Effect of Active Vitamin D₃ on Age-Related Immunological Changes

Yoshinobu NAKAO, Toshimitsu MATSUI, Tamio KOIZUMI, Toshitaro NAKAGAWA, Yuko KATAKAMI, and Takuo FUJITA

The Third Division, Department of Medicine, Kobe University
School of Medicine, Kobe 650, Japan

It is well known that immune functions of humans and experimental animals change with age. Diminished proliferation of lymphocytes, in response to stimulation with mitogens and alloantigens, impaired delayed type hypersensitivity reaction, and decreased activity of cytotoxic effector cells suggests an age-related decrease of T cell functions. Moreover, there is increasing evidence of several diseases being associated with age-related immunological changes, i.e. malignant neoplasms, chronic autoimmune diseases, decrease of resistance to infectious diseases. The immune system of mammals is a sophisticated, complex, multicellular system which has evolved as a mechanism for the defence of the host.

On the other hand, the immune cells differentiate from hematopoietic stem cells, and proliferate to maintain adequate populations for the specific immune functions. Of interest is evidence indicating the presence of common precursor cells between hematopoietic cells and bone cells. Therefore, it is important that both cell lineages are differentiated in the bone marrow cavity where microenvironmental homeostasis is essential for the multicellular cooperative functions. Thus, bone is important not only for the skeletal system but also for the major metabolic pool of calcium and phosphates. With aging, calcium metabolism and serum levels of calcium regulating hormones are changed. Increasing tendency of bone resorption becomes apparent with age.

Moreover, many investigations indicated that extra- and intra-calcium ions have an important role in the proliferation and functional differentiation of immune cells. Therefore, age-related changes of calcium metabolism might be in association with that of immune functions. To clarify these problems, we examined the effects of active vitamin D on age-related immunological changes and osteopenic processes.

Materials and Methods

T cell subsets in osteoporosis. The study was conducted on 32 subjects between the ages of 70 and 88 years, 16 males and 16 females, along with 7 young males and females 24–32 years of age, as controls. Lateral X-ray pictures of the dorsal and lumbar spine revealed one or more compression fractures in 13 (5 females and 8 males) of the 32 elderly subjects but not in 19 (11 females and 8 males) of these and young controls. Radial mineral content was measured with a Norland Cameron apparatus, and expressed as g/cm². Blood samples were obtained from the cubital vein in the morning on these fasting over-night, and cells were separated by centrifugation. After isolation of mononuclear cells on the Ficoll-Hypaque gradient, monoclonal antibodies (OKT4, OKT8) were used to stain the lymphocytes, using an immunofluorescent technique, and counted by flow cytometry (1). OKT4 to OKT8 ratio was calculated as a value for the helper-inducer versus suppressor-cytotoxic T cell proportional change. In 6 patients with compression fracture, 0.5 μg/day 1α,25-dihydroxyvitamin D₃ (1αOHD₃) was administered daily for 2 months and lymphocyte count and subsets were determined again by the same method. The results were statistically analyzed by Student’s t test.

Effects of active vitamin D on activated T cells. Human peripheral blood mononuclear cells were isolated from heparinized blood obtained from healthy adult volunteers by Ficoll-Hypaque density gradient centrifugation. The mononuclear cells (1 × 10⁶/ml) were activated with PHA-P (15 μg/ml,
Difco Laboratories, Detroit, Mich.) in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum (FCS), (Flow Laboratories, Rockville, Md.). After 48 h activation, the cells were washed twice with the medium and resuspended (5 × 10^6 cells/ml) in the medium with or without vitamin D analogues, i.e. 1,25(OH)\(_2\)D\(_3\), 1,24R(OH)\(_2\)D\(_3\), 1a(OH)D\(_3\), 25OH\(_2\)D\(_3\), 24,25(OH)\(_2\)D\(_3\) and 1,25(OH)\(_2\)D-26,23-lactone (kindly provided by Teijin Institute for Bio-Medical Research, Tokyo, Japan). After another 48 h incubation, cell surface antigens were analyzed using monoclonal antibodies (OKT3, OKT4, OKT8, OKT9, OKIa1; Ortho Diagnostics, Raritan, N.J., B1, Mo2; Coulter Electronics, Hialeah, FL, Leu7; Becton Dickinson Co., Mountain View, CA and monoclonal anti-Tac antibody was kindly provided by Dr. T. Uchiyama, Kyoto University) as described previously (2, 3). Briefly, antigens were detected by exposing the cells to primary antibodies for 30 min at 4°C, washing and then adding fluorescein isothiocyanate-conjugated goat anti-mouse IgG, F(ab')\(_2\) fragment specific (Cappel Laboratories, Cochranville, Pa.) for another 30 min. Quantitative fluorescence measurements were made in FACS model IV (Becton Dickinson Co.). The cell numbers were counted using a hematocytometer.

PHA-activated mononuclear cells were cultured with various concentrations of vitamin D analogues in the presence or absence of 20% interleukin 2 (IL 2; lectin and interferon-free IL 2, Electronics, Inc., Silver Spring, Md.) for 48 h. [\(^{3}H\)]Thymidine (6.7 Ci/mmol, New England Nuclear, Boston, Mass.) incorporation during the last 3 h of the 48 h culture were assayed as previously reported (2, 3).

**Induction of differentiation in HL-60 cells.**

Human myeloid leukemia cell line, HL-60 was kindly provided by Dr. R.C. Gallo (National Cancer Institute, Bethesda, Md.). The cells (1 × 10^6/ml) were seeded in 10 ml of the RPMI 1640 medium and cultured with different concentrations of vitamin D analogues, 12-O-tetradecanoyl phorbol-13-acetate (TPA) and retinoic acid (RA), dissolved in ethanol. The final concentration of ethanol was less than 0.1% and control culture medium containing 0.1% ethanol had no effect on HL-60 cells. Duplicate cultures were carried out for each of the 4 culture days. Different concentrations of agents were added to the culture medium.

Morphological assessment of the cells was made on cytopsin slide preparations stained with Wright-Giemsa solution. To measure the phagocytic capacity of HL-60 cells, the cells were incubated for 1 h with immunobeads coated with rabbit anti-human immunoglobulins (Bio-Rad Laboratories) at 37°C under 5% CO\(_2/95%\) air and the number of phagocytic cells among at least 500 viable cells was counted under a phase-contrast microscope (Nikon, Tokyo).

A nitroblue tetrazolium (NBT) reduction test was performed by incubating 2 × 10^6 cells/ml for 25 min at 37°C, with an equal volume of 0.2% NBT dissolved in phosphate-buffered saline (PBS), containing various inducers. The cells containing intracellular blue-black deposits were regarded as positive and were counted by phase-contrast microscopy (Nikon).

To evaluate the phenotypic differentiation of HL-60 cells quantitatively, cell surface antigenic changes were measured as reported previously (2, 3). Monoclonal anti-human monocyte antibodies, Mo2, OKM1, 63D3 (Bethesda Research Laboratory, Gaithersburg, Md.) and monoclonal anti-human transferrin receptor antibody (Ortho Diagnostic System) were used in a FACS system (2, 3). Results were expressed as the proportion in percentage of cells with a fluorescence intensity above that of control cells stained without monoclonal antibodies in the total population.

Effect of recombinant human \(\gamma\)-interferon (provided by Shionogi Pharmaceutical Co., Osaka, Japan) on growth of HL-60 cells and phenotypic differentiation with combination treatment of 1,25(OH)\(_2\)D\(_3\) was tested as follows. HL-60 cells (1 × 10^6 cells/ml) were cultured in the presence or absence of \(\gamma\)-interferon (1,000 U/ml) and 1,25(OH)\(_2\)D\(_3\) for 4 days (4).

For the study of calcium ion requirement for differentiation-induction of HL-60 cells, a calcium-free RPMI 1640 medium was specially prepared (Flow Laboratory). Calcium-free FCS was prepared by treatment with Chelex 100 (Bio-Rad), according to the procedure of Tupper and Zorgniotti (5). CaCl\(_2\) (400 mm stock solution) was added by the electrode method (SERA 250, Horiba Co., Kyoto, Japan) to determine their free Ca\(^{2+}\) content. Before the addition of low Ca\(^{2+}\) medium, the cells were washed with Ca\(^{2+}\) and Mg\(^{2+}\)-free
PBS. After 4 days culture, with or without differentiation-inducers, cell numbers and phenotypic changes were measured [6].

To evaluate the specific effect of calmodulin antagonists, i.e. W7, W13 (Wako Pure Chemical Industries, Osaka, Japan), on the induction of HL-60 cell differentiation with 1,25(OH)2D3, phagocytic activity and antigenic phenotype changes were assayed [7].

Intracellular free Ca2+ measurements. Cytosolic free Ca2+ levels were measured with a fluorescent indicator, quin 2-AM (Wako Pure Chemical Industries). Cells (2 × 10⁶ cells/ml) were suspended in RPMI 1640 medium containing 1 mM CaCl₂ and 20 mM HEPES and loaded by incubation with quin 2-AM, at a final concentration of 50 µM. After 20 min, 37°C incubation in this concentration, the cells were diluted ten-fold with a warm medium and then incubated for 60 min. After loading, the cells were washed with a medium containing 10% FCS and kept in the medium at room temperature, until used. Before use, a portion of the cells (4 × 10⁶ cells) was centrifuged in a microcentrifuge (Model 59, Fisher Scientific Co.) and resuspended in 2 ml of simplified saline containing 0.1 mM CaCl₂, 1 mM MgSO₄ and 20 mM HEPES and transferred to the cuvet. Fluorescence intensity was recorded with a spectrofluorometer (Model 650-60, Hitachi, Japan). The excitation and emission wave length were 339 nm with 4 nm slits, and 500 nm with 10 nm slits, respectively. Addition of Ca²⁺ was made from either 200 mM or 160 mM CaCl₂ stock solution. At the end of each experiment, the fluorescence signal was calibrated by equilibrating the internal quin 2-AM with a known concentration of Ca²⁺. The final medium contain 1 mM Ca²⁺. So Fmax was obtained after dye release with 100 µM digitonin (Sigma Chemical Co., St. Louis, Mo.). Fmin was determined by setting Ca²⁺ to 1 nm by adding 2 mM EGTA. The Ca²⁺ concentration, corresponding to fluorescence F emitted by trapped quin 2-AM, was calculated by the equation:

\[ [Ca^{2+}] = 115 \text{nm} \left( F - F_{\text{min}} \right) / \left( F_{\text{max}} - F \right) \] (8).

Results and Discussion

Among the age-related decreases in immune functions, relative impairment of T cell activities is the most predominant findings. By using monoclonal antibodies specific for T cell subsets, and the fluorescent-activated cell sorter system, the proportional changes of helper/inducer versus suppressor/cytotoxic T cell ratio (OKT4/OKT8) have been extensively studied. An increase of the OKT4/OKT8 ratio has been reported to occur in old age and other autoimmune diseases [9]. In our present study, the OKT4/OKT8 ratio was 2.04 in 13 patients with spinal osteoporosis and compression fracture, which is significantly higher than 1.4 in 19 control subjects of the same age group without compression fracture. The bone mineral content (BMC) was lower in those with osteoporosis than in non-osteoporotics. In addition, the administration of 0.5 μg/day 1αOHD₃ in 7 of

![Fig. 1. OKT4/OKT8 ratio in patients without and those with spinal compression fracture. The OKT4/OKT8 ratio was significantly higher in those with compression fracture (osteoporotics). The bone mineral content (BMC; g/cm²) was lower in osteoporotics than in non-osteoporotics, as expected. Mean values ±SE.](image_url)

![Fig. 2. OKT4/OKT8 ratio before and after oral administration of 0.5 μg/day 1αOHD₃ for 2 months in 7 osteoporotics. A significant decrease (p < 0.005) was noted. Mean values ±SE.](image_url)
the osteoporotics for 2 months caused a significant decrease in the OKT4/OKT8 ratio to the level of age-matched non-osteoporotic controls. In elderly persons, decrease of suppressor T cells is thought to be the natural course and the cause of the increased OKT4/OKT8 ratio. Thus, our data may indicate an accelerated age-related decrease of T cell functions, especially that of suppressor T cells. It is, however, undecided whether such changes are due to an increase of the helper T cells or decrease of the suppressor T cells in the case of osteoporosis, since prompt decrease of the elevated OKT4/OKT8 ratio in osteoporotics to the level of non-osteoporotics or young control subjects in response to administration of 1αOHD₃ remains to be explained. Alternatively, active vitamin D-sensitive subset of T cells could be more associated with the immunological background of osteoporotics. It is, thus, tempting to suggest that osteoclasts are under some kind of immune controls and abnormalities of T cells reflect themselves on osteoclast activity or bone resorption (10). Furthermore, in view of the recent report on the effect of solar exposure showing the probable decrease of the OKT4/OKT8 ratio (11), a pharmacologically immunomodulating effect of vitamin D derivative might be considered.

Therefore, we examined the direct effect of active vitamin D analogues on the each T cell subset. Since presence of specific receptor activity to vitamin D has been demonstrated in PHA-activated T cells, and not in resting T cells (12),

![Graph](image)

**Fig. 3.** Dose-response effect of 1,25(OH)₂D₃ on de novo DNA synthesis in PHA-activated T cells. (a) [³H]Thymidine incorporation; changes in cell surface antigens, (b) OKT4 and (c) OKT9-positive cells were analyzed in the presence (●) and absence (○) of IL 2. Results are presented as percentage of control cultures which contain 0.1% ethanol. Data are means ±SD of five donors.

![Graph](image)

**Fig. 4.** Regulation of T cell subsets by various vitamin D₃ analogues. ○, 1,25(OH)₂D₃; ●, 1,24R(OH)₂D₃; ×, 1,24S(OH)₂D₃; △, 24,25(OH)₂D₃; ▲, 25OHD₃; □, 1αOHD₃.
we studied the effects of active vitamin D analogues on mitogen, PHA-activated T cells (Figs. 3 and 4). The active vitamin D metabolite, 1,25(OH)₂D₃ suppressed the PHA-induced T cell proliferation dose-dependently (0.1–100 nM). The antiproliferative effect of 1,25(OH)₂D₃ on OKT4, OKT9-positive cells occurred over a physiological range, with a parallel change between cell number and [³H]thymidine incorporation. However, the addition did not decrease the percentage of OKT8-positive, Tac-positive (IL 2 receptor expressing), OKIa1 (HLA-DR framework antigen)-positive or Mo2 (monocyte antigen)-positive cells. Such effects persisted after the removal of monocytes by nylon column (13). When γ-interferon-free purified IL 2 was added along with 1,25(OH)₂D₃, the antiproliferative activity of the vitamin was abolished and the OKT4, OKT9-positive population increased to nearly the control level, suggesting that the action of 1,25(OH)₂D₃ might be mediated by a decrease of IL 2 secretion. Tsoukas et al. have recently reported the evidence that 1,25(OH)₂D₃ inhibited the secretion of IL 2 from mitogen activated human T cells (14). If 1,25(OH)₂D₃ suppressed T cell proliferation by inhibiting IL 2 production rather than by inhibiting the post-receptor responsiveness to IL 2, then the addition of IL 2 to 1,25(OH)₂D₃-treated lectin-stimulated T cells would be expected to overcome the 1,25(OH)₂D₃ suppression of the T cell proliferative response. In fact, we found that IL 2 replaced not only 1,25(OH)₂D₃-induced suppression of human lymphocyte proliferation, but also the inhibition of OKT4 and OKT9 expression (Fig. 3). The inhibitory effect of 1,25(OH)₂D₃ on transferrin receptor expression depends on the suppression of IL 2 production by 1,25(OH)₂D₃.

The antiproliferative effect of OKT4, OKT9-positive cells was shared by other vitamin D metabolites to some extent. 1,24R(OH)₂D₃ was almost as effective as 1,25(OH)₂D₃, but other metabolites such as 24,25(OH)₂D₃, 25(OH)D₃ were much less effective. The relative efficiencies in suppression were related to their relative potencies to differentiate HL-60 cells to monocytoid cells (3, 7), and affinities for the vitamin D receptors found in intestines (15). Our data indicates relatively higher sensitivity to active vitamin D is appreciable in OKT4-positive subset rather than in OKT-8-positive subset of T cells (13). Together with the following data indicating the effectiveness of active vitamin D on the proliferation of certain types of adult T cell leukemia cell lines, it is noteworthy. Since ATL cells are thought to be a malignant

![Diagram](image_url)

**Fig. 5.** Regulatory factors produced by activated T cell. CFU-s-SA, colony forming unit-s-stimulating activity; CSF, colony stimulating factor; PSF, P cell stimulating factor; ODF, osteoclast differentiation factor.
Fig. 6. Effect of 1,25(OH)2D3 on de novo DNA synthesis in T cell leukemia cells. Control; 1,25(OH)2D3, 10-8 M; 1,25(OH)2D3, 10-7 M.

Form of OKT4-positive nitrogen activated T cells (Tac+, HLA-DR+), it is of interest to confirm whether HTLV-transformed OKT4-positive T cells and mitogen-activated OKT4-positive T cells exhibit similar phenotypes or not. Activated T cells produce a variety of lymphokines (Fig. 5). T cell-derived bone resorbing factors could be some of them. This proposal is well supported by the evidence that ATL patients are complicated frequently with hypercalcemia and bone changes indicating increased bone resorption (16). Therefore, we examined the effects of active vitamin D on ATL cells. Antiproliferative effects of active vitamin D on certain cell lines of ATL was observed, whereas other T cell leukemia cell lines were not sensitive (Fig. 6). Among the ATL cell lines, HTLV-transformed cells are more sensitive than primary ATL leukemia cells. This means the HTLV-transformed cells might remain normal phenotypes of OKT4-positive mitogen-activated T cells. The effect of active vitamin D on the T cell-mediated bone resorption is currently under investigation in our laboratory.

Although the precursor of human osteoclasts have not been clearly demonstrated, the hematopoietic stem cells are thought to be the progenitor cells. Certain leukemia cells such as HL-60 and U937 have a potentiality to differentiate into monocytoid cells with osteoclast-like activity (17). Moreover, these cells are differentiated by lymphokines produced by T cells and ATL cells. Thus, it is important to elucidate the mechanism of differentiation in HL-60 cells with various inducers. As reported previously (3, 4), vitamin D metabolites induce the phenotypic differentiation of HL-60 cells, cultured in serum-free medium. Upon exposure to 10-10-10-7 M 1,25(OH)2D3, 1,24S(OH)2D3, 1,24R(OH)2D3, monocyte/granulocyte associated plasma membrane antigens of HL-60 cells detected by monoclonal antibodies, OKM1, 63D3 and Mo2, quantitated by FACS analysis, were increased dose-dependently (Fig. 7). Our data and others indicate that active vitamin D may act directly on the possible precursor of osteoclasts, as well as indirectly through the regulation of T cell-mediated bone resorbing mechanisms. In the microenvironment of bone marrow cavity,
many other cytokines are committed to cell differentiation. In addition, the osteoclast activating factor, osteoclast differentiation factor, macrophage fusion factor and interleukin 1 (IL 1) have been suggested as the bone-resorbing cytokines (19, 20). We demonstrated the important cooperative effect of γ-interferon on the differentiation induction in 1,25(OH)₂D₃-treated HL-60 cells (4). As shown in Fig. 8, treatment with γ-interferon (1,000 U) increased monocyte-associated cell surface antigens detected by monocyte-specific monoclonal antibodies in a dose-dependently manner. These antigenic changes were accompanied by a functional differentiation, determined by the increase of phagocytic capability and superoxide generation (3). The result is additional evidence

![Fig. 8](image_url)
Fig. 9. Effect of changing extracellular Ca\(^{2+}\) concentration on cytoplasmic free Ca\(^{2+}\). A typical recording of quin 2-AM fluorescence of HL-60 cells (upper panel) and the calculated concentrations of cytoplasmic free Ca\(^{2+}\). Data are expressed as means ± SD (n=9).

of T cell commitment of differentiation-induction in monocyte/osteoclast lineage.

Like other steroid hormones, precise mechanisms of vitamin D action to hematopoietic cells are not clearly elucidated. Even in the induction of cellular differentiation, heterogeneous pathways of action are proposed. In relation to the calcium metabolism, we examined the effect of deprivation in extracellular culture medium on the 1,25-(OH)\(_2\)D\(_3\)-induced phenotypic differentiation of HL-60 cells (6). 1,25(OH)\(_2\)D\(_3\) and retinoic acid-induced differentiation did not show Ca\(^{2+}\)-dependency, whereas TPA failed to induce the differentiation in 0.1 mM Ca\(^{2+}\)-containing medium (Table 1). Thus, the concentration of intracellular free Ca\(^{2+}\) was measured in the HL-60 cells by the quin 2-AM fluorescence method (Fig. 9). The increase in extracellular Ca\(^{2+}\) concentration from 0.1 to 0.1 mM caused an apparent increase in cytosolic free Ca\(^{2+}\). Furthermore, the relative increase from 0.1 to 0.6 mM Ca\(^{2+}\) was much greater than from 0.6 to 1.0 mM Ca\(^{2+}\). Thus, calcium dependency seems to be more specific for the mechanism of TPA-induced HL-60 cell differentiation. Concerning these data, we should recall the studies by Nishizuka and his colleagues (21). They proposed that there are two synergistic pathways of the calcium messenger system. One is modulated by Ca\(^{2+}\)-mobilization (i.e. calmodulin system), and the other is mediated by the activation of C-kinase. It is, therefore, of interest to know whether or not the mechanism of differentiation-induction is similar to tumor promotion. The difference in Ca\(^{2+}\)-requirement between TPA- and 1,25(OH)\(_2\)D\(_3\)-induced differentiation may be reflected in the presence of different mechanisms in phenotypic differentiation of HL-60 cells. Moreover, the inhibitor of calmodulin W7 and W13 did not have a significant effect on 1,25(OH)\(_2\)D\(_3\)-induced HL-60 cell differentiation (7) (Fig. 10).

Together with these data, one of the most critical differences between TPA-induced and 1,25(OH)\(_2\)D\(_3\)-induced differentiation could be the commitment of C-kinase. Though further investigations are underway, tentatively we may suggest heterogeneous pathways of vitamin D action in the induction of cell differentiation (6).

Although osteoporosis is a heterogeneous group of diseases, hematopoietic cells, especially T cell- and monocyte-lineage have very important roles in the regulation of bone metabolism and the control of Ca\(^{2+}\) and phosphate homeostasis in our body. These hematopoietic cells are differentiated from stem cells, under the regulatory effects of various hormones and cytokines. Among them active metabolites of vitamin D and T cell lymphokines have very important biological activities on the cell differentiation and bone resorption. These activities could be related to aging. What
is aging? Terminal differentiation of cells could be related to senescence of the entire body. It is, thus, worthwhile to examine the precise mechanisms of cell differentiation and proliferation, where calcium metabolism could be a critical factor (22). Our works, as well as those by others, are a part of the initial steps in elucidating age-related immunological changes and the associated diseases. Age-related osteopenia may be more than a metabolic disorder, or an immune-endocrine crossover disease. Furthermore, the microenvironment of the bone marrow cavity is a treasure space for the study on calcium metabolism, immune functions and aging.

REFERENCES

1) Nakao, Y., Matsuda, S., Fujita, T., Watanabe, S., Morikawa, S., Saida, T., and Ito, Y. (1980): Cancer Res., 42, 3843-3850.
2) Nakao, Y., Matsuda, S., Kimoto, H., Matsui, T., Kobayashi, N., Kishihara, M., Fujita, T., Watanabe, S., Ueda, K., and Ito, Y. (1982): Int. J. Cancer, 30, 687-695.
3) Matsui, T., Nakao, Y., Kobayashi, N., Kishihara, M., Ishizuka, S., Watanabe, S., and Fujita, T. (1984): Int. J. Cancer, 33, 193-202.
4) Matsui, T., Takahashi, R., Mihara, K., Nakagawa, T., Koizumi, T., Nakao, Y., Sugiyama, T., and Fujita, T. (1985): Cancer Res., 4366-4377.
5) Tupper, J.T., and Zorgniotti, F. (1977): J. Cell Biol., 75, 12-22.
6) Matsui, T., Nakao, Y., Nakagawa, T., Koizumi, T., and Fujita, T. (1986): Cancer Res., 46(2), in press.
7) Matsui, T., Nakao, Y., Kobayashi, N., Koizumi, T., Nakagawa, T., Kishihara, M., and Fujita, T. (1985): Cancer Res. 45, 311-316.
8) Tsien, R.Y., Pozzan, T., and Rink, J.T. (1982): J. Cell Biol., 94, 325-334.
9) Back, M.A., and Back, J.F. (1981): Int. J. Immunopharmacol., 3, 269-273.
10) Fujita, T., Matsui, T., Nakao, Y., and Watanabe, S. (1984): Mineral Electrolyte Metab., 10, 375-378.
11) Hersey, P., Bradley, M., Hasic, E., Haran, G., Edwards, A., and McCarthy, W.U. (1983): Lancet, 1, 545-547.
12) Provvedini, D.M., Tsoukas, C.P., Deftsos, L.J., and Manologas, S.L. (1983): Science, 221, 1181-1183.
13) Matsui, Y., Nakao, Y., Koizumi, T., Nakagawa, T., and Fujita, T. (1985): Life Sci., 37, 95-101.
14) Tsoukas, C.D., Provvedini, D.M., and Manologas, S.C. (1984): Science, 224, 1438-1440.
15) Neckers, L.M., and Cossman, J. (1983): Proc. Natl. Acad. Sci. U.S.A., 80, 3494-3498.
16) Nakao, Y., Maeda, S., Matsuda, S., Takubo, T., Masaoka, T., Shiozawa, S., Sugiyama, T., Ito, Y., Sarin, P.S., and Gallo, R.C. (1984): Cancer, 54, 259-265.
17) Wiktor-Jedrzejczak, W., Skelly, R.W., and Ahmed, A. (1981): in Immunologic Defect in Laboratory Animals, Vol. 1, ed. by Gershwin, M.F., and Marchant, B., Plenum Press, New York, p. 51.
18) Bar-Shavit, Z., Kahn, A.J., Reitsman, P., Trial, J., Astrin, S., Rothenberg, P., and Teitelbaum, S.L. (1984): in Endocrine Control of Bone and Calcium Metabolism, ed. by Cohn, D.V., Fujita, T., Potts, T., Jr., and Talmage, R.V., Excerpta Media, Amsterdam, pp. 384-387.
19) Suda, T., Abe, E., Miyaura, C., Tanaka, H., Shima, Y., Kunibayashi, T., Honma, Y., Hozumi, M., Momoi, T., and Nishi, Y. (1984): in Endocrine Control of Bone and Calcium Metabolism, ed. by Cohn, D.V., Fujita, T., Potts, T., and Talmage, R.V., Excerpta Medica, Amsterdam, pp. 308-315.
20) Oppenheim, J.J., and Gery, I. (1982): Immunol. Today, 3, 113-119.
21) Nishizuka, Y., Takai, Y., Kishimoto, A., Kikkawa, U., and Kaibuchi, K. (1984): Recent Prog. Hormone Res., 40, 301-341.
22) Kimoto, H., Nakao, Y., Kobayashi, N., Baba, Y., Sobue, K., Kakiuchi, S., and Fujita, T. (1983): Biochim. Biophys. Acta (Mol. Cell Biol.), 762, 24-30.