

## EFFECTS OF TAUTOMYCIN, A PROTEIN PHOSPHATASE INHIBITOR, ON RECYCLING OF MAMMALIAN CELL SURFACE MOLECULES

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The effects of tautomycin, a protein phosphatase inhibitor, on recycling of cell surface molecules were studied with transferrin receptor (TFR) of human myeloid leukemia K562 cells and with CD4 of murine thymocytes. Tautomycin increased expression of TFR of K562 cells whereas phorbol dibutylate (PDBu) decreased it. Tautomycin inhibited PDBu-induced down-regulation of CD4 although it did not induce up-regulation. Okadaic acid also inhibited down-regulation of CD4 which was induced by PDBu. The results suggest that certain inhibitors of protein phosphatases preferentially inhibit endocytosis of cell surface molecules.

Tautomycin is an antifungal antibiotic isolated from *Streptomyces spiroverticillatus*<sup>1,2</sup>. Tautomycin induced blebs on the surface of human myeloid leukemia K562 cells<sup>3</sup>. The morphological change, named bleb formation, was also induced by phorbol dibutylate (PDBu)<sup>3</sup>, cytochalasin D (CyD) and okadaic acid (OA)<sup>4</sup> suggesting that modulation of actin filament network and/or protein phosphorylation is involved in tautomycin-induced bleb formation<sup>4</sup>. Further studies on tautomycin revealed that tautomycin is a novel inhibitor of protein phosphatases and that the specificity of tautomycin to the enzymes was distinct from known inhibitors of protein phosphatases<sup>5,6</sup>. Cytoskeleton and protein phosphorylation are also involved in recycling of cell surface receptors for transferrin and epidermal growth factor (EGF)<sup>7,8</sup>. In fact, phorbol esters are known to affect expression of a number of important regulatory molecules on cell surface including the receptors for EGF<sup>8</sup> and transferrin<sup>9</sup> and CD4<sup>10</sup>. In the present paper, we studied the effects of tautomycin on phorbol ester-induced down-regulation of transferrin receptor (TFR), on K562 cells, and the effect of CD4 on murine thymocytes.

### Materials and Methods

#### Chemicals

Tautomycin was isolated as described by CHENG *et al.*<sup>1</sup> Staurosporine was also isolated from the culture broth of an actinomycetes. Okadaic acid and dinophysistoxin-I were kindly donated by Dr. H. FUJIKI (National Cancer Center Research Institute, Japan). Cytochalasin D and PDBu were purchased from Sigma Chem. H-7, H-8 and W-7 were purchased from Seikagaku Kogyo Co., Ltd. Japan. K252a and KT5926 were kindly gifted by Dr. H. KASE (Kyowa Hakko Kogyo Co., Ltd.).

#### Cell Culture

Cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 0.05 mM 2-mercaptoethanol, 50 mg/liter kanamycin and 8 mg/liter tylosin at 37°C in 5% CO<sub>2</sub> atmosphere.

### Analysis of Surface Molecules

Expression of surface molecules was quantitated using a flow cytometer (Epics C, Coulter Electronics, Inc. Healeah, Florida, U.S.A.). For analysis of TFR, K562 cells were treated with drugs at 37°C and were washed twice with cold buffer A (HANK's balanced salt solution containing 0.2% sodium azide and 2% FCS). Then the cells were incubated with mouse anti-human TFR antibody (final dilution 1:20; Becton Dickinson) at 4°C for 45 minutes. After being washed for three times with buffer A, the cells were incubated with FITC-conjugated anti-mouse immunoglobulin antibody (final dilution 1:20; Cappel) at 4°C for an additional 45 minutes. For analysis of CD4, thymocytes from C3H mice (Nippon Charles River Co., Ltd.) were cultured with drugs at 37°C and were washed twice with cold buffer A. Then the cells were incubated with phycoerythrin-conjugated anti-L3T4 antibody (final dilution 1:10; Becton Dickinson) at 4°C for 45 minutes.

## Results

### Effects of Tautomycin on Transferrin Receptor

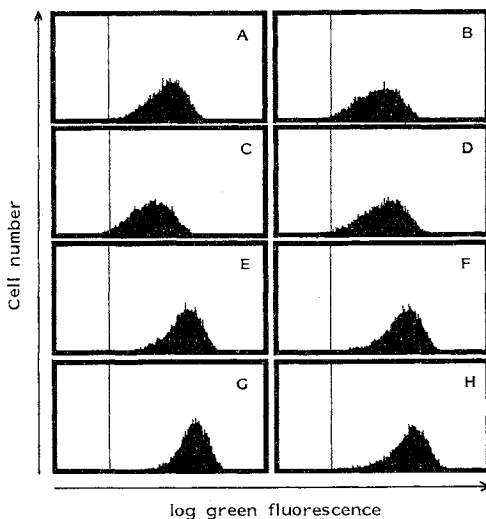
Effects of tautomycin on TFR of K562 cells were analyzed by a flow cytometer using indirect fluorescent antibody technique (Fig. 1). As reported previously<sup>10~12</sup>, phorbol ester which is an activator of protein kinase C (PKC)<sup>13</sup> induced down-regulation of TFR. An inhibitor of protein kinase, staurosporine<sup>14</sup>, inhibited the PDBu-induced down-regulation. On the contrary, tautomycin increased surface expression of TFR, and the expression was not inhibited by staurosporine. Simultaneous treatment of K562 cells with tautomycin and PDBu resulted in enhancement of up-regulation of TFR.

### Effects of Tautomycin on CD4 on Murine Thymocytes

As has been reported<sup>15~17</sup>, PDBu induced down-regulation of CD4 on murine thymocytes (Fig. 2).

Fig. 1. Effects of tautomycin on PDBu-induced down-regulation of transferrin receptor.

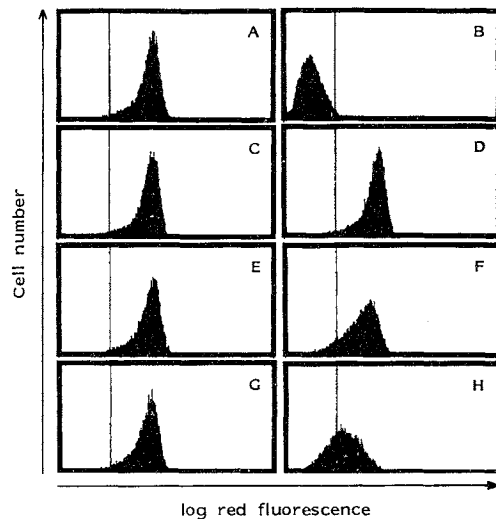
K562 cells were cultured for 1 hour in the absence (A, C, E, G), or the presence of staurosporine (100 ng/ml; B, D, F, H). PDBu (1 µg/ml; C, D), tautomycin (30 µg/ml; E, F) or tautomycin and PDBu (G, H) were added simultaneously.



Expression of TFR was determined as described in Materials and Methods.

Fig. 2. Effects of tautomycin on PDBu-induced down-regulation of CD4.

Thymocytes were cultured for 1 hour in the absence (A, C, E, G) or the presence of PDBu (100 ng/ml; B, D, F, H). Staurosporine (100 ng/ml; C, D), tautomycin (30 µg/ml; E, F) or both of staurosporine and tautomycin (G, H) were added to the culture.



Expression of CD4 was determined as described in Materials and Methods.

Although tautomycin alone had no effect on expression of CD4, tautomycin as well as staurosporine inhibited PDBu-induced down-regulation of CD4. Only partial inhibition was observed when the cells were treated with tautomycin and staurosporine simultaneously.

#### Effects of Tautomycin on Re-expression of CD4

To determine whether tautomycin or staurosporine can induce re-expression of CD4, thymocytes, CD4 of which had disappeared by the treatment with PDBu for 1 hour, were washed and cultured in the absence of PDBu for 1 hour (Fig. 3). The second culture without PDBu allowed partial re-expression of CD4. Addition of tautomycin or staurosporine during the period of second culture had no effect on the expression of CD4. The results demonstrate that tautomycin and staurosporine inhibit down-regulation but do not affect re-expression of CD4.

#### Effects of Okadaic Acid and Dinophysistoxin-1 on PDBu-Induced Down-regulation of CD4

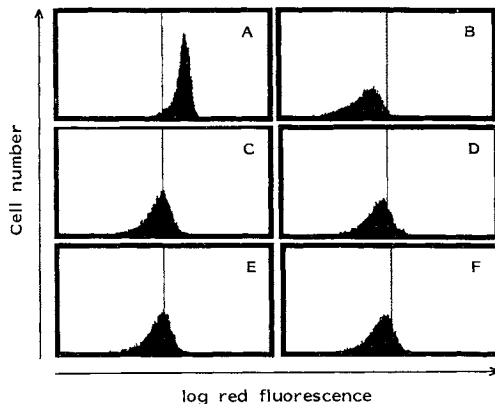
Similar to tautomycin, okadaic acid and dinophysistoxin-1 inhibit protein phosphatases<sup>18)</sup> and induce bleb formation of K562 cells<sup>4)</sup>. As shown in Fig. 4, they also inhibited PDBu-induced down-regulation of CD4 suggesting that inhibition of protein phosphatases is involved in the inhibitory effect of tautomycin on PDBu-induced down-regulation of CD4. IC<sub>50</sub> of okadaic acid, dinophysistoxin-1 and tautomycin was 0.6, 0.2 and 16  $\mu$ g/ml, respectively. Although cytochalasin D also induced bleb formation similar to tautomycin<sup>3)</sup> it did not inhibit the down-regulation (data not shown).

#### Effects of Protein Kinase Inhibitors on PDBu-Induced Down-regulation of CD4

KT5926<sup>19)</sup>, an inhibitor of myosin light chain kinase was unable to inhibit PDBu-induced down-regulation of CD4. W-7 which is a calmodulin specific inhibitor<sup>20)</sup>, inhibited the down-regulation. H-7 and H-8, inhibitors of protein kinases<sup>21)</sup>, inhibited the down-regulation. The effect of H-8 was more

Fig. 3. Effects of tautomycin on re-expression of CD4.

After incubation for 1 hour in the absence or the presence of PDBu 1  $\mu$ g/ml, thymocytes were washed followed by incubation for additional 1 hour in the absence (A, C) or the presence of PDBu (B), tautomycin (D), staurosporine (E) or tautomycin and staurosporine (F).



Expression of CD4 was determined as described in Materials and Methods. Concentration of agents was the same as in Fig. 2.

Fig. 4. Effects of okadaic acid and dinophysistoxin-1 on expression of CD4.

Thymocytes were cultured with PDBu (100 ng/ml) in the absence ( $\blacktriangle$ ) or the presence of dinophysistoxin-1 ( $\blacksquare$ ), okadaic acid ( $\square$ ) or tautomycin ( $\circ$ ). Percent CD4<sup>+</sup> cells was determined by a flow cytometer.  $\Delta$ : percent positive cells of untreated thymocytes.

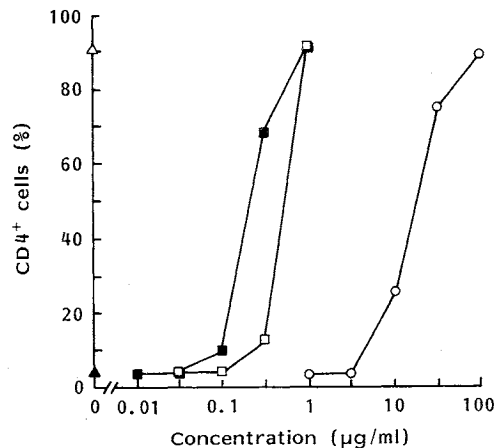
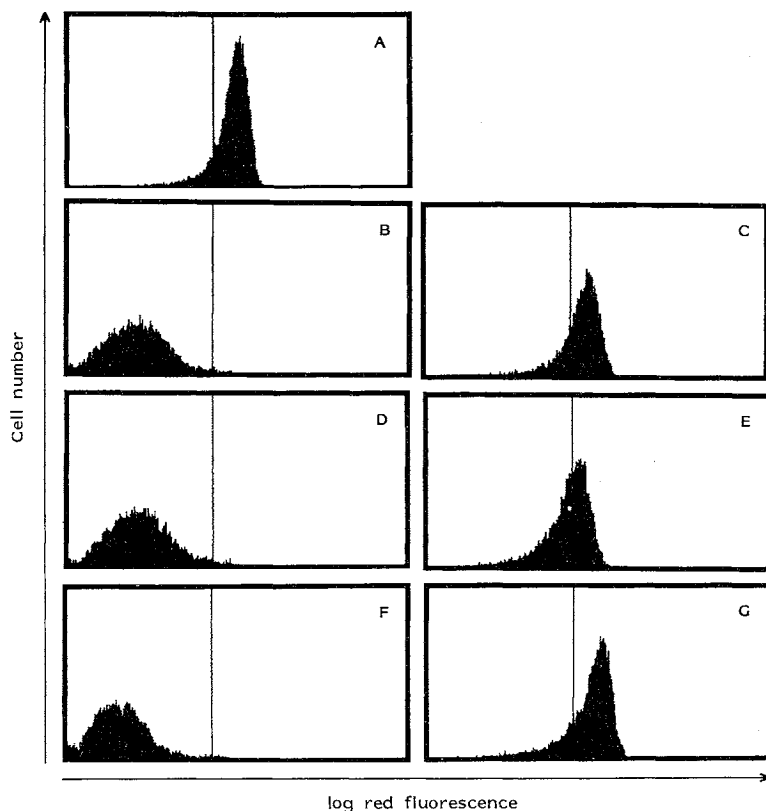


Fig. 5. Effects of kinase inhibitors on PDBu-induced down-regulation.

Thymocytes were cultured for 1 hour in the absence (A) or the presence of PDBu (1  $\mu$ /ml; B~G). W-7 (100  $\mu$ M; C), K252a (1  $\mu$ g/ml; D), H-7 (1 mM; E), KT5926 (1  $\mu$ g/ml; F), or H-8 (1 mM; G) was added to the culture.



Expression of CD4 was determined by a flow cytometer.

significant than that of H-7.

Although staurosporine<sup>14)</sup> and K252a<sup>22)</sup> inhibited activity of multiple protein kinases, K252a, unlike staurosporine, did not inhibit the down-regulation at the concentration up to 1  $\mu$ g/ml. The same concentration of K252a completely inhibited bleb formation of K562 cells which was induced by PDBu (data not shown). This suggests that different kinases are involved in the down-regulation and the bleb formation.

The results are presented in Fig. 5.

### Discussion

In 1984 KLAUSNER *et al.* reported that phorbol ester, an activator of protein kinase C, induced rapid internalization of TFR, independently of its binding to the ligand<sup>12)</sup>. Since hyperphosphorylation of proteins could result from inhibition of protein phosphatases as well as from activation of protein kinases, we examined the effect of tautomycin on expression of TFR. Our results showed that tautomycin, in contrast to PDBu, induced up-regulation of TFR.

It is possible that tautomycin induced the apparent up-regulation by inhibiting internalization of TFR. This possibility was further studied with CD4 of thymocytes and we found that tautomycin as well as

staurosporine did not induce up-regulation of CD4 but inhibited PDBu-induced down-regulation. However, tautomycin did not inhibit re-expression of CD4 which once disappeared after the treatment with PDBu. It is reported that it takes 48 hours for internalized CD4 to be re-expressed on the cell surface<sup>16</sup>). In contrast, recycling time of TFR is about 10 minutes<sup>23</sup>). Thus, it is assumed that up-regulation of tautomycin is observed only with surface antigens whose recycling time is as short as TFR. These results suggest that tautomycin inhibits endocytosis but not exocytosis of these surface antigens.

Cytochalasin D, an inhibitor of actin polymerization, did not inhibit PDBu-induced down-regulation of CD4 as reported previously<sup>24</sup>). Therefore, unlike bleb formation induced by tautomycin, the inhibiting effects of tautomycin on PDBu-induced down-regulation of CD4 may not be mediated through modulation of actin filaments. Okadaic acid and dinophysistoxin-1 blocked the down-regulation at lower concentration than tautomycin. The IC<sub>50</sub> of okadaic acid against type-1 and type-2A protein phosphatases are 224 nM and 10 nM, respectively, whereas the values for tautomycin are both 32 nM. Therefore, it is likely that type-2A phosphatase is involved in the regulation of CD4 expression.

H-8 inhibited the down-regulation more significantly than H-7. H-8 inhibits cyclic AMP dependent protein kinase (PKA), and cyclic-GMP-dependent protein kinase (PKG), more significantly than PKC, whereas H-7 inhibits PKC more significantly<sup>21</sup>). Thus, some kinase other than PKC may be involved in PDBu-induced down-regulation as BIGBY *et al.* have already suggested<sup>24</sup>). This interpretation was further supported by the obligation that K252a did not inhibit the down-regulation. W-7, an antagonist of calmodulin, inhibited the down-regulation as BIGBY *et al.* reported<sup>24</sup>). We suggest that myosin light chain kinase, a calmodulin-dependent kinase, may not be related to the down-regulation because KT5926, an inhibitor of the kinase, did not affect the reaction. Further studies are required to identify the kinase involved.

Protein phosphorylation activates or inactivates various kinases and turns on/off switches of various cascades of protein phosphorylation<sup>25</sup>). Competition between staurosporine and tautomycin observed in Fig. 2 suggests that balanced reactions among protein kinases and protein phosphatases regulate the expression of surface antigens. Further study using tautomycin, specific inhibitor of protein phosphatases type-1 and -2A as well as okadaic acid<sup>18</sup>) and calyculin A<sup>26</sup>) will provide insights into the modulation of surface molecules by protein phosphorylation.

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