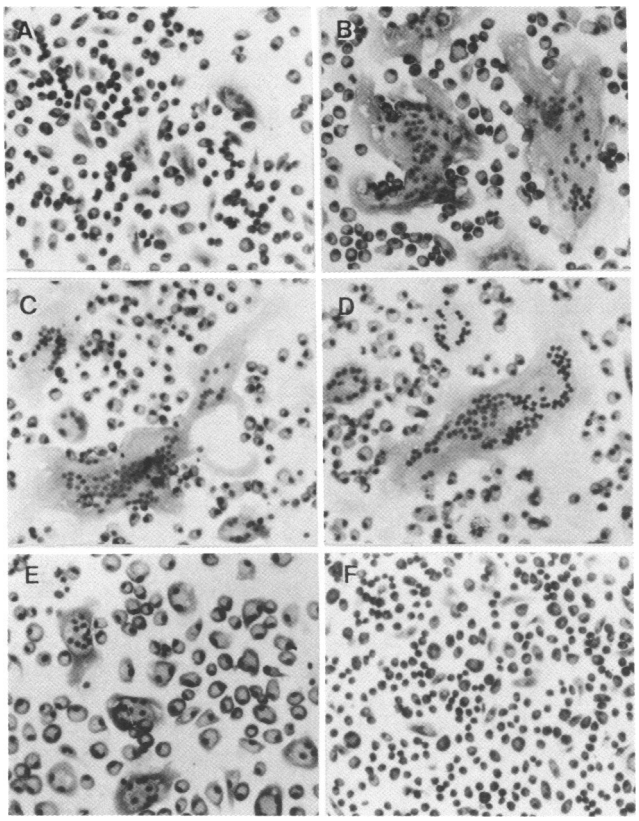

FIG. 2. Morphology of multinucleated giant cells formed from mouse alveolar macrophages. Macrophages were incubated with conditioned media treated for 3 days with vehicle (*A*), 12 nM $1\alpha,25(OH)_2D_3$ (*B*), and 15 μg of PHA (*C*) or Con A (*D*) per ml. Macrophages were also incubated directly with 12 nM $1\alpha,25(OH)_2D_3$ (*E*) or vehicle (*F*). In each assay, alveolar macrophages were incubated for 3 days. (May/Grünwald/Giemsa; ×100.)

trations 1,000-fold higher than $1\alpha,25(OH)_2D_3$.

Comparison of the Direct Effect of $1\alpha,25(OH)_2D_3$ and the Spleen Cell-Mediated Indirect Effect on Fusion of Alveolar Macrophages. Fig. 5 shows the dose–response effect on fusion of alveolar macrophages of conditioned media obtained from spleen cell cultures treated with various derivatives of vitamin D_3. Similar but more sensitive dose–response curves than those in Fig. 4 were obtained. As much as 80–90% of the alveolar macrophages fused to form multinucleated giant cells. Fig. 6 shows the difference between the direct effect and the spleen cell-mediated indirect effect of $1\alpha,25(OH)_2D_3$. Conditioned

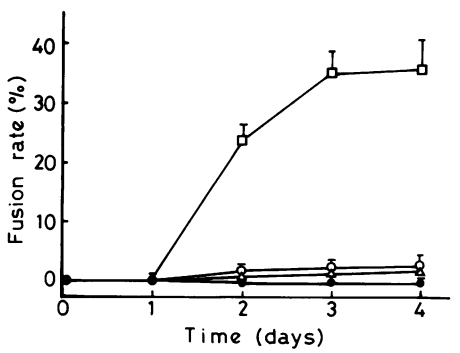

FIG. 3. Time course of change in fusion of macrophages induced by 15 μg of PHA or Con A per ml or 12 nM $1\alpha,25(OH)_2D_3$ added directly to the assay system. ○, PHA; △, Con A; □, $1\alpha,25(OH)_2D_3$; ●, control. Data are means ± SEM of three to nine experiments.

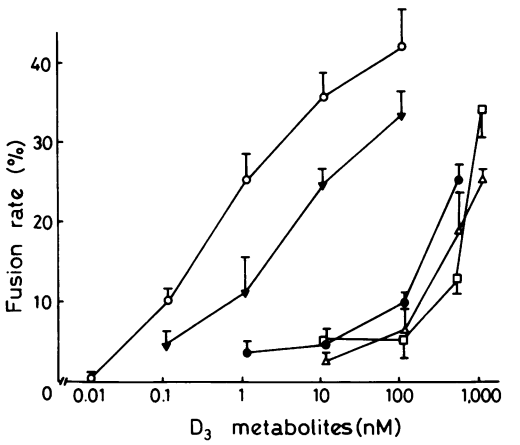

FIG. 4. Dose–response effect on multinucleated giant cell formation of vitamin D_3 metabolites directly added to the fusion assay system. Various concentrations of vitamin D_3 metabolites were added to the fusion assay system of alveolar macrophages and incubated for 3 days. ○, $1\alpha,25(OH)_2D_3$; ▼, $1\alpha,24R,25(OH)_3D_3$; ●, $1\alpha(OH)D_3$; △, $25(OH)D_3$; □, $24R,25(OH)_2D_3$. The control value was 2.6 ± 1.5%. Data are means ± SEM of three or four experiments.

media from spleen cells treated with the physiological plasma concentration (0.12 nM) of $1\alpha,25(OH)_2D_3$ caused as much as 75% of the alveolar macrophages to fuse, forming giant cells, whereas the same concentration of $1\alpha,25(OH)_2D_3$ directly added to the assay system induced fusion of only 10% of the macrophages. The spleen cell-mediated indirect effect of $1\alpha,25(OH)_2D_3$ was always greater than the direct effect of the vitamin at each concentration. The giant cells directly induced by $1\alpha,25(OH)_2D_3$ were relatively small and contained only a limited number of nuclei (Fig. 2*E*). Alveolar macrophages incubated for 3 days with vehicle did not fuse at all (Fig. 2*F*).

Purification of a Fusion Factor Obtained from Spleen Cells Treated with $1\alpha,25(OH)_2D_3$. To search for the identity of the fusion factor, mixed spleen cells and T cells isolated from the spleen cells were separately incubated with 12 nM $1\alpha,25(OH)_2D_3$ for 3 days. Each conditioned medium obtained was applied to a Sephadex G-100 column. Similar elution profiles were obtained in both the mixed spleen cell cultures and the T cell cul-

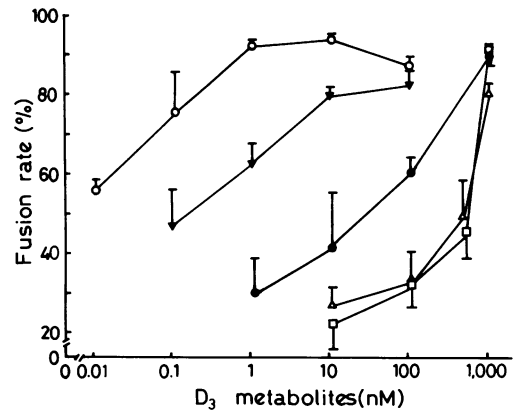

FIG. 5. Dose–response effect on multinucleated giant cell formation of conditioned media from spleen cells treated with various vitamin D_3 metabolites. Spleen cells were incubated with vitamin D_3 metabolites for 3 days. The conditioned media were added to the fusion assay system, and the fusion rate was assayed on day 3. ○, $1\alpha,25(OH)_2D_3$; ▼, $1\alpha,24R,25(OH)_3D_3$; ●, $1\alpha(OH)D_3$; △, $25(OH)D_3$; □, $24R,25(OH)_2D_3$. The control value was 22.5 ± 3.3%. Data are means ± SEM of three to seven experiments.

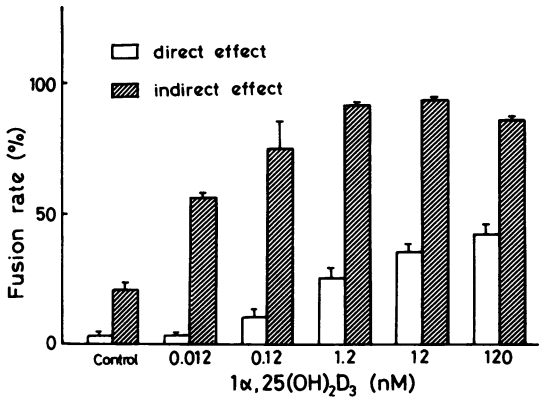

FIG. 6. Difference between the direct effect and the spleen cell-mediated indirect effect of $1\alpha,25(OH)_2D_3$ on multinucleated giant cell formation. The direct effect (□) was examined by adding graded concentrations of $1\alpha,25(OH)_2D_3$ to the fusion assay system. The indirect effect (▨) was examined by incubating alveolar macrophages with conditioned media from spleen cell cultures treated with various concentrations of $1\alpha,25(OH)_2D_3$ for 3 days. In each case, the fusion rate was assayed on day 3. Data are means ± SEM of three to six experiments.

tures (data not shown). In each case, a major peak with fusion activity was eluted at the M_r of 37,000–70,000, whereas $1\alpha,25(OH)_2[^3H]D_3$ added to the conditioned medium bound to vitamin D transport protein in serum and migrated to the same position as that of human albumin (M_r 66,000).

DISCUSSION

The present study clearly indicates that, like PHA and Con A, the naturally occurring hormone $1\alpha,25(OH)_2D_3$ induces a very high rate of fusion of mouse alveolar macrophages. However, the way $1\alpha,25(OH)_2D_3$ acts to induce cell fusion is different from the PHA and Con A action. $1\alpha,25(OH)_2D_3$ induced fusion both by a direct mechanism and by a spleen cell-mediated indirect mechanism, whereas PHA and Con A induced fusion by the indirect mechanism only (Figs. 1 and 3). The inducing potency of $1\alpha,25(OH)_2D_3$ added directly to the assay system of alveolar macrophages was relatively low: only 35% of the cells fused by 12 nM $1\alpha,25(OH)_2D_3$. But when conditioned media of spleen cells treated with $1\alpha,25(OH)_2D_3$ were added to the assay system, as little as 0.012 nM $1\alpha,25(OH)_2D_3$ significantly induced fusion of alveolar macrophages and as much as 80% of the macrophages were caused to fuse by vitamin at 1.2 nM (Fig. 5). Of the vitamin D_3 derivatives tested, $1\alpha,25(OH)_2D_3$ was the most potent, followed successively by $1\alpha,24R,25(OH)_3D_3$, $1\alpha(OH)D_3$, $25(OH)D_3$, and $24R,25(OH)_2D_3$ (Figs. 4 and 5). The order of the potency was very similar to the affinity of vitamin D_3 derivatives in binding to the specific cytosol receptors found in intestines (16) and human myeloid leukemia cells (HL-60) (17, 18). This suggests that $1\alpha,25(OH)_2D_3$ induces the production of a MFF by spleen cells and promotes fusion of mouse alveolar macrophages by a receptor-mediated mechanism.

It has been reported that PHA and Con A induce production of lymphokines such as the macrophage activating factor (19), the macrophage migration inhibitory factor (20, 21), and the MFF (12, 22). The spleen cell-mediated indirect action of $1\alpha,25(OH)_2D_3$ was much more potent than the direct action of the vitamin (Fig. 6), prompting us to examine the production of MFF by spleen cells treated with $1\alpha,25(OH)_2D_3$. The peak with fusion activity in alveolar macrophages was successfully separated from the $1\alpha,25(OH)_2D_3$ remaining in the conditioned media of spleen cells treated with the vitamin. The M_r of the MFF induced by $1\alpha,25(OH)_2D_3$ was estimated to be 37,000–70,000. A similar MFF with the same molecular weight was also found in the conditioned media of spleen cells treated with PHA or Con A (data not shown). Postlethwaite *et al.* (22) have also reported that PHA induced production of a MFF with similar molecular weight by human lymphocytes.

It has been postulated that multinucleated osteoclasts are formed by fusion of cells of the mononuclear macrophage-monocyte series (4, 5). We have reported that $1\alpha,25(OH)_2D_3$ is a potent natural modulator in inducing mouse myeloid leukemia cells (M1) to differentiate into macrophages (23). In addition, the present study provides strong evidence that the vitamin induces fusion of mouse alveolar macrophages both directly and by a spleen cell-mediated indirect mechanism. Dexamethasone, a potent stimulator of differentiation of M1 cells into macrophages (23, 24), did not in any way induce fusion of mouse alveolar macrophages at 0.1–1,000 nM. It has been reported that monocytes isolated from the peripheral blood (25) and bone marrow mononuclear cells (26, 27) fuse to form multinucleated giant cells that resorb bone. Preliminary experiments showed that $1\alpha,25(OH)_2D_3$ induces fusion of monocytes isolated from human peripheral blood (data not included). A lymphokine called osteoclast-activating factor has been isolated in the peripheral blood of patients with myeloma and lymphoma (28) and in PHA-stimulated human lymphocytes (29–31). It is not known whether the MFF isolated from the conditioned media of spleen cells treated with $1\alpha,25(OH)_2D_3$ is identical with osteoclast-activating factor. It also remains to be elucidated whether giant cells induced by the vitamin resorb bone.

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