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Posttranslational Modification of Interleukin-2 is a Late Event During Activation of Human T Lymphocytes by Ionophore A23187 and Phorbol Ester

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Human peripheral blood lymphocytes secrete high titers of interleukin-2 (IL-2) after stimulation by Ca^{2+}-ionophore A23187/phorbol 12-myristate-13-acetate. During the first 30 hours of incubation cells secrete only the nonglycosylated IL-2 M form of the lymphokine, the glycosylated forms IL-2 $N_{1,2}$ being detected only after prolonged culture times (30-48 h). After recultivation of cells for a second 48 h period (without additional mitogen), the glycosylated and nonglycosylated IL-2 forms are secreted at a constant ratio of 7:3 throughout. The detection of glycosylated IL-2 is parallelled by an increase in cellular glycosyltransferase activities involved in formation of sialylated oligosaccharides O-linked to proteins.

One of the most important immunoregulatory events during T cell activation (initiated by antigenic stimulation or induced with plant lectins like concanavalin A (ConA) or phytohemagglutinin (PHA)) is the secretion of interleukin 2 (IL-2) which, after binding to its cell surface receptor is an absolute requirement for T cell proliferation [1].

We have previously reported [2] that human IL-2, secreted by normal peripheral blood lymphocytes after stimulation with calcium ionophore A23187/-phorbol 12-myristate-13-acetate (PMA), consists of a mixture of glycosylated protein forms (IL-2 $N_{1,2}$) and a nonglycosylated form (IL-2 M). IL-2 N_1 contains an O-linked NeuAcα2-3Galß1-3GalNAc oligosaccharide chain attached to threonine in position 3 of the polypeptide [2]. The IL-2 N_2 oligosaccharide bears an additional NeuAc which is α2-6 linked to GalNAc.

In the present work we have used different induction protocols for IL-2 biosynthesis in human peripheral blood T lymphocytes. We have analysed the patterns of secreted IL-2 and the time dependent changes in cellular glycosyltransferase activities that are involved in the formation of O-linked carbohydrate structures such as found in human IL-2.

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Material and Methods

CMP-N-acetyl[4,5,6,7,8,9-^{14}C]neuraminic acid (308mCi/mmol), UDP-D[^{14}C]galactose (258mCi/mmol) and UDP[^{14}C]N-acetylgalactosamine (60 mCi/mmol) were purchased from Amersham-Buchler (FRG, Braunschweig). Unlabelled nucleotide sugars, bovine submaxillary mucin, concanavalin A, phytohemagglutinin, ionophore A23187, and phorbol 12-myristate 13-acetate were from Sigma Chemicals Co. (FRG, München). Basic myelin protein was from Calbiochem. (FRG, Frankfurt).

Cell culture: Human mononuclear cells (≥ 90% T cells) were isolated from blood of healthy volunteers and were cultivated in RPMI 1640 medium at a cell density of $3x10^6$ cells/ml. The medium contained additionally 25mM N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid (pH 7.2), 0.5mM $CaCl_2$ and 10 μg each of neomycin and streptomycin. Cells were induced for IL-2 biosynthesis in the presence of either 75ng/ml A23187, 10μg/ml ConA, or 10μg/ml PHA in the presence of 20ng/ml PMA.

IL-2 assay: IL-2 bioactivity was measured as described [3] and units were related to the IL-2-Standard Reagent Preparation provided by the NCI Biological Response Modifiers Program.

Monoclonal antibodies against human IL-2: Female Balb c mice were immunized with purified human IL-2 [2]. Spleen cells were fused with X63 AG8653 cells. Five hybrid subclones were isolated which stably produced anti-hu-IL-2 IgG. Antibodies derived from culture supernatants or ascites fluid recognized all glycosylated and nonglycosylated human IL-2 forms in Western blots as well as recombinant human IL-2 from *E. coli* or mammalian cell lines. However, antibodies did not inhibit IL-2 bioactivity nor were they suited for immunoaffinity chromatography of IL-2 or for immunoprecipitation.

SDS-PAGE and Western Blotting: Proteins from IL-2 containing culture supernatants were precipitated with 5 vol. of -20°C acetone and resolubilized in SDS sample buffer [4] containing 5mM dithioerythreitol. Sample equivalents corresponding to 20-50μl (A23187/PMA stimulated cells) or up to 1 ml culture supernatant (lectin/PMA stimulated cells) were run on 15% SDS-polyacrylamide gels as described [4]. Protein transfer onto nitrocellulose was conducted using a modified method of Towbin [5]. After incubation with a mixture of monoclonal antibodies raised against purified human IL-2, IL-2 protein was visualized by horseradish peroxidase conjugated rabbit anti-mouse IgG.

Preparation of cell lysates: Cells were collected by centrifugation at 800xg for 5 min., washed with 50 vol. of ice-cold phosphate buffered saline and stored as frozen pellets for 1-3 months at -70°C. 10^9-10^{10} cells were thawed in 10mM MES buffer, pH 6.1, containing 1% Triton X-100 and were further disrupted in a Dounce homogenisator equipped with a tight fitting glass pestle (8 strokes). Particles were removed by centrifugation at 6500xg for 10 min. The supernatant was used for enzyme activity measurements.

Enzyme assays: **UDP-GalNAc**:polypeptide(α1-0)N-acetylgalactosaminyltransferase was assayed in a final volume of 100μl containing 100mM HEPES, pH 7.0, 20mM $MnCl_2$, 0.5% Triton X-100, 15μM UDP[^{14}C]GalNAc [0.2μCi], 40μg of basic myelin protein and cell lysate corresponding to 0.5-$1x10^7$ cells.

UDP-Gal:GalNAc-O-protein ß1-3 galactosyltransferase was tested as described [6].

CMP-NeuAc:R-GalNAc-O-protein α2-6 sialyltransferase was assayed using bovine submaxillary asialo-mucin as acceptor.

CMP-NeuAc:Galß1-3GalNAc-O-protein α2-3 sialyltransferase was assayed using Galß1-3GalNAC-protein (prepared as described [7]) as substrate.

CMP-NeuAc:Galß1-4R α2-3/6 sialyltransferase activity was determined with asialo-transferrin as acceptor. Assays contained in a final volume of 100μl 100mM N-morpholino etanesulfonic acid, pH 6.1, 25μM CMP-[^{14}C]NeuAc (0.07μCi), 0.5% Triton X-100 and 300-500μg of appropriate acceptor protein. After desired times aliquots from incubation mixtures were withdrawn and protein bound radioactivity was determined after precipitation with 1% phosphotungstic acid in 0.5N HCl (PTA) at 0°C and collected on glass fiber filters (Whatman GF/C, 2.4cm diam.). Filters were washed with 3x5 ml cold PTA and 10 ml of cold methanol. After drying filters were counted for

radioactivity in toluene based liquid scintillation coctail. [^{14}C]sialylated oligosaccharides were liberated from acceptor proteins by ß-elimination in the presence of $NaBH_4$ and were identified by HPLC-chromatography on NH_2-bonded phase according to [8].

Results

Glycosylated human IL-2 forms N_1, N_2, and nonglycosylated IL-2 M can be distinguished based on their different mobilities in SDS-PAGE as shown in Fig.1. Mono-sialylated IL-2 N_1 and di-sialylated IL-2 N_2 migrate as a closely spaced doublet of 16.5-17kd, the IL-2 M form has an apparent molecular mass of 14.5kd [2]. Upon limited digestion with *Vibrio cholerae* sialidase (Fig. 1, lane B) a protein band corresponding to mono-sialylated IL-2 is still detected and a new protein band of approximately 15.2kd is formed, representing the Galß1-3GalNAc-derivative of IL-2. Exhaustive sialidase treatment (as well as mild acid treatment) converts all of the IL-2 form into the new 15.2 kd band (Fig. 1, lane C).

IL-2 secretion by T cells after activation by calcium ionophore/PMA or lectins/PMA: T lymphocytes stimulated with optimal concentrations [9] of A23187/PMA secreted higher amounts of IL-2 activity than cells treated with PHA/PMA or ConA/PMA (Fig. 2). When A23187/PMA stimulated cells were resuspended in fresh medium after 40-48 h, they continued to secrete IL-2 (without addition of mitogen) for a second 48 h period at a rate comparable to the first incubation (Fig. 2, dotted line).

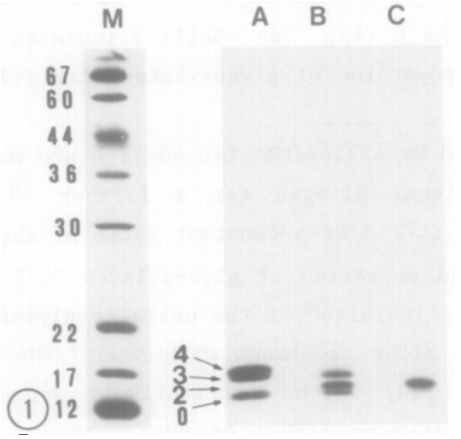

Figure 1. SDS/PAGE of purified human IL-2 from peripheral blood lymphocytes. (M) molecular weight standards; (A) untreated IL-2 (4=NeuAc α2-3 Galß1-3(NeuAcα2-6)GalNAc-O-IL-2; 3=NeuAc-α2-3Gal 1-3GalNAc-O-IL-2; 2=Galß1-3GalNAc-O-IL-2; 0=non-glycosylated IL-2); (B) after limited sialidase treatment; (C) after exhaustive sialidase treatment.

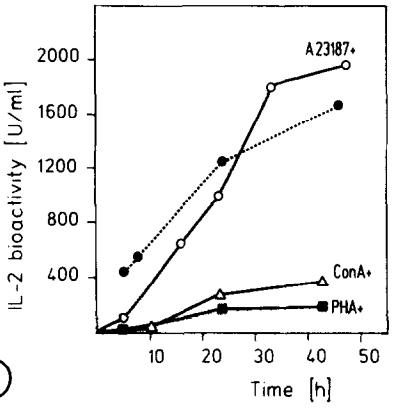

Figure 2. IL-2 secretion by human T cells after stimulation by o-o-o A23187/PMA; ▪-▪-▪ PHA/PMA; Δ-Δ-Δ ConA/PMA: •-•-• IL-2 secretion by cells that were recultivated in fresh mitogen-free medium after a 48 h prestimulation period.

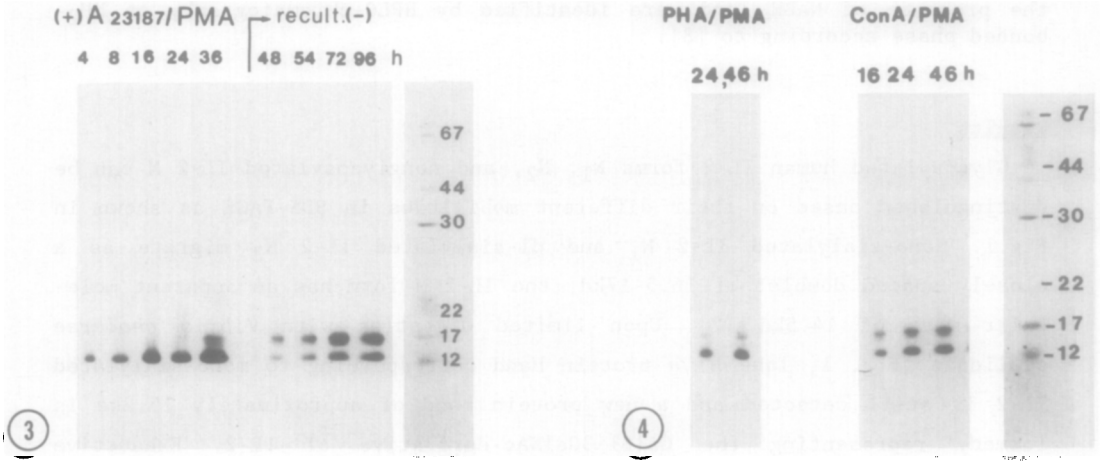

Figure 3. Western blot analysis of IL-2 from supernatants of stimulated T cells in the presence of A 23187/PMA (left) and cells that were subsequently incubated for a second period in the absence of mitogen (right).

Figure 4. Western blot analysis of IL-2 secreted by T cells after stimulation by PHA/PMA and ConA/PMA.

Detection of IL-2 protein forms by Western blotting: Immuno blot analysis of IL-2 secreted by T cells after stimulation by A23187/PMA revealed that during the first 24 h in culture, cells secrete almost exclusively the non-glycosylated IL-2 M form (Fig. 3A). The glycosylated IL-2 $N_{1,2}$ proteins appeared in the medium only after 30-40 h of incubation. When incubated in the presence of PHA/PMA, T lymphocytes synthesized IL-2 N and IL-2 M at a constant ratio of about 2:8 for up to 48 h (Fig. 3B). Cells stimulated by ConA/PMA typically secreted a higher proportion of glycosylated IL-2 (Fig. 3C).

T lymphocytes that were prestimulated by A23187/PMA for 40-48 h and were recultivated in medium without additional mitogen for a further 48 h released glycosylated IL-2 N forms and IL-2 M at a constant ratio of about 7:3 (compare Fig. 3B). That the delayed secretion of glycosylated IL-2 by ionophore/PMA stimulated cells is due to inhibiton of the cellular glycosylation machinery seems unlikely, since after treatment with A23187/PMA of genetically engineered mouse L cells [10] which constitutively secrete glycosylated and nonglycosylated human IL-2 no effect on the synthesis of either protein form was observed (H.S. Conradt and H. Hauser, manuscript in preparation).

The results of the Western blot analysis confirms our previous finding that no glycosylation intermediates (Galß1-3GalNAc- or GalNAc-O-IL-2) are secreted by stimulated human T cells [2] which would have been readily detected by the methodology applied (compare Fig. 1, lane B)(Fig. 4).

Table 1 Glycosyltransferase Activities in Lysates from Induced and Noninduced T Cells

Transferase[a]	Sugar Incorporation (cpm/10 cells/hour)[b]						
	noninduced	A23187/PMA				ConA/PMA	
		24h	40h	72h	96h	24h	48h
1.GalNAc-T	1900	2030	6800	7600	7100	2500	2800
2.ß1-3Gal-T	3100	2800	9400	12000	n.a.	n.a.	n.a.
3.α2-3NeuAc-T[c]	2750	2600	11000	13500	12300	3100	4800
4.α2-6NeuAc-T	n.d.	820	1100	1470	1800	950	1050
5.α2-3/6NeuAc-T	1750	1650	3400	4500	n.a.	1000	2900

a. Acceptor proteins used for reaction: 1= basic myelin protein; 2= bovine asialo-mucin; 3= Galß1-3GalNAc-protein; 4= bovine asialo-mucin; 5= asialo-transferrin.
b. Breakdown of nucleotide sugars was < 15 % during incubation; transfer to endogenous acceptors subtracted; n.d.= not detected, n.a.= not assayed.
c. [^{14}C]NeuAcα2-3Galß1-3GalNAc-ol quantitated after ß-elimination in the presence of $NaBH_4$ and identified by HPLC on NH_2-phase [8].
*values for lysates prepared from cells stimulated by A23187/PMA for 48h and recultivated for a second time period (in the absence of mitogen).

Glycosyltransferase activities in induced and noninduced human T lymphocytes: As shown in Table 1, the activities of glycosyltransferases involved in formation of the NeuAcα2-3Galß1-3(NeuAcα2-6)GalNAc carbohydrate structure of human IL-2 are detectable in lysates of noninduced cells except for the GalNAc-protein α2-6 sialyltransferase activity (with asialo-mucin as substrate). Stimulation of cells by A23187/PMA, however, led to significantly increased enzyme levels at 48 h after induction. Similar high levels of all glycosyltransferase activities were measured in lysates of cells that were recultivated for an additional 48 h period in the absence of mitogen (values 72 and 96 h in Table 1). Cell lysates from ConA/PMA stimulated lymphocytes contained about 1/2 of the glycosyltransferase activities at 48 h as compared to ionophore/PMA induced cells. Comparable values were obtained for PHA/PMA induced lymphocytes (data not shown).

Discussion

Activation of T cells by mitogenic lectins or antigens involves a series of intra- and intercellular reactions which ultimately lead to differentiation and proliferation of lymphocytes. Early events during T cell activation include a rapid rise in cytosolic calcium concentration and activation of

protein kinase C [1] followed by expression of several proliferation relevant genes, e.g. IL-2 (2-3 h after induction), IL-2 receptor (6 h) and immunoregulatory signal factors like Interferon-γ (16-36 h) (compare Ref. 11-16). These reactions can also be triggered by stimulation of cells with calcium ionophore A23287 in combination with phorbol myristrate acetate [13,14].

From the results reported in the present paper we conclude that after exposure of T cells to lectins and PMA only lymphocyte subsets bearing certain cell surface receptors which specifically bind ConA or PHA are induced to synthesize IL-2 (resulting in rather low levels of secreted IL-2). The ratio of glycosylated IL-2 N and nonglycosylated IL-2 M detected in culture supernatants therefore reflects the carbohydrate synthesizing capacity of the individual stimulated T cell population.

The less specific mitogen A3187/PMA triggers IL-2 synthesis and secretion also in additional T cell subpopulations including a great majority of cells lacking the proper glycosyltransferases which are involved in IL-2 carbohydrate biosynthesis [17]. High levels of IL-2 are secreted and detected as early as at 3 h after induction, however preponderantly as the nonglycosylated IL-M form. Cells first acquire the capacity to glycosylate substantial amounts of IL-2 36-48 h after stimulation as is evident by the detection of the IL-2 N forms in Western blots as well as the detection of significantly increased levels in glycosyltransferase activities at that time. However, a significant amount of nonglycosylated IL-2 obviously escapes glycosylation. Similarly, when human IL-2 is produced in genetically engineered heterologous mammalian cell lines, IL-2 N and IL-2 M forms are secreted at about the same ratio from ionophore/PMA stimulated T cells at 48-96 h (H. Hauser and H.S. Conradt, manuscript in preparation) with the exception for chinese hamster ovary cells [9] which produce less than 10% of nonglycosylated IL-2 M.

Secretion of IL-2 by T lymphocytes is an early event in activation, (detected as early as at 3 h after stimulation) the maturation of the cellular glycosylation machinery is obviously a late event during T cells activation by A23187/PMA. Polypeptide: (α1-0)GalNAc-transferase activity, the enzyme that initiates O-glycosylation of proteins shows significantly increased levels only at late times after stimulation of the cells. Since mixing experiments with lysates from induced and noninduced cells did not reveal any inhibitory activity in the latter we speculate that elevated enzyme levels are due to novo protein synthesis.

It will be interesting to investigate whether the enzymes involved in formation of N-linked oligosaccharides will be subjected to similar changes in activity. A good candidate for the analysis of this phenomenon at the level of N-glycosylated secretory proteins from activated human T cells will

be the human IFN-γ protein which has been shown to be secreted in at least three different posttranslationally modified forms by stimulated lymphocytes [18].

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