

## ISOLATION AND CHARACTERIZATION OF CLONES OF A HUMAN LEUKEMIA CELL LINE (HL-60 CELLS) RESISTANT TO $1\alpha,25$ -DIHYDROXYVITAMIN $D_3$ AND DIMETHYL SULFOXIDE

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### ABSTRACT

We have previously demonstrated that HL-60 cells pretreated with  $1\alpha,25$ -dihydroxyvitamin  $D_3$  at the stage of commitment were induced to differentiate into macrophage-like cells by dimethyl sulfoxide (DMSO) at the stage of promotion (15). To investigate the effect of DMSO on the differentiation of HL-60 cells induced by  $1\alpha,25$ -dihydroxyvitamin  $D_3$ , three variant clones (DRHL-1, DRHL-2 and DRHL-3 cells) resistant to  $1\alpha,25$ -dihydroxyvitamin  $D_3$  were isolated from a DMSO-resistant cell clone (DRHL) of HL-60 cells. These variant clones were unsusceptible to  $1\alpha,25$ -dihydroxyvitamin  $D_3$  or DMSO, when based on the criteria of cell growth, expression of antigens for human monocyte specific (OKM5) or human granulocyte specific (80H5) monoclonal antibodies, NBT-reducing activity, phagocytosis, nonspecific esterase activity, and cathepsin B and L activities. The variant clones, DRHL-1 and DRHL-3, which were unsusceptible to  $1\alpha,25$ -dihydroxyvitamin  $D_3$  or DMSO alone, were induced to differentiate into macrophage-like cells on simultaneous treatment with  $1\alpha,25$ -dihydroxyvitamin  $D_3$  and DMSO. Furthermore, DRHL-1 but not DRHL-3 differentiated into macrophage-like cells on 1-day treatment with  $1\alpha,25$ -dihydroxyvitamin  $D_3$  followed by 3-day treatment with DMSO. The other variant, DRHL-2, did not differentiate with any combination of  $1\alpha,25$ -dihydroxyvitamin  $D_3$  and DMSO. These results suggest that DMSO promotes the differentiation of HL-60 cells initiated by  $1\alpha,25$ -dihydroxyvitamin  $D_3$  into macrophages at various stages of differentiation.

A human promyelocytic leukemia cell line (HL-60 cells) has been demonstrated to be suitable for studying the chemically induced differentiation of myeloid cells *in vitro* (5). HL-60 cells are developmentally bipotential, *i.e.*, capable of differentiating into mature

granulocyte-like cells with dimethyl sulfoxide (DMSO) (6) or with retinoic acid (3, 9), and into monocyte/macrophage like-cells with TPA (19, 20).

$1\alpha,25$ -Dihydroxyvitamin  $D_3$  has been demonstrated to induce the differentiation of HL-60 cells into a monocyte/macrophage lineage by Suda and other workers (1, 11, 14, 22). HL-60 cells were found to contain cytosolic receptors for  $1\alpha,25$ -dihydroxyvitamin  $D_3$  (11, 22)

**Abbreviations:** NBT, nitroblue tetrazolium; DMSO, dimethyl sulfoxide; TPA, 12-*O*-tetradecanoyl phorbol 13-acetate

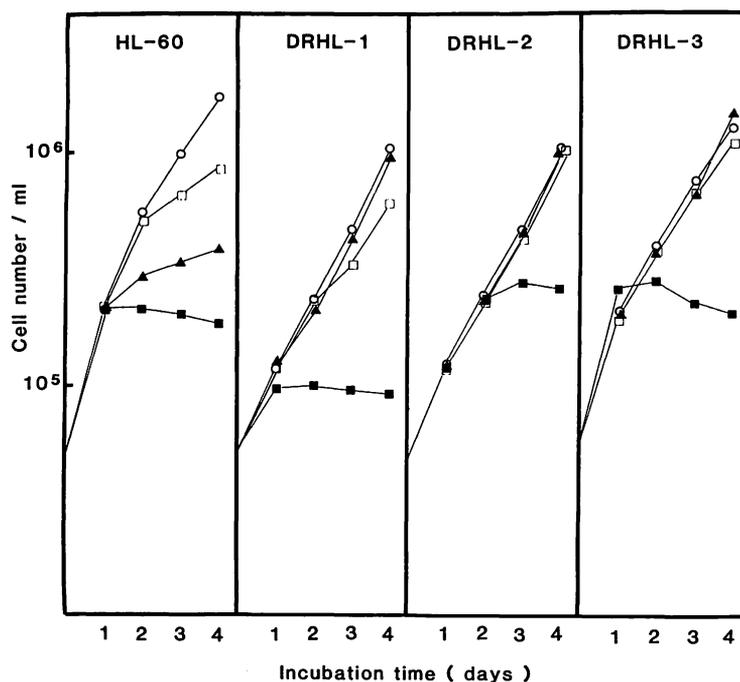


Fig. 1 Effect of  $1\alpha,25$ -dihydroxyvitamin  $D_3$ , TPA or DMSO on the growth of parental cells and the variant clones. Cells were treated with 1.25% DMSO ( $\blacktriangle$ ), 50 ng/ml  $1\alpha,25$ -dihydroxyvitamin  $D_3$  ( $\square$ ), or 10 ng/ml TPA ( $\blacksquare$ ). As a control ( $\circ$ ), cells were cultivated in the medium without any addition. On days indicated, cells were harvested and the cell numbers were determined with a Coulter Counter. Each point represents the mean for three determinations.

and  $1\alpha,25$ -dihydroxyvitamin  $D_3$ -treated HL-60 cells exhibited certain features characteristic of monocytes/macrophages, such as non-specific esterase activity, synthesis and secretion of lysozymes, high phagocytic activity and the presence of monocyte-specific cell surface antigens (1, 11, 13).

Recently, we found that the differentiation of HL-60 cells induced by  $1\alpha,25$ -dihydroxyvitamin  $D_3$  could be separated into two stages with DMSO, *i.e.*, commitment and promotion stages; HL-60 cells pretreated with  $1\alpha,25$ -dihydroxyvitamin  $D_3$  at the stage of commitment differentiated into the monocyte/macrophage lineage on subsequent treatment with DMSO at the stage of promotion (15). For further clarification of this phenomenon, we isolated variant cells resistant to  $1\alpha,25$ -dihydroxyvitamin  $D_3$  from DMSO-resistant (DRHL) cells (10). This paper deals with the characterization of such variants resistant to both  $1\alpha,25$ -dihydroxyvitamin  $D_3$  and DMSO.

## MATERIALS AND METHODS

### Chemicals

DMSO was purchased from E. Merck (Darmstadt, F.R.G.).  $1\alpha,25$ -Dihydroxyvitamin  $D_3$  was generously donated by Dr T. Suda (Showa University, Tokyo). TPA was obtained from Funakoshi Yakuhin (Tokyo); nitroblue tetrazolium (NBT) and  $\alpha$ -naphthyl butyrate were from Sigma (St. Louis, MO); latex particles (0.80  $\mu$ m in diameter) were from Dow Chemicals (Indianapolis, IN); benzoxycarbonyl phenylalanylarginyl-7-(4-methyl)-coumarylamide was from the Peptide Research Foundation (Osaka); mouse monoclonal antibodies, OKM5 and 80H5, were from Ortho Diagnostic Systems (Tokyo) and Immunotech (Marseille Cedex, France); FITC-conjugated rabbit antimouse IgG and IgM were from E. Y. Laboratories (San Mateo, CA); and [ $^3$ H] $1\alpha,25$ -dihydroxyvitamin  $D_3$  (130–180 Ci/mmol) was obtained from Amersham (Buckinghamshire, U.K.). Other chemicals used were all of ana-

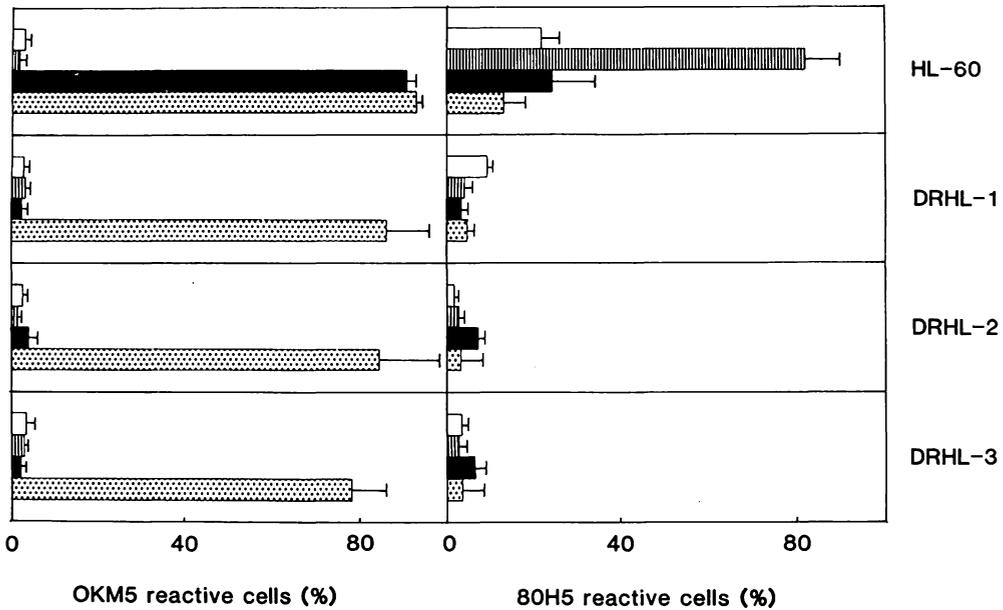


Fig. 2 Induction of antigens for human monocyte specific (OKM5) or human granulocyte specific (80H5) monoclonal antibodies. The percentage of reactive cells was determined as described under Materials and Methods after 4-day incubation without any differentiation inducer (□) and with 1.25% DMSO (▨), 50 ng/ml 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> (■) or 10 ng/ml TPA (▩). Values are the means  $\pm$  SE for three determinations.

lytical grade.

#### Cells and Culture Conditions

HL-60 cells, originally isolated by Dr R. C. Gallo (National Cancer Institute, Bethesda, MD), were provided by Dr M. Terada (National Cancer Research Institute, Tokyo). The cells were cultivated in RPMI-1640 medium (Nissui Seiyaku, Tokyo) supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 units/ml) and streptomycin (100  $\mu$ g/ml) (Gibco Laboratories, NY) in a humidified atmosphere of 5% CO<sub>2</sub> in air at 37°C.

HL-60 cells resistant to DMSO (DRHL cells) were isolated as described previously (10). DRHL cells were further cultivated in a medium containing 50 ng/ml 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> for 4 weeks in order to isolate 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> resistant cells. Surviving cells were cloned by means of limiting dilution, and three clones (DRHL-1, DRHL-2 and DRHL-3 cells) were obtained.

#### Cell Growth

Cells were inoculated at  $5 \times 10^4$  cells/ml into a

medium containing 1.25% DMSO, 50 ng/ml 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>, or 10 ng/ml TPA, and incubated at 37°C. At intervals, the number of cells was determined with a Coulter Counter (Coulter Electronics, Hialeah, FL).

#### Characterization of Differentiated Cells

To characterize the differentiated cells, two monoclonal antibodies, OKM5 specific for human monocytes (21) and 80H5 specific for human granulocytes (12), were used. Expression of these two antigens was detected by means of the indirect immunostaining method using FITC-conjugated rabbit anti-mouse IgG or IgM. NBT reduction activity was assayed as described by Collins *et al.* (7). Phagocytic activity was determined as described by Rabellino *et al.* using latex particles (18). Nonspecific esterase activity was determined with  $\alpha$ -naphthyl butyrate as a substrate (27). Cathepsin B and L activities were measured by means of an assay based on the method of Burnett *et al.*, with benzoxycarbonyl phenylalanylarginyl-7-(4-methyl)-coumarylamide as a substrate (4). The protein concentration of samples was determined by the

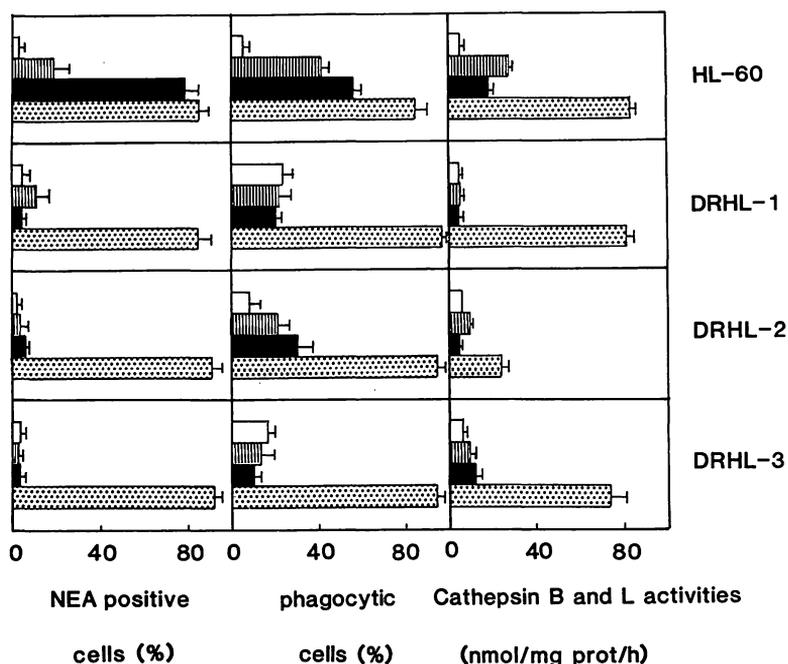


Fig. 3 Induction of nonspecific esterase (NEA), phagocytotic, and cathepsin B and L activities in lysates of parental HL-60 cells and the variant clones. Without differentiation inducer (□); 1.25% DMSO (▨); 50 ng/ml  $1\alpha,25$ -dihydroxyvitamin  $D_3$  (■); or 10 ng/ml TPA (▤). Positive cells were determined as described under Materials and Methods. Cathepsin B and L activities were assayed with a fluorimetric substrate, benzoxycarbonyl phenylalanylarginyl-7-(4-methyl)-coumarylamide, and expressed as nmol/mg protein/h. Values are the means  $\pm$  SE for three determinations.

method of Bradford with bovine serum albumin as a standard (2).

#### *Binding Assay for $1\alpha,25$ -dihydroxyvitamin $D_3$*

The binding activity of  $1\alpha,25$ -dihydroxyvitamin  $D_3$  was measured with the hydroxylapatite batch system (24). Parental HL-60 cells and their variants were sedimented at 3,000  $g$  for 5 min, resuspended in TEK buffer (0.15 M KCl, 2 mM EDTA and 10 mM Tris-HCl, pH 7.4), and disrupted by sonication (two 30 sec bursts). After centrifugation at 160,000  $g$  for 30 min, the cytosol was incubated with [ $^3H$ ] $1\alpha,25$ -dihydroxyvitamin  $D_3$  at concentrations indicated (5–20 nM) in the presence or absence of 50 nM nonradioactive  $1\alpha,25$ -dihydroxyvitamin  $D_3$ . Bound and free [ $^3H$ ]- $1\alpha,25$ -dihydroxyvitamin  $D_3$  were separated on hydroxylapatite. The specific binding of [ $^3H$ ] $1\alpha,25$ -dihydroxyvitamin  $D_3$  was taken as

the difference between the total and nonspecific binding.

## RESULTS

#### *Insusceptibility of the Variant Clones to $1\alpha,25$ -dihydroxyvitamin $D_3$ and DMSO*

Different from that of the parental HL-60 cells, the growth of the variant clones, DRHL-1, DRHL-2 and DRHL-3, was not inhibited by either  $1\alpha,25$ -dihydroxyvitamin  $D_3$  or DMSO (Fig. 1). In the variants, none of the macrophagic and granulocytic markers, such as OKM5, nonspecific esterase activity (NEA), phagocytosis and 80H5, were induced by  $1\alpha,25$ -dihydroxyvitamin  $D_3$  or by DMSO (Figs. 2 and 3).

In contrast, TPA inhibited the cell growth (Fig. 1) and induced the macrophagic markers in the variants as well as in the parental HL-60 cells (Figs. 2 and 3).

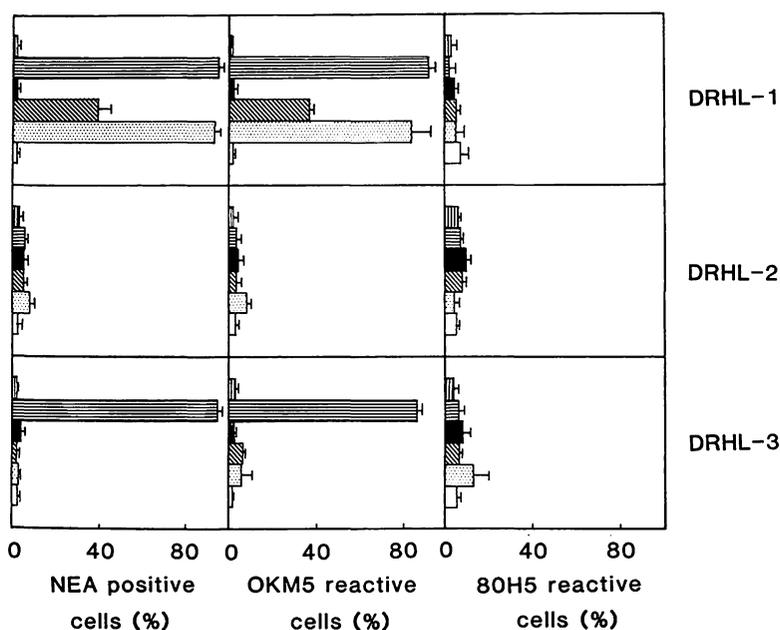


Fig. 4 Susceptibility of the variant clones to combinational treatment with DMSO and  $1\alpha,25$ -dihydroxyvitamin D<sub>3</sub>. Cells were incubated with 1.25% DMSO for 4 days (▨); with 1.25% DMSO and 50 ng/ml  $1\alpha,25$ -dihydroxyvitamin D<sub>3</sub> for 4 days (▧); with 50 ng/ml  $1\alpha,25$ -dihydroxyvitamin D<sub>3</sub> for 4 days (■); first with  $1\alpha,25$ -dihydroxyvitamin D<sub>3</sub> for 1 day and then with DMSO for 3 days (▩); first with DMSO for 1 day and then with  $1\alpha,25$ -dihydroxyvitamin D<sub>3</sub> for 3 days (▤); and without any addition for 4 days (□). After 4-day incubation, percentage of nonspecific esterase activity (NEA), and OKM5- or 80H5-positive cells was determined as described under Materials and Methods.

#### *Induction of Differentiation of the Variant Clones on Simultaneous Treatment with $1\alpha,25$ -dihydroxyvitamin D<sub>3</sub> and DMSO*

Though refractory to  $1\alpha,25$ -dihydroxyvitamin D<sub>3</sub> or DMSO alone, the DRHL-1 and DRHL-3 cells differentiated into NEA- and OKM5-positive cells on simultaneous treatment with  $1\alpha,25$ -dihydroxyvitamin D<sub>3</sub> and DMSO for 4 days. The other variant, DRHL-2, was refractory even to such combinational treatment. Reactivity with 80H5 was not induced by the combinational treatment in any of the variants.

As the next step, successive combined treatment of the variant clones was investigated. DRHL-1 cells were induced to differentiate into NEA- and OKM5-positive cells on 1-day  $1\alpha,25$ -dihydroxyvitamin D<sub>3</sub> treatment followed by 3-day DMSO treatment, although the percentage of the marker-positive cells was lower than that in the simultaneously treated cells. Conversely, DRHL-1 cells were

also induced to differentiate into macrophage-like cells by 1-day DMSO treatment followed by 3-days  $1\alpha,25$ -dihydroxyvitamin D<sub>3</sub> treatment. On the other hand, DRHL-3 cells, which responded to the simultaneous treatment, did not respond to such a treatment schedule (Fig. 4).

#### *Binding of [<sup>3</sup>H] $1\alpha,25$ -dihydroxyvitamin D<sub>3</sub> to the Cytosol Protein*

The cytosolic receptor activity of the variant clones was compared with that of the parental HL-60 cells (Figs. 5 and 6). There were no remarkable differences in the number of cytosolic receptors and dissociation constants ( $K_d$ ), calculated by the Scatchard method, between HL-60, DRHL-1, DRHL-2 and DRHL-3 cells (88, 121, 104 and 121 fmol/mg protein, and 50.0, 70.7, 40.0 and 100 pM, respectively; Fig. 6, A-D). When the Hill plot was used to analyze the data, positive cooperativity was found in the binding of  $1\alpha,25$ -dihydroxyvita-

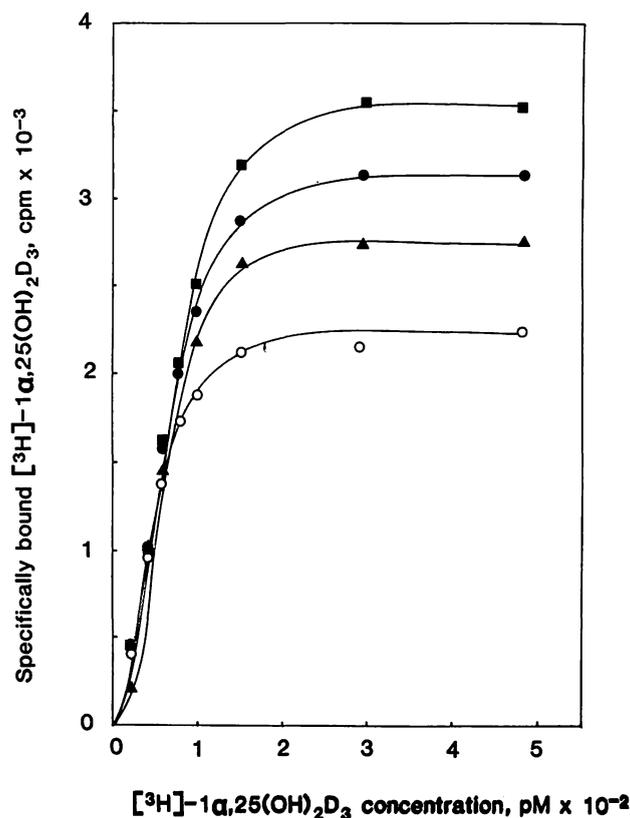


Fig. 5 Saturation analysis of the specific [<sup>3</sup>H]1α,25-dihydroxyvitamin D<sub>3</sub> binding in the cytosol of parental cells (○), and of variant clones, DRHL-1 (●), DRHL-2 (▲) and DRHL-3 (■). Binding activity was assayed as described under Materials and Methods.

min D<sub>3</sub> to the receptors of parental HL-60 cells and the variants, DRHL-1, DRHL-2 and DRHL-3; when calculated from the slopes of the linear lines, the Hill coefficients ( $n_H$ ) were 1.64, 1.83, 1.89 and 1.93, respectively (Fig. 6, E-H). On the other hand, the dissociation constants ( $K_D$ ) of HL-60, and DRHL-1, DRHL-2 and DRHL-3 were 170, 438, 529 and 773 pM, respectively.

## DISCUSSION

In the differentiation of the human promyelocytic leukemia cell line, HL-60, a model of chemically induced cell differentiation, the presence of two stages controlled by different agents has been demonstrated (28). During the commitment phase, various inducer-mediated reversible cellular changes take

place, and the cells are eventually induced into terminal differentiation. For instance, HL-60 cells primed with 10 nM retinoic acid were induced into differentiation by 10 nM prostaglandin E<sub>2</sub> or 1 nM cholera toxin (16). Fibach *et al.* also demonstrated that serine proteases induced the differentiation of HL-60 cells pretreated with DMSO (8).

The differentiation of HL-60 cells induced by 1α,25-dihydroxyvitamin D<sub>3</sub> was also demonstrated to be separated into two stages in our previous study, *i.e.*, cells committed by 1α,25-dihydroxyvitamin D<sub>3</sub> were induced to differentiate into a monocyte/macrophage lineage by DMSO (15).

For a more detailed study of the effects of DMSO on the 1α,25-dihydroxyvitamin D<sub>3</sub>-induced differentiation, we isolated three variant clones, DRHL-1, DRHL-2 and DRHL-3,

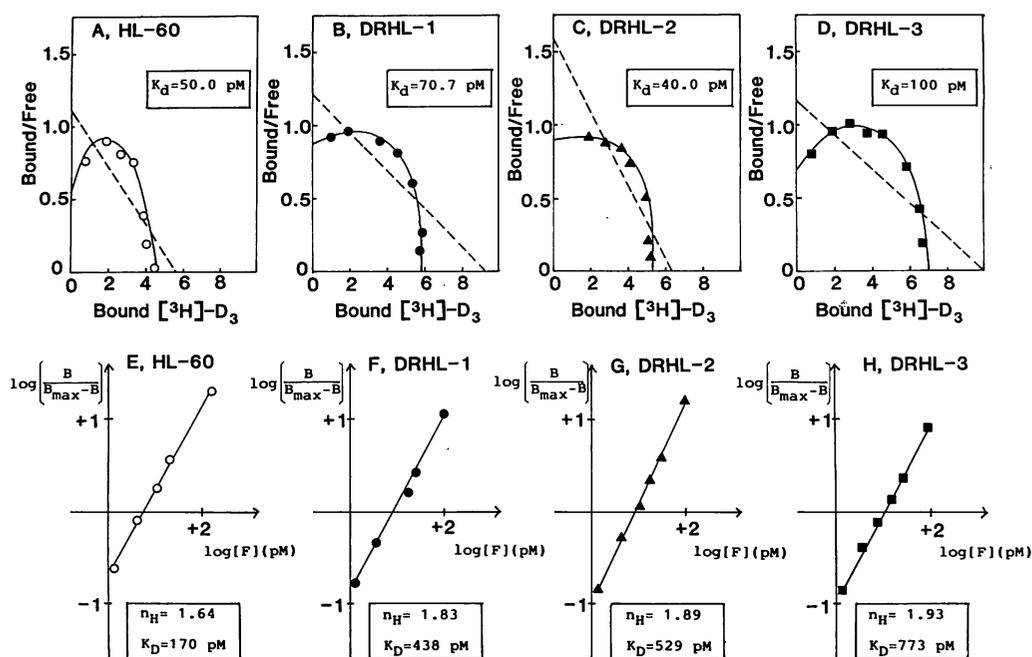


Fig. 6 Scatchard and Hill plots of the saturation of receptors for  $1\alpha,25$ -dihydroxyvitamin D<sub>3</sub> in parental HL-60 cells (—○—, A and E) and the variant clones (—●—, B and F: DRHL-1; —▲—, C and G: DRHL-2; and —■—, D and H: DRHL-3). The dashed lines in A-D represent the calculated linear regression slopes of Scatchard plots using all the available data. The curved lines represent the best fit in A-D.  $n_H$ , Hill coefficient;  $K_d$  and  $K_D$ , dissociation constants;  $B_{max}$ , maximum binding capacity; F, free ligand; B, ligand specifically bound to the receptor; Bound [<sup>3</sup>H]-D<sub>3</sub>, bound [<sup>3</sup>H] $1\alpha,25$ -dihydroxyvitamin D<sub>3</sub>, pM  $\times 10^{-1}$

resistant to  $1\alpha,25$ -dihydroxyvitamin D<sub>3</sub> from DMSO resistant (DRHL) cells (10). Unlike the parental HL-60 cells, all these variants did not differentiate in the presence of either DMSO or  $1\alpha,25$ -dihydroxyvitamin D<sub>3</sub> alone (Figs. 1-3). However, DRHL-1 and DRHL-3 differentiated on simultaneous treatment with both agents, while DRHL-2 failed to respond such combined treatment with the two agents (Fig. 4). As to the two stages of differentiation of HL-60 cells, the differences in the insusceptibility of the three variants could be explained as follows: DRHL-1 cells can differentiate into macrophages with 50 ng/ml  $1\alpha,25$ -dihydroxyvitamin D<sub>3</sub> when 1.25% DMSO is present at the stage of promotion. DRHL-3 cells differentiate into macrophages with  $1\alpha,25$ -dihydroxyvitamin D<sub>3</sub> in the presence of DMSO at the stages of both commitment and promotion. DRHL-2 cells can not differentiate with  $1\alpha,25$ -dihydroxyvitamin D<sub>3</sub>, even when incubated with DMSO at the stages of both commitment and promotion. Thus, DMSO may affect the various stages of

the differentiation of HL-60 cells induced by  $1\alpha,25$ -dihydroxyvitamin D<sub>3</sub>, and the differentiating direction of HL-60 cells into a monocyte/macrophage lineage by  $1\alpha,25$ -dihydroxyvitamin D<sub>3</sub> may be determined at the stage of commitment.

At present, the reason for the insusceptibility of the variant cells to  $1\alpha,25$ -dihydroxyvitamin D<sub>3</sub> remains unknown. Positive cooperativity (25, 26) in the binding of  $1\alpha,25$ -dihydroxyvitamin D<sub>3</sub> to the receptors in the variants ( $n_H = 1.83$ -1.93) may not be related to this insusceptibility, because a similar Hill coefficient was also found in parental HL-60 cells ( $n_H = 1.64$ ) (Fig. 6). As shown in Figs. 5 and 6, the number of receptors and their dissociation constants, calculated by either Scatchard or Hill plot, indicated that the receptors were not defective in the variant cells. The number of receptors in the variant cells was higher than that in the parental HL-60 cells. However, blocking of the binding of the ligand-receptor complex to nuclear proteins or DNA can not be ruled out, because such

blocking has been speculated to be involved in the pathogenesis in patients with vitamin D<sub>3</sub> dependent rickets type II, who show normal binding activities and numbers of receptors (17). DMSO may promote the differentiation of the HL-60 cell variants by removal of the blocking with conformational changes in DNA or nuclear proteins (23).

In conclusion, we isolated three variant clones that are insensitive to either DMSO or 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> alone. Defective points in the differentiation of the variant cells showed that DMSO promotes the differentiation of HL-60 cells at various stages of differentiation induced by 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>. However, the molecular events of the promotional effect of DMSO remain to be elucidated.

We are grateful to Drs T. Furuya and H. Sakuraba, Division of Pathological Chemistry, National Center for Nervous, Mental and Muscular Disorders, for their valuable discussions. We also wish to thank Professor Y. Nagai, Department of Cell Physiology, Medical Research Institute, Tokyo Medical and Dental University, for his continuous encouragement and the kind offer of facilities for the cell culture. This work was supported by grants-in-aid for Cancer Research and for Scientific Research from the Ministry of Education, Science and Culture, Japan.

Received for publication 29 May 1986; and in revised form 21 July 1986

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