Results

Part Ⅰ Effect of cytokinins on the differentiation of human myeloid leukemia cells

Effects of various adenine analogs on growth inhibition and differentiation of HL-60 cells.

The abilities of various adenine analogues containing cytokinins to inhibit the growth of human myeloid leukemia HL-60 cells were examined. The cells were cultured with various concentrations of the analogs for 5 days, and concentrations of the analogs that inhibited the number of viable cells by 50% (IC_{50}) were calculated (Table Ⅰ). Among the adenine analogs, cytokinins such as IPA, kinetin and 6-dimethyladenine were more potent than other adenine analogs in inhibiting cell growth. HL-60 cells are induced to differentiate into forms that resemble mature granulocytes or macrophages functionaly and morphologically by various compounds (41). Then, I examined the abilities of various adenine analogs to induce the differentiation of HL-60 cells (Fig. Ⅰ-1). NBT-reducing activity, a typical functional marker of differentiation, was significantly induced by several adenine analogs. Among the adenine analogs, cytokinins such as IPA, kinetin and BA were more potent in inducing NBT-reduction of HL-60 cells. Since IPA was the most potent among the compounds tested (Fig. Ⅰ-1), IPA was used for further analysis. The IPA-induced differentiation of HL-60 cells was confirmed by examining other differentiation-associated properties, such as

lysozyme activity which is preferentially synthesized by mature granulocytes and macrophages (Fig. Ⅰ-2A) and expression of differentiation-associated antigen CD11b which is a leukocyte-type integrin expressed on the surface (Fig. Ⅰ-2B). Morphological examination of HL-60 cells treated with IPA using May- Grünwald -Giemsa staining revealed that IPA induced morphological differentiation of HL-60 cells into granulocytes, which showed nuclear condensation, defects of staining properties (Fig. Ⅰ-3). Similar results were obtained in other myeloid leukemia ML-1, NB4 and U937 cells (data not shown) indicating that cytokinins are potent inducers of differentiation of myeloid leukemia cells.

The effect of urea-cyokinins on growth inhibition and differentiation of HL-60 cells.

Although some phenylurea or diphenylurea analogs have been reported to have cytokinin-like activities (11, 76, 85 and 102), there was an argument among plant physiologists whether the compounds that has the urea structure, different from the adenine one, is cytokinin or not. However, it has been cleared that some phenylurea compounds have the similar biological activities characteristics to cytokinins, and common highly specific binding protein and receptors, indicating that the some phenylurea compounds have intrinsic cytokinin properties (58). Then, cytokinins can be structurally classified into at least two categories: the adenine cytokinins and the urea cytokinins. Therefore, I examined the differentiation-inducing effects of urea cytokinins on HL-60 cells. Although 1,3-diphenylurea did not essentially affect the growth and differentiation of the cells, N-phenyl-N'-(1,2,3-thiadiazol-5-yl)-urea (thidiazuron) and N-(2-chloro-4-pyridyl)-N'-phenylurea

(4-CPPU) inhibited the cell growth and induced NBT reduction in a concentration-dependent manner (Fig. Ⅰ-4). These results indicate that some urea-cytokinins are also effective at inducing differentiation of human myeloid leukemia cells.

Commitment to differentiation and growth inhibition by cytokinins

In normal hematopoiesis, once a cell has differentiated as a granulocyte or erythrocyte or some other type of blood cells, there is no going back. The state of differentiation is not reversible. Therefore, at some stage in their development, the progeny of the pluripotent stem cell must become irreversibility committed or determined for a particular line of differentiation. This irreversible differentiation has been studied using leukemia cells by treatment with some differentiation inducers. There are the cases that the irreversible induction of differentiation requires continuous treatment with inducers for several days (27, 33, 104 and 121).

To determine whether cytokinin-induced differentiation was quickly committed, I examined the effect of pulse treatment with IPA or other typical inducers such as ATRA, and VD_3 on the differentiation of HL-60 cells. The cells were incubated with IPA for various lengths of time, washed with fresh medium, and incubated in medium without IPA. On day 6, NBT-reducing activities and cell numbers were measured. When the cells were incubated with IPA for 1 day and then cultured in the absence of the drug for 5 days, a significant induction of NBT-reducing activity and a growth-inhibitory effect were observed (Fig. Ⅰ-5). This 1-day treatment with IPA was similar to continuous treatment with regard to commitment to differentiation and growth inhibition in HL-60 cells, indicating that the effect of IPA on the induction of

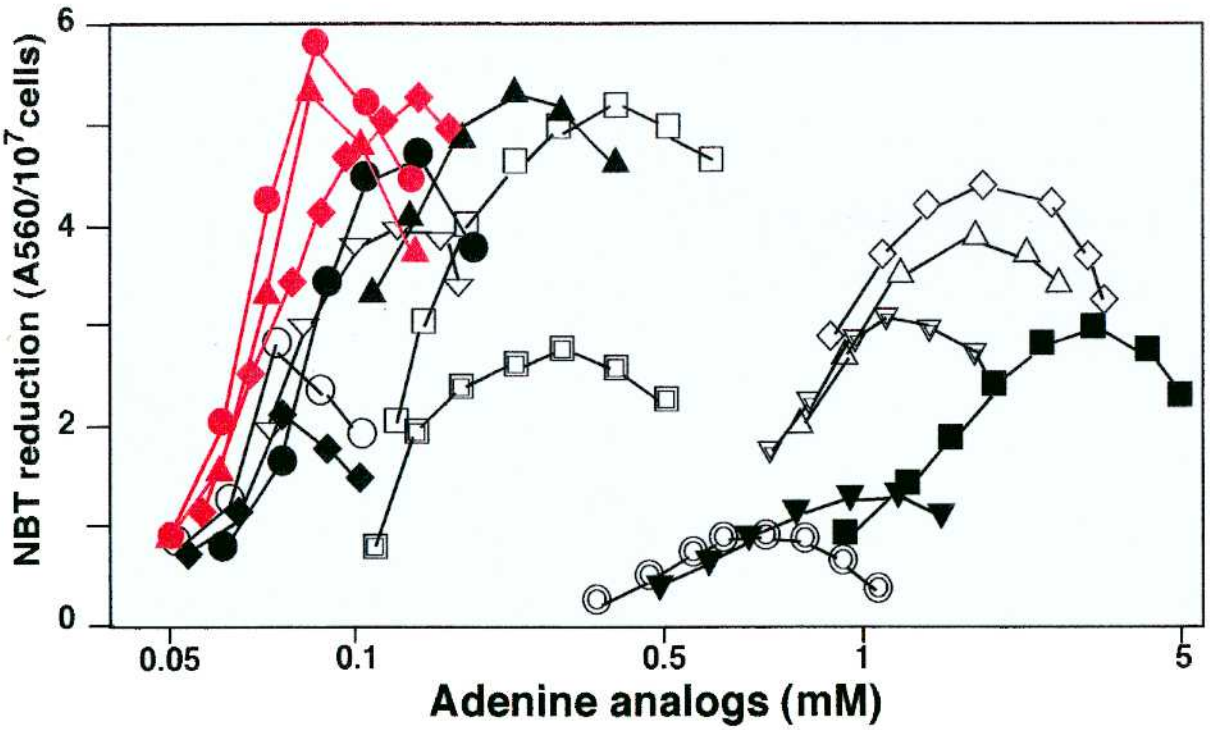
differentiation was irreversible. On the other hand, treatment with ATRA or VD_3 for 1 day was not sufficient to induce the differentiation of HL-60 cells (Fig. Ⅰ-5), suggesting that continuous exposure to these inducers is required to induce the differentiation of HL-60 cells.

Table Ⅰ. Effects of adenine analogs on growth of HL-60 cells.

Analog	Growth inhibition (IC_{50}, μM)
6-dimethylaminopurine	47.2
isopnetenyladenine	47.6
kinetin	48.8
6-anilinopurine	56.3
2,6-diaminopurine	61.3
6-benzyladenine	67.6
6-n-hexylaminopurine	87.5
adenine	261
olomoucine	326
6-methyladenine	327
trans-zeatin	516
6-methoxypurine	744
3-methyladenine	1172
2-aminopurine	1324
1-methyladenine	1713

Cells were cultured with various concentrations of the analogs for 5 days. Means of three separate experiments. IC_{50}, concentration of compound required for 50% inhibition of cell growth.

Figure Ⅰ-1. Effects of various analogs of adenine on induction of NBT reduction in HL-60 cells. Cells were incubated with various concentrations of the compounds for 5 days. Values are means of four separate experiments.



●, isopentenyl adenine; ▲, kinetin; ◆,6-benzyladenine; ○, 6-dimethyladenine;
▽, 6-n-hexylaminopurine;, 6-anilinopurine; ▣, adenine ▽, 3-methyladenine
□, 6-methyladenine; ◆, purine; ●, 2,6-diaminopurine; ■, 1-methyladenine;
▼, *trans*-zeatin; ◎,olomoucine ;◇,2-aminopurine; △,6-methoxyaminopurine

Figure Ⅰ-2. Effects of IPA on the induction of lysozyme activity (A) and CD11b expression (B) in HL-60 cells.

(A) Cells were cultured with 0 (◆), 0.05 (■) , 0.1 (▲), or 0.15 (●) mM IPA for various days for 5 days. Determination of the induced lysozyme activity was carried out as described in Materials and Methods. (B) Cells were cultured in the absence or presence of 0.1mM, or 0.15 mM IPA for 5 days. The expression was evaluated by immunofluorescent staining using anti-CD11b monoclonal antibody by flow cytometry.

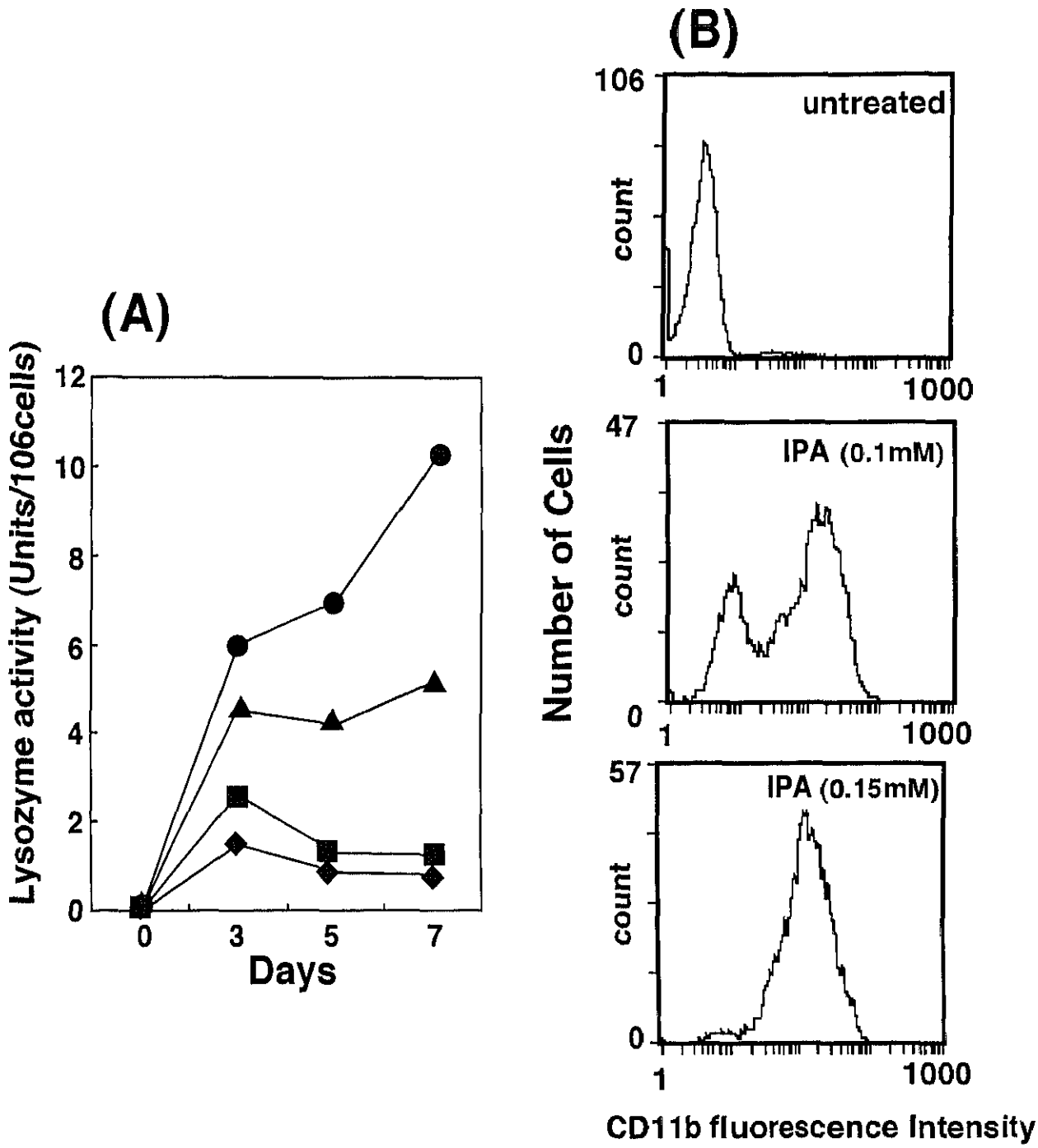
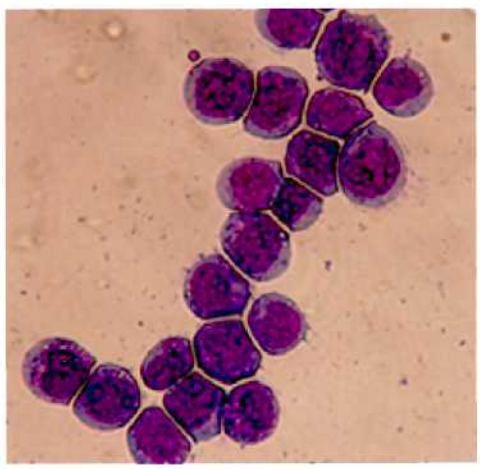

(A)
Lysozyme activity (Units/106cells)
12
10
8
6
4
2
0
0
3
5
7
Days
(B)
106
untreated
count
0
1
1000
47
IPA (0.1mM)
count
0
1
1000
57
IPA (0.15mM)
count
0
1
1000
Number of Cells
CD11b fluorescence Intensity

Figure Ⅰ-3. Morphologic changes in HL-60 cells by IPA.
Cells were cultured with (B) or without (A) 0.1 mM IPA for 6 days.

(A)



(B)

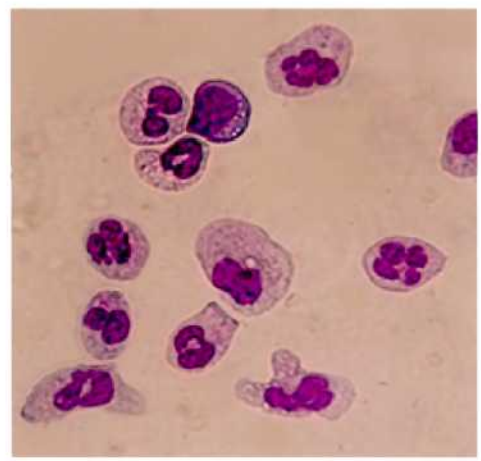


Figure Ⅰ-4. Effects of various urea-cytokinins on inhibition of growth (A) and induction of NBT reduction (B) in HL-60 cells.
Cells were cultured with various concentrations of 1,6-diphenylurea (▲), thidiazuron (■) or 4-CPPU (●) for 5 days. Results are given as the mean of three separate experiments.

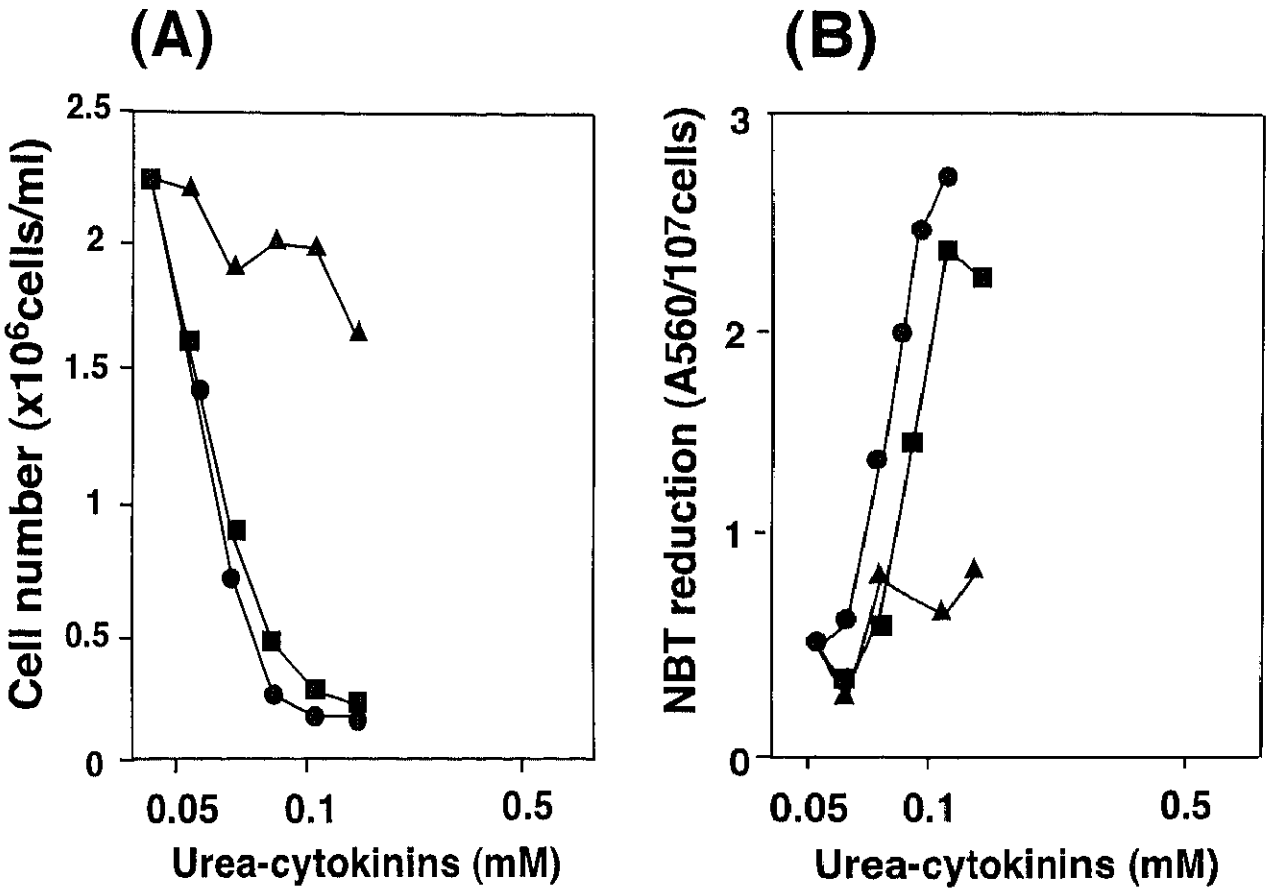
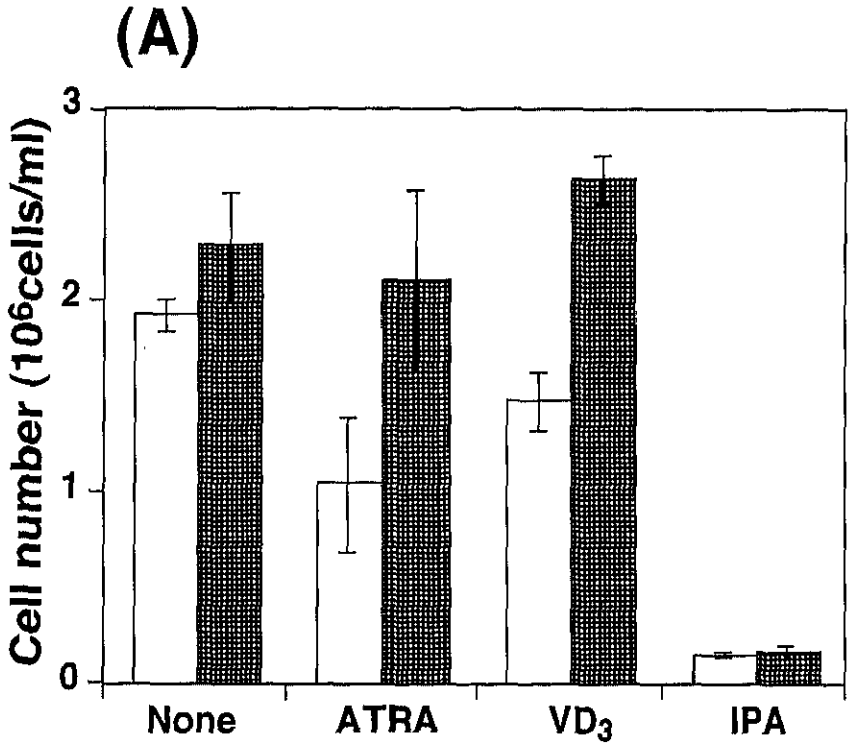
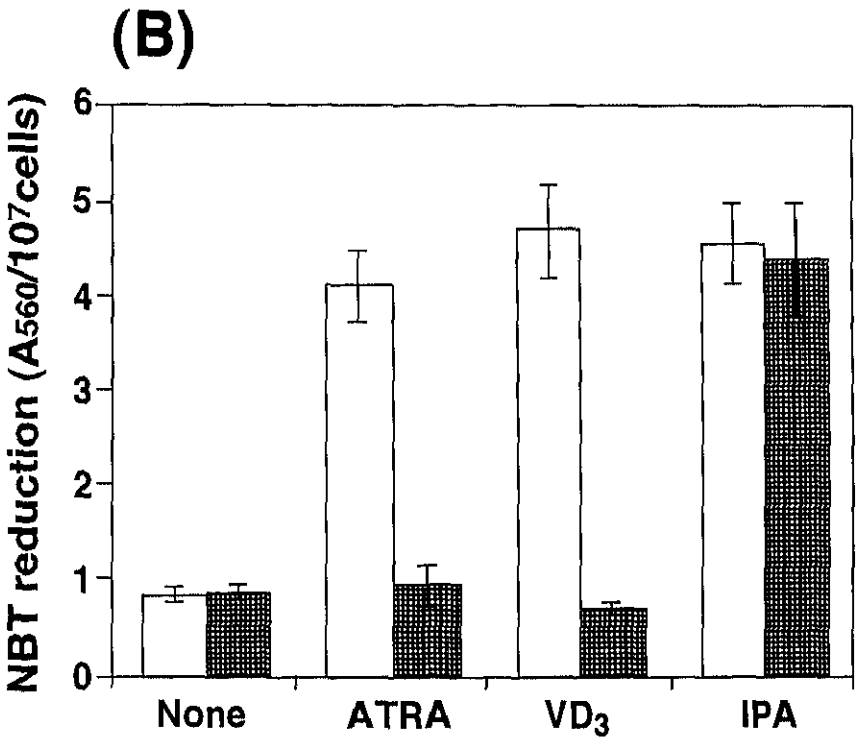

(A)
Cell number (x10^6cells/ml)
2.5
2
1.5
1
0.5
0
0.05
0.1
0.5
Urea-cytokinins (mM)
(B)
NBT reduction (A560/10^7cells)
3
2
1
0
0.05
0.1
0.5
Urea-cytokinins (mM)

Figure Ⅰ-5. One-day-treatment with IPA, but not with ATRA or VD_3, induced commitment to differentiation in HL-60 cells.

HL-60 cells were cultured with 0.1 mM IPA, 400 nM ATRA, or 24 nM VD_3 for 1 day. Closed bar: the cells were then washed with fresh medium and reincubated without the inducers for 5 days. Open bar: the cells were continuously treated with 0.1 mM IPA, 400 nM ATRA, or 24 nM VD_3 for 6 days. Results are given as the mean ± SD of three separate experiments.


(A)
Cell number (10^6cells/ml)
3
2
1
0
None
ATRA
VD_3
IPA


(B)
NBT reduction ($A_{560}/10^7$cells)
6
5
4
3
2
1
0
None
ATRA
VD_3
IPA