Increased Degradation of Newly Synthesized Interferon-γ (IFN-γ) in Anti CD3-Stimulated Lymphocytes Treated with Glycoprotein Processing Inhibitors

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As described previously (Kosuge T., Toyosima S., Biol. Pharm. Bull., 23, 1—5 (2000)), inhibitors of the glycoprotein processing enzymes, glucosidase I and II, induce decreased secretion of interferon-γ (IFN-γ) into culture supernatants of anti CD3-stimulated lymphocytes, and in the present study the mechanism has been investigated in further detail.

The processing inhibitors did not affect intracellular levels of IFN-γ but enhanced the degradation of newly synthesized IFN-γ in anti CD3-stimulated lymphocytes. Furthermore, since the stability of N-glycosylated proteins is known to be regulated by lectin family chaperones, such as calnexin, a type I transmembrane protein located in the endoplasmic reticulum (ER), and calreticulin, a soluble protein in the ER lumen, the effect of the processing inhibitors on the interaction of IFN-γ with calnexin and calreticulin was investigated. It was found that IFN-γ formed complexes with calnexin and calreticulin in anti CD3-stimulated lymphocytes. Total binding of IFN-γ to calnexin was not affected but that to calreticulin was increased in anti CD3-stimulated lymphocytes treated with the processing inhibitors. However, binding of newly synthesized IFN-γ to calreticulin was decreased in the lymphocytes under the same conditions as above. These results suggest that these glycoprotein processing inhibitors block the release of IFN-γ from already formed calreticulin complexes, which prevents the binding of newly synthesized IFN-γ to calreticulin and results in the enhancement of IFN-γ degradation.

Key words glycoprotein processing; interferon-γ(IFN-γ); chaperone; calnexin; calreticulin

It is known that regulation of cellular immune response is mediated by cytokines secreted from activated T cells. Most of the cytokines are glycoproteins and their carbohydrate moieties are thought to contribute to their functions, however, their role in the function is not well understood. Wall et al.1) and Bowlin et al.2) investigated the role of N-linked oligosaccharides on T cell glycoproteins by using drugs that inhibit enzymes in the processing pathway of N-linked oligosaccharides on glycoproteins, and found that sarsino- nine (SWN), an inhibitor of processing mannosidase II, enhanced the proliferative response of T cells to mitogens and interleukin-2 (IL-2) production. The precise mechanism of SWN-mediated enhancement of IL-2 production is not known, but it appears that the inhibition of N-linked oligosaccharides processing of IL-2 does not induce the enhancement of IL-2 production, since previous investigators have reported that no N-linked oligosaccharide linkage sequence is involved in the primary structure of murine IL-2.3) On the other hand, we have found recently that inhibitors of the processing enzymes, glucosidase I and II, induce the decreased secretion of cytokines with N-linked oligosaccharides, which results in the reduction of T cell function.4) This result suggests that the inhibition of N-linked oligosaccharide processing on cytokines affects the secretion process into culture supernatants of T cells. However, since intracellular cytokines in activated T cells treated with inhibitors of processing glucosidases I and II were not reduced or increased, it did not seem that the inhibitors directly suppressed cytokine secretion into the culture medium.

In the present study, the mechanism of the decreased secretion of interferon-γ (IFN-γ), a cytokine with N-linked oligosaccharides, in lymphocytes treated with the inhibitors of glycoprotein processing was further investigated. First, the effect of the inhibitors on IFN-γ degradation in activated lymphocytes was studied, and then the effect of the inhibitors on the interaction of IFN-γ with lectin family chaperones, such as calnexin, a type I transmembrane protein located in the endoplasmic reticulum (ER),5) and calreticulin, a soluble protein of the ER lumen,6) was studied, because lectin family chaperones are known to control the quality of N-glycosylated proteins, such as stability and intracellular transportation.

MATERIALS AND METHODS

Culture Medium RPMI 1640 (ICN, Aurora, OH) supplemented with 10% fetal calf serum (FCS), 5×10⁻⁴ M 2-mercaptoethanol (2-ME), 50 U/ml penicillin and 50 µg/ml streptomycin was used in all experiments for T cell stimulation. Methionine free RPMI 1640 (ICN, Aurora, OH) supplemented with 10% dialyzed-FCS, 5×10⁻³ M 2-ME, 50 U/ml penicillin and 50 µg/ml streptomycin and 150 mCi/ml TRAN ³⁵S-label (ICN, Aurora, OH) was used for ³⁵S-metabolic labeling of IFN-γ.

Antibodies and Reagents Anti-CD3ε (145-2C11, hamster IgG) antibody, which specifically stimulates T cells, was purchased from Hornby (Ontario, Canada). Anti-IFN-γ (R4-6A2, rat IgG₁) antibody as a capture antibody for immuno-precipitation and anti-IFN-γ(XMG1.2, rat IgG₁) antibody as a detection antibody for Western-blot analysis were purchased from Pharmingen (San Diego, CA). Anti-calnexin antibody was purchased from StressGen (Victoria, Canada) and anti-calreticulin antibody from Affinity Bioreagents (Golden, CO). The glycoprotein processing inhibitors, 1-deoxyxojirimycin (dNM), 1-deoxyxojirimycin (dMAN) and SWN were purchased from Sigma (St. Louis, MO) and N-methyl-1-deoxyxojirimycin (NMeDMN) from Wako (Osaka, Japan). Aprotinin and Nonidet P-40 were purchased from Wako and
Nacalai Tesque (Kyoto, Japan), respectively.

Stimulation of T Cells
The spleen cells or thymocytes from 6- to 10-week-old female C57BL/6 mice were stimulated with plate-coated anti-CD3 antibody (25 ng/well) in flat-bottom 24-well microplates. Cells (10^7 cells/well) were incubated at 37°C for 24 h in the presence or absence of a glycoprotein-processing inhibitor, dNM (3 mM), NMdNM (3 mM), dMAN (3 mM), and SWN (30 μM).

Measurement of Intracellular IFN-γ Degradation
After 24 h stimulation with plate-coated anti-CD3 antibody, spleen cells were washed with methionine-free RPMI 1640 medium. Then, the cells were cultured at 37°C for 24 h to pulse with [35S] TRAN. For chase experiments, the pulse-labeled cells were washed twice with 35S-free RPMI 1640 medium and cultured at 37°C for 2 more hours in RPMI 1640 medium. After three times wash with RPMI 1640 medium, the cells were lysed in a lysis buffer containing 2% Nonidet P-40, 1 mM EDTA and freshly added 10 μg/ml aprotinin in Tris-buffered saline, pH 7.8 (TBS). After incubation at 4°C for 1 h, nuclei were removed by centrifugation, and post-nuclear lysates were precleared with proteinG-Sepharose beads (Amersham Pharmacia Biotech, Uppsala, Sweden). The beads were removed by centrifugation, and the supernatants were incubated with anti IFN-γ antibody (R4-6A2) for 90 min at 4°C. Then, a 50% suspension of proteinG-Sepharose beads was added to the mixture of the supernatants and anti IFN-γ antibody, and incubated for 90 min at 4°C. After 5 times wash with TBS, the beads were boiled in a reducing gel buffer for 10 min at 95°C to elute proteins. Samples were analyzed by electrophoresis on 10% slab gels, and the gels were autoradiographed using a Bioimaging Analyzer BAS-2000. For semi-quantitative analysis of IFN-γ on the autoradiograph, the relative intensity of photo-stimulated luminescence (PSL) per given area of IFN-γ band was also calculated by BAS-2000.

Western Blot Analysis
After 24 h stimulation with anti-CD3 antibody, the cells were solubilized in lysis buffer as described above. The lysates were precleared as indicated above, and the supernatants were incubated with anti-catenin or anti-calreticulin antibodies. Immunoprecipitates analyzed by electrophoresis on 10% slab gels were transferred to Hybond ECL membranes (Amersham Life Science, Buckinghamshire, UK). After 1 h blocking, membranes were incubated with anti IFN-γ antibodies conjugated to biotin for 1 h, washed three times with TBS/0.05% Tween-20 (TBS/T), and incubated for 30 min with streptavidin-conjugated horseradish peroxidase (PharMingen, San Diego, CA). Following five times wash with TBS/T, the membranes were applied to a ECL Western blotting analysis system (Amersham Life Science).

Interaction of Calreticulin with Newly Synthesized IFN-γ
Spleen cells stimulated with plate-coated anti-CD3 antibody for 24 h were washed with methionine-free RPMI 1640 medium. The cells were then further incubated at 37°C for 24 h to label IFN-γ with [35S] TRAN. After three times wash with RPMI 1640 medium, the cells were solubilized in lysis buffer and the lysates were precleared as indicated above. The supernatants were incubated with anti-calreticulin antibody for 90 min at 4°C. Then, a 50% suspension of proteinG-Sepharose beads was added to the mixture of the supernatants and anti-calreticulin antibody, and incubated for 90 min at 4°C. After 5 times wash with TBS, the beads were boiled in a reducing gel buffer for 10 min at 95°C to elute proteins. Samples were analyzed by electrophoresis on 10% slab gels, and the gels were autoradiographed using a Bioimaging Analyzer BAS-2000. For semi-quantitative analysis of IFN-γ on the autoradiograph, PSL per given area of IFN-γ band was calculated by BAS-2000.

RESULTS AND DISCUSSION
Enhancement of Intracellular IFN-γ Degradation by Processing Glucosidase Inhibitors
Previously, we reported that inhibitors of processing glucosidase I and II caused the accumulation of glucose containing high-mannose-type oligosaccharides and induced decreased secretion of cytokines with N-linked oligosaccharides into culture supernatants of anti CD3-stimulated lymphocytes. If the inhibitors directly suppressed the cytokine secretion process, intracellular cytokines should be increased. However, the intracellular cytokines in anti CD3-stimulated lymphocytes treated with inhibitors were not increased. This result suggests that the inhibitors enhance cytokine degradation by an unknown mechanism. Therefore, to investigate the mechanism of the decreased secretion of IFN-γ, a cytokine with N-linked oligosaccharides, the effects of glycoprotein processing inhibitors on the degradation of newly synthesized IFN-γ was studied. For the measurement of IFN-γ degradation, anti CD3-stimulated lymphocytes were incubated in a medium containing [35S] methionine to label newly synthesized IFN-γ in the presence or absence of the inhibitors and after washing, chased for 2 h at 37°C in [35S] methionine free medium. Added inhibitors did not affect IFN-γ biosynthesis (Fig. 1A), but the glucosidase inhibitors enhanced IFN-γ degradation (Fig. 1B). This result was confirmed by comparing PSL of IFN-γ bands with each other (Table 1). NMdNM, an in-

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Fig. 1. Effect of Inhibitors of Glycoprotein Processing on Degradation of Intracellular IFN-γ in Anti CD3-Stimulated Lymphocytes
The cells were cultured to pulse with [35S] methionine for 24 h with an inhibitor, NMdNM (lane 2), dNM (lane 3), dMAN (lane 4) or SWN (lane 5), or without an inhibitor (lane 1). The pulsed cells were chased for 2 h in [35S] methionine free medium (B), or not chased (A).
hbinator of processing glucosidase I, and dNM, an inhibitor of processing glucosidase I and II, enhanced the degradation of newly synthesized IFN-γ but dMAN, an inhibitor of processing mannosidase I, or SWN, an inhibitor of processing mannosidase II, did not (Fig. 1B and Table 1B). This result corresponded with the previous result that processing glucosidase inhibitors induced decreased secretion of IFN-γ but processing mannosidase inhibitors did not. These results suggest that processing glucosidase inhibitors enhance the degradation of newly synthesized IFN-γ in the cell, which results in decreased IFN-γ secretion into culture supernatants.

**Binding of IFN-γ with Lectin Family Chaperones, Calnexin and Calreticulin**

Chaperones control the quality of newly synthesized proteins, such as stability and intracellular transportation. Especially, glycoproteins with N-linked oligosaccharides are known to interact with lectin family chaperones, such as calnexin and calreticulin. Therefore, our results suggested that processing glucosidase inhibitors inhibited N-linked oligosaccharide processing on IFN-γ and reduced its interaction with lectin family chaperones. The interaction of IFN-γ with calnexin and calreticulin was therefore investigated. When complexes of intracellular IFN-γ and chaperones were immunoprecipitated with anti-IFN-γ antibody and analyzed by western blot using anti calnexin or calreticulin antibody, calnexin and calreticulin were not detected on the immunoblotted membrane (data not shown). However, when the immunoprecipitations were performed by an anti calnexin or calreticulin antibody and anti IFN-γ antibody was used as a detection antibody in immunoblot analysis, IFN-γ was detected on the immunoblotted membrane (Fig. 2). IFN-γ was associated with both calnexin (Fig. 2A) and calreticulin (Fig. 2B) in anti CD3-stimulated lymphocytes. This result corresponds to the reports that calnexin and calreticulin share extensive sequence homology, and have similar binding selectivities to glycoproteins and physiological roles in ER.

Furthermore, the above result suggests that the decrease of IFN-γ association with calnexin and calreticulin reduces the stability of IFN-γ. Therefore, effects of processing glucosidase inhibitors on the binding of IFN-γ with calnexin and calreticulin were investigated. Contrary to our expectation, the inhibitors increased the binding of IFN-γ to calreticulin (Fig. 2B, lane 2 and 3) and did not affect that to calnexin (Fig. 2A, lane 2 and 3). Recently, it has been reported that calnexin and calreticulin differently interact with certain glycoproteins. Mouse MHC class I heavy chains only bind calnexin at the initial stage in ER, and at the late stage they only bind calreticulin. Also, the T cell receptor α subunit only binds calnexin in thymocytes, but in spleen T cells both calnexin and calreticulin. Our results also suggest that the characteristics of interaction of calnexin and calreticulin with IFN-γ are not always same. Detailed analysis of the interaction of IFN-γ with calnexin and calreticulin is now underway.

Moreover, recently, it has been reported that processing glucosidase inhibitors also block release of glycoproteins from already formed calnexin and calreticulin complexes. This observation suggests that the hydrolysis of glucose from glycosylated IFN-γ by glycosidases is needed for the release of the bound calnexin and calreticulin and the inhibitors block the release of IFN-γ from already formed calnexin and calreticulin complexes, which results in the blockage of newly synthesized IFN-γ binding to calnexin and calreticulin. To investigate this possibility, the effects of the processing glucosidase inhibitors on the binding of newly synthesized IFN-γ to calreticulin were studied. Newly synthesized IFN-γ was pre-labeled with [35S] methionine and its binding to calreticulin was compared in the presence and absence of the inhibitors. As shown in Fig. 3 and Table 2, the glucosidase inhibitors, NMDNM and dNM, suppressed the binding of newly synthesized IFN-γ to calreticulin.

In conclusion, we suggest that processing glucosidase inhibitors suppress the binding of newly synthesized IFN-γ to the lectin family chaperones by blocking the release of IFN-γ

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**Table 1. Effect of Inhibitors of Glycoprotein Processing on Degradation of Intracellular IFN-γ in Anti CD3-Stimulated Lymphocytes**

<table>
<thead>
<tr>
<th></th>
<th>None(1)</th>
<th>NMDNM(1)</th>
<th>dNM(1)</th>
<th>dMAN(1)</th>
<th>SWN(1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: Pulse: 24 h, Chase: 0 h</td>
<td>1489.2</td>
<td>1308.4</td>
<td>1311.4</td>
<td>1438.7</td>
<td>1443.5</td>
</tr>
<tr>
<td>B: Pulse: 24 h, Chase: 24 h</td>
<td>984.8</td>
<td>262.4</td>
<td>273.3</td>
<td>991.4</td>
<td>964.7</td>
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</table>

a) None, NMDNM, dNM, dMAN and SWN correspond to lanes 1, 2, 3, 4 and 5 in Fig. 1A (A) and 1B (B), respectively. b) PSL represents the relative intensity of photo-stimulated luminescence per given area of IFN-γ band in BAS-2000.

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**Fig. 2. Effect of Inhibitors of Glycoprotein Processing on Association of Calnexin and Calreticulin with IFN-γ in Anti CD3-Stimulated Lymphocytes**

Immunoprecipitations were performed by anti calnexin antibody (αCNXx) (A), or anti calreticulin antibody (αCRT) (B), and anti IFN-γ antibody (αIFN-γ) was used as a detection antibody. Lane 1, control; lane 2, NMDNM; lane 3, dNM; lane 4, dMAN.

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**Fig. 3. Effect of Inhibitors of Glycoprotein Processing on Binding of Newly Synthesized IFN-γ with Calreticulin in Anti CD3-Stimulated Lymphocytes**

The cells were cultured to pulse with [35S] methionine for 24 h with an inhibitor, NMDNM (lane 2), dNM (lane 3) or dMAN (lane 4), or without an inhibitor (lane 1). Immunoprecipitations were performed by anti calreticulin antibody.
Table 2. Effect of Inhibitors of Glycoprotein Processing on Binding of Newly Synthesized IFN-γ with Calreticulin in Anti CD3-Stimulated Lymphocytes

<table>
<thead>
<tr>
<th></th>
<th>None (a)</th>
<th>NMdNM (b)</th>
<th>dNM (c)</th>
<th>dMAN (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSL of IFN-γ</td>
<td>2809.7</td>
<td>504.2</td>
<td>447.6</td>
<td>2380.4</td>
</tr>
</tbody>
</table>

(a) None, NMdNM, dNM and dMAN correspond to lanes 1, 2, 3 and 4 in Fig. 3, respectively. 
(b) PSL represents the relative intensity of photo-stimulated luminescence per given area of IFN-γ band in BAS-2000.

from already formed complexes with chaperones and enhance the degradation of newly synthesized IFN-γ.

REFERENCES