Effect of Inhibitors of Glycoprotein Processing on Cytokine Secretion and Production in Anti CD3-Stimulated T Cells

Takashi KOSUGE,^a Toshiki TAMURA,^b Hideo NARIUCHI,^b and Satoshi TOYOSHIMA^{*,a}

Department of Biochemistry, Faculty of Pharmaceutical Sciences, Hoshi University,^a 2–4–41 Ebara, Shinagawa-ku, Tokyo 142–8501, Japan and Department of Allergology, The Institute of Medical Science, the University of Tokyo,^b 4–6–1 Shiroganedai, Minato-ku, Tokyo 108–0071, Japan. Received June 10, 1999; accepted September 14, 1999

We have investigated the effect of inhibitors of glycoprotein processing on cytokine secretion and production in anti CD3-stimulated T cells to elucidate the role of carbohydrate in the triggering of T cell function. The inhibitors of glycoprotein processing, especially mannosidase inhibitors, enhanced the anti CD3-induced production of interleukin-2 (IL-2), which is a cytokine without the linkage sequence of *N*-linked oligosaccharides. On the other hand, *N*-methyl-1-deoxynojirimycin (NMdNM, an inhibitor of processing glucosidase I), 1-deoxynojirimycin (dNM, an inhibitor of processing glucosidase I and II) and bromoconduritol (BCD, an inhibitor of processing glucosidase II) inhibited the secretion of interleukin-4 (IL-4), interferon- γ (IFN- γ), or interleukin-5 (IL-5) into culture supernatants of anti CD3-stimulated T cells, which had *N*-linked oligosaccharides. Mannosidase inhibitors, 1-deoxymannojirimycin (dMAN, an inhibitor of processing mannosidase I) and swainsonine (SWN, an inhibitor of processing mannosidase II) did not inhibit the secretion or production of IL-4, IFN- γ and IL-5. To confirm the inhibition of *N*-linked oligosaccharide processing in the cytokines by the above inhibitors, the binding of IFN- γ to lectins with various sugar-binding specificities was investigated. All inhibitors reduced the binding of IFN- γ to PHA E4, which had a high affinity to bi- or tri-antennary complex type *N*-linked oligosaccharides with bisecting *N*-acetylglucosamine. Similarly, all inhibitors reduced the binding of IFN- γ to PHA L4, which had high affinity to tri- or tetra-antennary complex type *N*-linked oligosaccharides with β 1-6-linked branching. SWN and dMAN increased the binding of IFN- γ to concanavalin A (ConA), which had a high affinity to bi-antennary complex type, hybrid type and high-mannose type *N*-linked oligosaccharides. These results suggest that the processing inhibitors used here inhibit the *N*-linked oligosaccharide processing of cytokines, and the inhibition of processing enzyme glucosidases I and II induces a decreased secretion of cytokines with *N*-linked oligosaccharides.

Key words glycoprotein processing; cytokine; anti CD3 antibody; T cell

It has been known that T cells largely regulate immune responses by secreting cytokines. Most of the cytokines are glycoproteins but the role of their carbohydrate moieties is not well understood and is of considerable interest in understanding how the sugar chain contributes to glycoprotein function.

In the present study, we investigated the role of carbohydrate moieties of cytokines by using drugs that inhibit enzymes in the processing pathway of *N*-linked oligosaccharides on glycoproteins. The addition of an *N*-linked oligosaccharide to proteins is initiated by the transfer of a large oligosaccharide, Glc₃Man₉GlcNAc₂, to certain asparagine residues on the protein from a lipid carrier as a Glc₃Man₉GlcNAc₂-diphosphodolichol. The transferred oligosaccharide is processed by glucosidases, mannosidases and glycosyltransferases prior to final maturation.^{1,2)}

Wall *et al.* reported that swainsonine (SWN) enhanced the proliferative response of T cells to antigen and to mitogen.³⁾ In addition to the proliferation, SWN has been reported to enhance concanavalin A (Con A)-induced lymphocyte interleukin-2 (IL-2) receptor expression and IL-2 production.^{4,5)} These reports suggest that the accumulation of glycoproteins bearing high-mannose or hybrid types of *N*-linked oligosaccharide enhances IL-2 production and IL-2R expression and results in the increased proliferative response in T cells. The mechanism of this enhancement is unknown. However, it seems that the accumulation of high-mannose oligosaccharide-containing glycoproteins, except IL-2, induces increased responsiveness to Con A, since IL-2 does not have the linkage sequence (Asn-X-Ser/Thr) of *N*-linked oligosaccha-

rides,⁶⁾ and Con A is a mitogenic lectin with high affinity to high-mannose type *N*-linked oligosaccharides. Therefore, in addition to IL-2, we studied the effect of various kinds of processing enzyme inhibitors on the production and secretion of cytokines bearing *N*-linked oligosaccharides such as interferon- γ (IFN- γ), IL-4 and IL-5 in anti CD3-stimulated T cells, and found that the inhibitors of processing glucosidases and mannosidases enhanced anti CD3-induced IL-2 production, and that the inhibitors of processing glucosidase I and II caused an accumulation of glucose-containing highmannose-type oligosaccharides and suppressed the secretion of IL-4, IFN- γ and IL-5.

MATERIALS AND METHODS

Culture Medium RPMI 1640 (ICN, Aurora, OH, U.S.A.) supplemented with 10% fetal calf serum (FCS), 5×10^{-5} M 2-ME, 50 U/ml penicillin and 50 mg/ml streptomycin was used in all experiments for T cell stimulation.

Antibodies and Reagents Anti-CD3 ϵ (145-2C11, hamster IgG) ascites, which specifically stimulate T cells, was purchased from Hornby (Ont., Canada). For sandwich ELISA, anti-IL-2 (JES6-1A12, rat IgG2a), anti-IFN- γ (R4-6A2, rat IgG1), anti-IL-4 (11B11, rat IgG1) and anti-IL-5 (TRFK5, rat IgG1) antibodies as capture antibodies, and anti-IL-2 (JES6-5H4, rat IgG2a), anti-IFN- γ (XMG1.2, rat IgG1), anti-IL-4 (BVD6-24G2, rat IgG1) and anti-IL-5 (TRFK4, rat IgG2a) antibodies as detection antibodies were purchased from PharMingen (San Diego, CA, U.S.A.). The compounds, 1-deoxynojirimycin (dNM),^{7,8)} 1-deoxymanno-

* To whom correspondence should be addressed.

jirimycin (dMAN)^{7,9} and swainsonine (SWN)^{4,7,10} were purchased from Sigma (St. Louis, MO, U.S.A.), bromoconduritol (BCD) from Toronto Research Chemicals, Inc. (Ont., Canada) and *N*-methyl-1-deoxynojirimycin (NMdNM)^{11,12} from Wako (Osaka, Japan). Neuraminidase (from *Streptococcus* sp.), PNA-agarose, ConA-agarose, PHA E4-agarose and PHA L4-agarose were purchased from Seikagaku Corp. (Tokyo, Japan).

Stimulation of T Cells The spleen cells from 6- to 10-week-old female C57BL/6 mice were stimulated with plate-coated anti-CD3 antibody, a selective stimulator for T cells, in flat-bottomed 24-well microplates. The dose of anti-CD3 to induce the maximum response of the T cells was determined to be 25 ng/well in preliminary experiments. Cells (10^7 cells/well) were incubated at 37 °C for 2 d in the presence or absence of a glycoprotein-processing inhibitor, dNM (3 mM), NMdNM (3 mM), BCD (0.5 mM), dMAN (3 mM) or SWN (30 μ M).

Mouse helper T cell clones, 35-9D (Th1 cell clone) and D10.G4.1 (Th2 cell clone), were established as described previously.^{13,14} T cell clones (3×10^5 cells/ml) were stimulated with plate-coated anti CD3 (25 ng/well) in the presence or absence of a glycoprotein-processing inhibitor, dNM (0.5 mM), NMdNM (0.5 mM), BCD (0.1 mM), dMAN (0.5 mM) or SWN (6 μ M). Since T cell clones were more sensitive to the processing inhibitors than spleen cells, they were added to T cell clone cultures at a lower concentration.

Measurement of Secreted and Intracellular Cytokines in Anti CD3-Stimulated T Cells After 2 d incubation, the cells were lysed with freezing and thawing. Then, the cell lysates were centrifuged and the supernatants were collected as soluble cell lysates, which contained intracellular components. Cytokines in the culture supernatants and the soluble cell lysates were assayed by ELISA.

Binding of IL-2 to PNA and IFN- γ to Various Lectins After 2 d incubation of anti CD3-stimulated T cells with glycoprotein processing inhibitors, culture supernatants were collected. The culture supernatants were incubated with PNA,¹⁵ ConA,¹⁶ PHA E4¹⁷ and PHA L4¹⁸ agarose gels at 4 °C for 12 h. Before incubation with PNA-agarose, the culture supernatants were treated with neuraminidase (37.5 mU/ml). After centrifugation, IL-2 and IFN- γ in the supernatants were measured by ELISA.

RESULTS AND DISCUSSION

Inhibitors of Glycoprotein Processing Enhance IL-2 Production in Anti CD3-Stimulated T Cells It has been reported that SWN, an inhibitor of processing mannosidase II, enhances ConA-induced IL-2 production, IL-2 receptor expression and T cell proliferation.³⁻⁵ Since ConA is a mitogenic lectin with high affinity to bi-antennary complex type, hybrid type and high-mannose type *N*-linked oligosaccharides, the accumulation of oligosaccharides having an oligomannosyl core [Man₅(GlcNAc)₂] characteristic of neutral oligosaccharides and one or more NeuNAc-Gal-GlcNAc sequences characteristic of complex chains in SWN-treated cells¹⁹ may enhance the cellular response to ConA, suggesting that the SWN-induced enhancement of IL-2 production may be observed only in ConA-stimulated T cells. To examine this possibility, we investigated the effects of various in-

Fig. 1. Effect of Inhibitors of Glycoprotein Processing on Secreted- and Intracellular-IL-2 in Anti CD3-Stimulated T Cells

Spleen cells were stimulated with plate-coated anti CD3 antibody (2.5 ng/well) for 2 d alone or in the presence of NMdNM (1 mM), dNM (3 mM), BCD (0.5 mM), dMAN (3 mM) or SWN (30 μ M). Secreted IL-2 in culture supernatants and intracellular IL-2 in soluble cell lysates were measured by ELISA. Each column represents the mean $\pm$ S.D. of 3 experiments.

hibitors of glycoprotein processing on IL-2 production in anti CD3-stimulated T cells. Total IL-2 produced in anti CD3-stimulated cells was estimated by measuring secreted IL-2 into culture supernatants and intracellular IL-2 in the supernatants of cell lysates with ELISA. All inhibitors used in this experiment enhanced the secretion of IL-2 into culture supernatants by about two times (Fig. 1). Intracellular IL-2 was also increased by the treatment of lymphocytes with these inhibitors, especially processing mannosidase inhibitors, dMAN and SWN (Fig. 1). Although ConA-induced IL-2 production was enhanced only by SWN, an inhibitor of mannosidase II,⁴ anti CD3-induced IL-2 production was enhanced by all inhibitors used in the present experiment, inhibitors of glucosidase I, glucosidase I and II, mannosidase I and mannosidase II (Fig. 1). The results suggest that the processing glycosidase inhibitors enhance IL-2 production in anti CD3-stimulated T cells. The precise mechanism of this inhibitor-mediated enhancement of IL-2 production has been unknown and it does not seem that the inhibition of *N*-linked oligosaccharide processing of IL-2 induced the enhancement of IL-2 production, since previous investigators reported that no linkage sequence of *N*-linked oligosaccharides was involved in the primary structure of murine IL-2,⁶ and we also found that murine IL-2 did not bind *N*-linked oligosaccharide-binding lectins such as ConA, PHA E4 and PHA L4 (data not shown). These results suggest that the accumulation of high-mannose oligosaccharides-containing glycoproteins induces increased responsiveness to anti CD3 antibody, or, the inhibitors of processing glucosidases not only inhibit the maturation of *N*-linked oligosaccharides but also inhibit the maturation of *O*-linked oligosaccharides.

Therefore, to investigate whether the inhibitors affected *O*-linked oligosaccharide maturation, the binding ability of secreted IL-2 bearing *O*-linked oligosaccharides to peanut lectin (PNA), which bound *O*-linked oligosaccharides but not *N*-linked oligosaccharides,¹⁵ was studied (Fig. 2). The inhibitors did not affect the binding ability of IL-2 to PNA, suggesting that the inhibitors did not have an effect on *O*-linked oligosaccharide maturation of IL-2.

Inhibitors of Processing Glucosidases Suppress the Secretion of Cytokines Having *N*-linked Oligosaccharides in Anti CD3-Stimulated T Cells As shown in Fig. 1, in-

Fig. 2. Effect of Inhibitors of Glycoprotein Processing on IL-2 Binding to PNA-Agarose

Spleen cells were cultured as described in the legend of Fig. 1. The culture supernatants were treated with neuraminidase and then incubated with PNA-agarose. After centrifugation, the % of bound IL-2 was determined by ELISA. Each column represents the mean $\pm$ S.D. of 3 experiments.

Fig. 3. Effect of Inhibitors of Glycoprotein Processing on Secreted- and Intracellular-IL-4 in Anti CD3-Stimulated T Cells

Secreted- and intracellular-IL-4 were measured as described in the legend of Fig. 1. Each column represents the mean $\pm$ S.D. of 3 experiments.

hibitors of glycoprotein processing enhanced the total production of IL-2, which did not have *N*-linked oligosaccharides, by an unknown mechanism. Then, the effect of processing inhibitors on the production of IL-4, which is a cytokine with *N*-linked oligosaccharides,²⁰⁾ was investigated. Glucosidase inhibitors, NMdNM (an inhibitor of processing glucosidase I), dNM (an inhibitor of processing glucosidase I and II) and BCD (an inhibitor of processing glucosidase II), suppressed the secretion of IL-4 into the culture supernatants of anti CD3-stimulated T cells, but mannosidase inhibitors, dMAN (an inhibitor of processing mannosidase I) and SWN (an inhibitor of processing mannosidase II) did not. None of these inhibitors affected the intracellular IL-4 (Fig. 3). The results suggest that the glucosidase inhibitors suppress the secretion of IL-4 which have *N*-linked oligosaccharides in anti CD3-stimulated T cells.

Then, the effects of the processing inhibitors on the secretion and production of IFN- γ and IL-5, which are known to have *N*-linked oligosaccharides,^{21,22)} were investigated. The results were quite similar to those of IL-4 (Figs. 4 and 5). NMdNM, dNM and BCD suppressed the secretion of both cytokines but did not affect the intracellular cytokines. On the other hand, the processing mannosidase inhibitors did not affect the secreted or intracellular cytokines. These results suggest that the accumulation of glucosylated high-mannose type sugar chains by glucosidase inhibitor treatment induce the suppression of the secretion of cytokines having *N*-linked

Fig. 4. Effect of Inhibitors of Glycoprotein Processing on Secreted- and Intracellular-IFN- γ in Anti CD3-Stimulated T Cells

Secreted- and intracellular-IFN- γ were measured as described in the legend of Fig. 1. Each column represents the mean $\pm$ S.D. of 3 experiments.

Fig. 5. Effect of Inhibitors of Glycoprotein Processing on Secreted- and Intracellular-IL-5 in Anti CD3-Stimulated T Cells

Secreted- and intracellular-IL-5 were measured as described in the legend of Fig. 1. Each column represents the mean $\pm$ S.D. of 3 experiments.

oligosaccharides.

Recently, helper T cells have been separated into two subtypes, Th1 and Th2, according to their different profiles of cytokine production.²³⁾ IL-2 and IL-4 are cytokines selectively produced by Th1 and Th2 cells, respectively. Therefore, the processing inhibitors may selectively induce the enhancement of Th1 cytokine production but the processing glucosidase inhibitors may induce the suppression of Th2 cytokine secretion. However, glucosidase inhibitors suppressed the secretion of both IFN- γ and IL-5, which were Th1 cytokine and Th2 cytokine, respectively, suggesting that the processing inhibitors did not selectively act on Th1 and Th2 cells.

Inhibitors of Processing Glycosidases Suppress the Secretion of Cytokines Having *N*-Linked Oligosaccharides in Anti CD3-Stimulated Helper T Cell Clones CD3 forms a complex with T cell receptor (TcR) on T cells and transduces the activation signal of antigen-binding to TcR into T cells. Therefore, anti CD3 might selectively stimulate T cells and induce cytokine production in T cells. To confirm this possibility, the effect of glycoprotein-processing inhibitors on IFN- γ , a Th1 cytokine, and IL-4, a Th2 cytokine, secretions in anti CD3-stimulated mouse helper T cell clones, 35-9D (Th1 cell clone) and D10. G4. 1 (Th2 cell clone) was investigated. The results were quite similar to those in spleen cells (Fig. 6). The glucosidase inhibitors suppressed the secretion of IFN- γ and IL-4 into the culture supernatants of anti CD3-stimulated Th1 clone and Th2 clone, respectively, but the

Fig. 6. Effect of Inhibitors of Glycoprotein Processing on Secretion of IFN- γ (A) and IL-4 (B) in Anti CD3-Stimulated Th1 and Th2 Cell Clones, Respectively

Secreted IFN- γ and IL-4 were measured as described in the legend of Fig. 1. Each column represents the mean $\pm$ S.D. of 3 experiments.

Fig. 7. Effect of Inhibitors of Glycoprotein Processing on IFN- γ Binding to Con A-, PHA E4- and PHA L4-Agarose

Spleen cells were cultured as described in the legend of Fig. 1. The culture supernatants were incubated with Con A-, PHA E4- and PHA L4-agarose, and the % of bound IFN- γ to Con A ($\square$), PHA E4 ($\square$) and PHA L4 ($\blacksquare$) was determined. Each column represents the mean $\pm$ S.D. of 3 experiments.

mannosidase inhibitors did not. From these results, we conclude that the inhibition of glucosidases in the processing of *N*-linked oligosaccharides decreases the secretion of cytokines having *N*-linked oligosaccharides in T cells.

Effect of Processing Inhibitors on IFN- γ -binding to Various Lectins To confirm that the processing inhibitors suppress *N*-linked oligosaccharide processing of cytokines, we investigated the binding ability of IFN- γ in the culture supernatants from anti CD3-stimulated T cells to various lectins, PHA E4, PHA L4 and ConA. PHA E4 has high affinity to bi- or tri-antennary complex type *N*-linked oligosaccharides with bisecting *N*-acetylglucosamine. PHA L4 has a high affinity to tri- or tetra-antennary complex-type *N*-linked oligosaccharides with β 1-6-linked branching. Con A has high affinity to bi-antennary complex-type, hybrid-type and high-mannose type *N*-linked oligosaccharides.¹⁶⁻¹⁸⁾ The

binding of IFN- γ to PHA E4 and PHA L4 was decreased, but that to ConA was increased (Fig. 7). As described above, PHA E4 and PHA L4 have high affinity to complex-type *N*-linked oligosaccharides and ConA has high affinity to high-mannose-type *N*-linked oligosaccharides. Therefore, these results suggest that the inhibitors suppress *N*-linked oligosaccharide processing in IFN- γ secreted from anti CD3-stimulated T cells. Since processing inhibitors suppress the sugar chain processing of all glycoproteins having *N*-linked oligosaccharides, the *N*-linked oligosaccharide processing of IL-4 and IL-5 were thought to also be suppressed by the processing inhibitors used here.

In the present study, we showed that the inhibitors of glycoprotein processing caused an accumulation of immature *N*-linked oligosaccharides in IFN- γ , a cytokine having *N*-linked oligosaccharides, by using lectins with various specificities, and that the accumulation of glucose-containing high-mannose-type oligosaccharides induced a decreased secretion of cytokines having *N*-linked oligosaccharides. The mechanism of glucosidase inhibitor-mediated suppression of cytokine secretion is unknown. In some preliminary experiments, we found that intracellular IFN- γ forms a complex with unidentified proteins, which may be lectin-like chaperons such as calnexin²⁴⁻²⁷⁾ and which may have a role in the secretion and/or stability of cytokines containing *N*-linked oligosaccharides. The identification of the proteins forming a complex with IFN- γ is under investigation.

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