Glycoconjugate Journal (1998) **15:** 69–74

EGC2
Development of glycosylated human interleukin-1*a*, neoglyco IL-1*a*, by coupling with D-galactose monosaccharide: biological activities *in vitro*

Sachi Nabeshima[1]*, Taku Chiba[1], Yutaka Takei[1], Shiro Watanabe[2], Harumi Okuyama[2] and Kikuo Onozaki[1]

[1]*Departments of Hygienic Chemistry and* [2]*Biological Chemistry, Faculty of Pharmaceutical Sciences, Nagoya City University, Tanabe, Mizuho, Nagoya 467 Japan*

In the previous study, galactose with C9 spacer was chemically coupled to human recombinant (rh) IL-1*a* in order to study the effect of glycosylation on its activities, and to develop IL-1 with less deleterious effects. In this study we examined a variety of IL-1 activities *in vitro*, including proliferative effect on T cells, antiproliferative effect on myeloid leukemic cells and melanoma cells, stimulatory effects on IL-6 synthesis by melanoma cells and PGE_2 synthesis by fibroblast cells Galactose-introduced IL-1*a* (Gal-IL-1*a*) exhibited reduced activities from 10 to 10000 times compared with unmodified IL-1*a* in all the activities performed *in vitro*. The competitive binding of ^{125}I-IL-1*a* to mouse T cells and pre-B cells with unlabeled IL-1*a* s suggests a decrease in binding affinities of Gal-IL-1*a* to both type I and type II IL-1 receptors. Therefore, reduced activities of Gal-IL-1*a* are due, at least partially, to the decrease in their receptor binding affinities.

***Keywords*: neoglycoprotein, interleukin 1, cytokine**

Introduction

Human IL-1 is a nonglycosylated cytokine mainly produced by macrophages and monocytes, although murine IL-1 may be glycosylated. IL-1 exhibits pleiotropic effects on various cell types [1]. IL-1 is potentially useful for therapy because of its antitumor effect and protective effect on animals against microorganism infection, radiation and chemotherapy. IL-1 also may be used against hyperglycemia. However, because of its proinflammatory activity, IL-1 exhibits serious deleterious effects, including endogenous pyrogen activity, induction of other proinflammatory cytokines and prostanoids, tissue injury and hypotension.

Few attempts have been made to chemically introduce carbohydrates into a biological active protein. Before our study only superoxide dismutase has been coupled with carbohydrates to alter the tissue distribution [2, 3]. In a previous study, we chemically synthesized D-Manα(1-6)Man [$Man_2\alpha$(1-6)] conjugated-human recombinant interleukin-1α (IL-1α), neoglyco IL-1α, to determine the effect of glycosylation on its activities and to develop IL-1 with less deleterious effects [4]. $Man_2\alpha$(1-6)-IL-1α exhibited impairment in biological activities in all the experiments *in vitro* and in receptor binding capacity compared with intact IL-1α. However $Man_2\alpha$(1-6)-IL-1α exhibited exhibited selective activities *in vivo* [5]. $Man_2\alpha$(1-6)-IL-1α exhibited very low activities in pyrogenicity and induction of serum α_1-acid glycoprotein and IL-6 compared with untreated IL-1α. However, $Man_2\alpha$(1-6)-IL-1α possessed the same potency as untreated IL-1α in reduction of serum levels of glucose and triglyceride, and the recovery of peripheral white blood cells in 5-fluorouracil-treated mice. Furthermore, the tissue distribution of $Man_2\alpha$(1-6)-IL-1α in mice differed from that of intact IL-1α [6]. As the level of glycosylated IL-1α decreased as compared to that of untreated IL-1α in kidney, in contrast, it increased in liver; these differences in tissue distribution were considered to contribute to the selective activities *in vivo*.

Lectins specific to galactose/*N*-acetyl galactosamine (Gal/GalNAc) have been isolated from rat [7, 8] and human hepatocyte [9], and are transmembrane proteins that participate in receptor-mediated endocytosis. The macrophage lectin specific to Gal/GalNAc is immunologically related to the hepatic lectin [10]. In addition, it has been reported that Gal/GalNAc-specific C-type lectin is expressed on the cell surface of inflammatory macrophages and on activated tumoricidal macrophages. The binding of activated or

*To whom correspondence should be addressed. Tel: + 81-52-836-3419; Fax: + 81-52-836-3419; E-mail: konozaki@phar.nagoya-cu.ac.jp.

0282–0080

inflammatory macrophages to tumor cells through the Gal/GalNAc-specific lectin is suggested to play an important role in the tumoricidal process [11, 12]. Therefore, it was expected that introduction of galactose into IL-1 may alter the activity through its interaction with Gal/GalNAc-specific lectin *in vivo*. In this report, we studied the biological activities of Gal-IL-1α and its receptor binding ability.

Materials and methods

Reagents

RPMI 1640, bovine serum albumin (BSA), and polymixin B were purchased from Sigma Chemical Co. (St-Louis, MO). Eagle's MEM was from Nissui Pharmaceutical Co. (Tokyo, Japan). Fetal bovine serum (FBS) was from JRH Biosciences (Lenexa, KS). Human recombinant IL-1α (rhIL-1α) (2×10^7 U ml^{-1}) was provided by Dr M. Yamada of Dainippon Pharmaceutical Co. (Osaka, Japan). Human recombinant IL-6 (rhIL-6) was provided by Dr Y. Akiyama of Ajinomoto Co. (Osaka, Japan).

Cell culture

D10H.2 is a subclone of the mouse T cell line D10(N4)M provided by Dr S. Hopkins (University of Manchester) [13] and its proliferation depends on IL-1 in the absence of any exogenous cytokine or mitogens. D10H.2 cells were maintained in culture medium (RPMl 1640, 100U ml^{-1} penicillin G, 100 μg ml^{-1} streptomycin, 15 mM HEPES) supplemented with 5×10^{-5} M 2-mercaptoethanol, 10% heat-inactivated FBS, and 5 U ml^{-1}rhIL-1α. Murine hybridoma MH60.BSF2 cells provided by Dr T. Hirano (University of Osaka) were maintained in culture medium supplemented with 10% FBS and 1 U ml^{-1}rhIL-6 [14]. Murine pre-B lymphocyte 70Z/3.12 was purchased from the American Type Culture Collection and maintained in culture medium supplemented with 5×10^{-5} M 2-mercaptoethanol and 10% FBS. A375-6 is an IL-1 sensitive subclone of human melanoma cell line A375 which was originally given by Dr R. Ruddon (NCl, Rethesda, MD). The mouse lymphoma cell line EL-4 6.1 C10 was provided by Dr T. Akahoshi (University of Kitazato). The mouse myeloid cell line M1 was provided by Dr K. S. Akagawa (National Institute of Health, Tokyo, Japan), and IL-1 sensitive clone M1-3b was obtained by limiting dilution. A375-6, EL-4 6.1 C10, and M1-3b were maintained in culture medium supplemented with 10% FBS. Human fibroblast cell line TIG-1 were provided by the Japanese Cancer Research Resorces Bank (Tokyo, Japan). TIG-1 cells were maintained in culture medium (Eagle's MEM) supplemented with 10% FBS.

Assay for D10H.2 proliferation

Cultured D10H.2 cells were washed three times with IL-1 free culture medium. Fifty microliters of cell suspension (2×10^5 cells per ml) were added to each flat-bottomed well of a 96 well microtiter plate (Falcon, Lincoln, NJ). Fifty microliters of medium containing IL-1s were added, and then the cells were cultured for 72h at 37 °C in 5% CO_2 in air. Proliferation of the cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [15].

Assay for M1-3b growth inhibition

Cultured M1-3b cells were washed three times with culture medium. Fifty microliters of cell suspension (2×10^5 cells per ml) with 5 μg ml^{-1} polymixin B were added to each flat-bottomed well of a 96 well microtiter plate (Falcon, Lincoln, NJ). Polymixin B was added to the culture to prevent the effect of contaminant endotoxin which inhibits M1-3b cell proliferation. Fifty microliters of medium containing IL-1s were added, and then the cells were cultured for 72 h at 37 °C in 5% CO_2 in air. Proliferation of the cells was determined by MTT method [15]. The percentage of cell growth was calculated as follows:

$$\% \text{ of Control} = \frac{\text{O.D.595 of cells cultured in the presence of samples}}{\text{O.D.595 of cells cultured in the presence of medium alone}} \times 100$$

Assay for A375-6 growth inhibition

A375-6 cells were detached from culture dish with 0.02% EDTA-PBS. The cells were washed with the culture medium and 100 μl of cell suspension (4×10^4 cells per ml) were added to each well of a 96 well microtiter plate. After 24 h culture at 37 °C in 5% CO_2 in air, 100 μl of medium containing IL-1s were added, and the plates were incubated for another 72 h under the same conditions. The cell growth was determined by the crystal violet-staining method [16]. After solubilization of the dye-staining the absorbance at 595 nm was determined using an ELISA autoreader (Bio-Rad Laboratories, Richmond, CA). The percentage of cell growth was calculated as the same method as M1-3b.

Assay for IL-6 activity produced by A375-6 cells

A375-6 cells were cultured at 1×10^5 cells per ml for 24 h with varying concentrations of IL-1s. IL-6 activity in the supernatant was determined using the proliferation-IL-6 of dependent MH60·BSF2 cells as described [14]. IL-6 activity was expressed as the equivalent amount of rhIL-6.

Assay for PGE_2 released from TIG-1 cells

TIG-1 cells were cultured at 1×10^5 cells per ml for 24 h with varying concentrations of IL-1s. Prostaglandin E_2 (PGE_2) in the supernatants was converted into PGB_2 by alkaline treatment, and quantitated by competitive immunoassay using a rabbit anti-PGB_2 antibody and a biotin-PGB_2 conjugate as tracer [17].

^{125}I-IL-1α binding assay

(3-[^{125}I]iodotyrosyl)IL-1α (81.01 TBq mmol^{-1}) was purchased from Amersham (Aylesbury, UK). EL-4 6.1 C10 cells (1×10^6) or 70Z/3.12 cells (7.3×10^5) were incubated at 4 °C for 1 h in a total volume of 0.2 ml RPMI 1640 containing 1 mg ml^{-1} BSA, 22.75 pg and 2.8 ng of ^{125}I-IL-1α, respectively, and varying concentrations of unlabeled rhIL-1αs. The free and bound radioactivity was separated by the binding oil column method [18] and measured with a γ-counter (Aloka, Tokyo, Japan).

Determination of protein content

The amount of protein was determined using a protein assay kit (Bio-rad, Richmond, CA) with bovine serum albumin as a standard.

Results

IL-1 proliferative effect on T cells

Biological activities of Gal-IL-1α were compared with those of untreated IL-1α, and control (treated) IL-1α. T cell proliferation-stimulating activity was determined by using the IL-1-dependent mouse T cell clone D10H.2 [13]. The cells were cultured for 4 days with or without IL-1s, and then the cell proliferation was determined. As shown in Figure 1, control IL-1 exhibited the same or a greater strength of activity than untreated IL-1. In contrast, Gal-IL-1 exhibited about 1/30 activity of untreated IL-1.

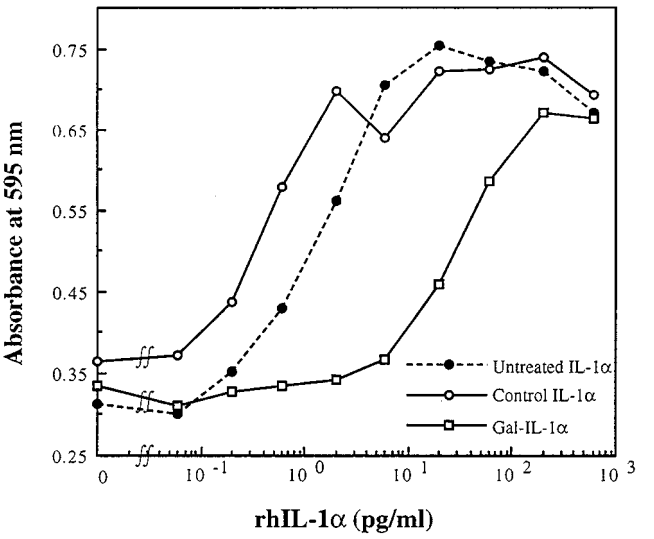

Figure 1. Effect of IL-1 on proliferation of D10H.2 cells. D10H.2 cells were cultured at 37 °C for 4 days with or without varying doses of IL-1*a* s. After culture, cell proliferation was determined by the MTT method.

IL-1 antiproliferative effect on melanoma cells and myeloid leukemic cells

IL-1 inhibits the growth of mouse myeloid leukemic cells M1 and human melanoma cells A375-6 [19, 20]. To determine the IL-1 antiproliferative activity, M1 cells and A375-6 cells were treated with or without IL-1s for 4 and 3 days, respectively, and then cell proliferation was determined. As shown in Figure 2A, control IL-1 exhibited the same

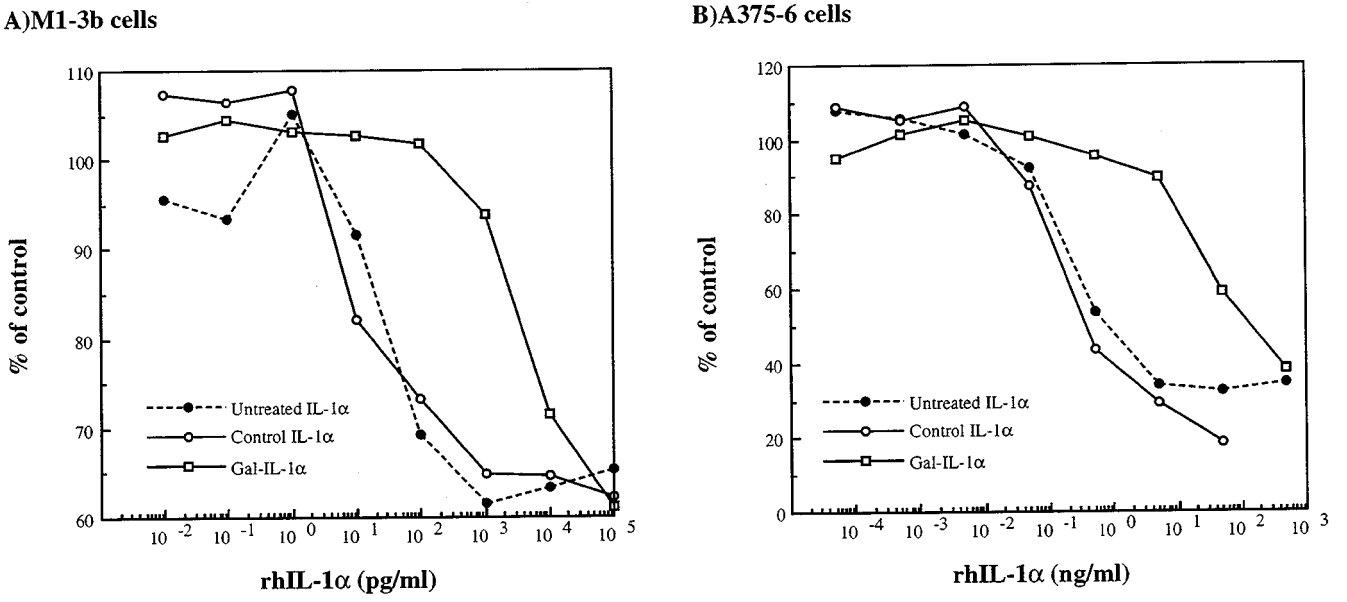

Figure 2. Antiproliferative effect of IL-1 on M1 cells(A), and A375-6 cells(B). (A) Mouse myeloid leukemic cells, M1-3b, were cultured at 37 °C for 4 days with or without varying doses of IL-1*a* s. After culture, cell proliferation was determined by MTT method. (B) Human melanoma cells, A375-6, were cultured at 37 °C for 3 days with or without varying doses of IL-1*a* s. After culture, cells were stained with crystal violet.

strength of activity as untreated IL-1. Gal-IL-1 exhibited activity at about 1/100 of untreated IL-1. Control IL-1 also exhibited the same strength of activity as untreated IL-1 in inhibition of A375-6 cell proliferation (Figure 2B). The activity of Gal-IL-1 decreased to about 1/100 of untreated IL-1.

IL-1 induction of IL-6 by melanoma cells

To evaluate the IL-6 inducing activity of IL-1 (21), A375-6 cells were cultured with or without IL-1s for 24 h, and then the amount of IL-6 in the culture supernatants was determined. As shown in Figure 3, control IL-1 exhibited the same potency as untreated IL-1. Gal-IL-1 exhibited activity at about 1/10 of untreated IL-1.

IL-1 stimulation of PGE_2 production by fibroblast cells

To evaluate the stimulating activity of PGE_2 production by fibroblast cells [22], human fibroblast cell line TIG-1 cells were cultured with or without IL-1s for 24 h, and then the amount of PGE_2 in the culture supernatants was determined (Figure 4). Control IL-1 activity was the same as that of untreated IL-1. Gal-IL-1 exhibited activity below 1/10 000 of untreated IL-1.

Competitive binding of ^{125}I-IL-1α to T cells and B cells with unlabeled IL-1s

T cells and B cells preferentially express Type 1 and Type II IL-1 receptor (IL-1R) respectively [23, 24]. To determine the ability of IL-1s to bind type 1 IL-1R the competitive binding of ^{125}I-IL-1α and unlabeled IL-1 to mouse T cell line cells (EL-4) was examined. As shown in Figure 5A, control IL-1 exhibited binding activity at the same level of untreated IL-1. The binding activity of Gal-IL-1 was about 1/500. Using mouse preB cell line 70/3.12 cells, the binding activity of IL-1 to Type II IL-1R was examined. As shown in Figure 5B, the binding activity of control IL-1 to Type II IL-1R was again at the same level as untreated IL-1. In contrast, the activity of Gal-IL-1 was about 1/100 of untreated IL-1.

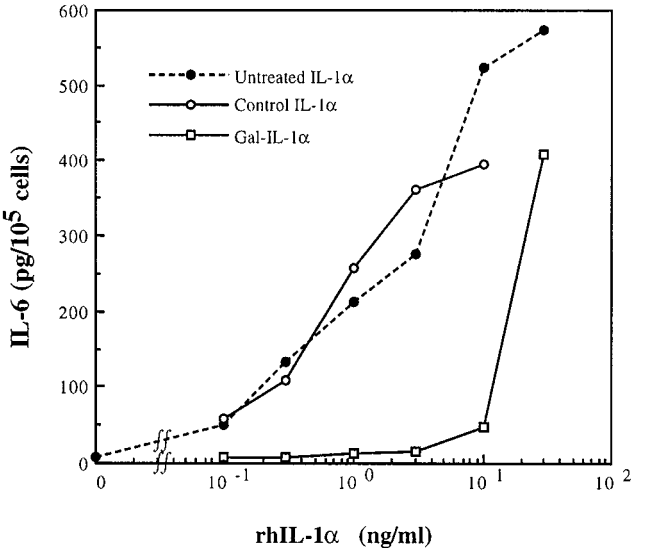

Figure 3. Effect of IL-1 on the IL-6 production by A375-6 cells. A375-6 cells were cultured with or without varying doses of IL-1*a* s. After 24 h culture, IL-6 activity in the supernatants was determined by the proliferation of IL-6 dependent MH60·BSF2 cells.

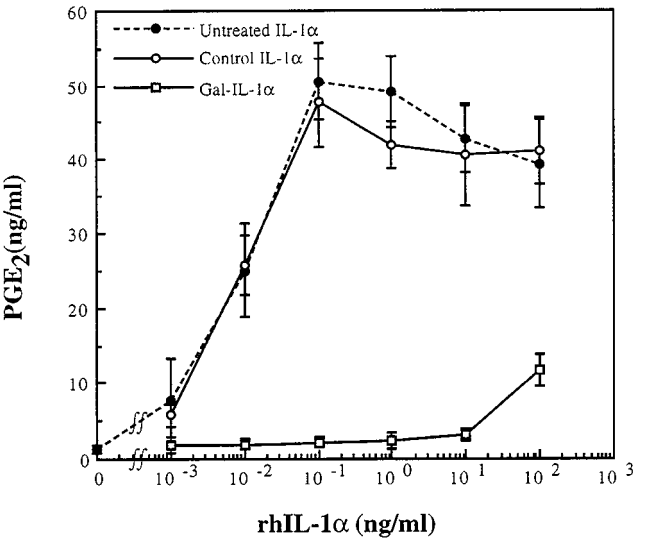

Figure 4. Effect of IL-1 on the PGE_2 production by TIG-1 cells. Human fibroblast cells, TIG-1, were cultured with or without varying doses of IL-1*a* s. After 24 h culture, the amount of PGE_2 in the supernatants was determined by EIA,

Discussion

In this study Gal-IL-1 exhibited a reduction in all the biological activities examined *in vitro*. However, as control IL-1 exhibited the same strength of activities as untreated IL-1, the coupling condition did not appear to affect the IL-1 activity. This was the same in the case of mannose dimer introduction into rhIL-1α [4]. It is of note, however, that the magnitude of the reduction of biological activities of Gal-IL-1 varied depending on the assay. Least reduction was 1/10 in IL-6 induction by melanoma cells, and most pronounced reduction was $<1/10000$ in PGE_2 induction by fibroblast cells. Similar varied reduction of biological activities was also observed in mannose dimer-introduced IL-1α. In that case, however, the least reduction was 1/10 to 1/30 in T cell proliferation activity, and most marked reduction was IL-6 induction by melanoma cells (Table 1). Of particular interest is the stimulation of IL-6 production by melanoma cells. The reduction was maximum in $Man_2\alpha$(1-6)-IL-1 and least

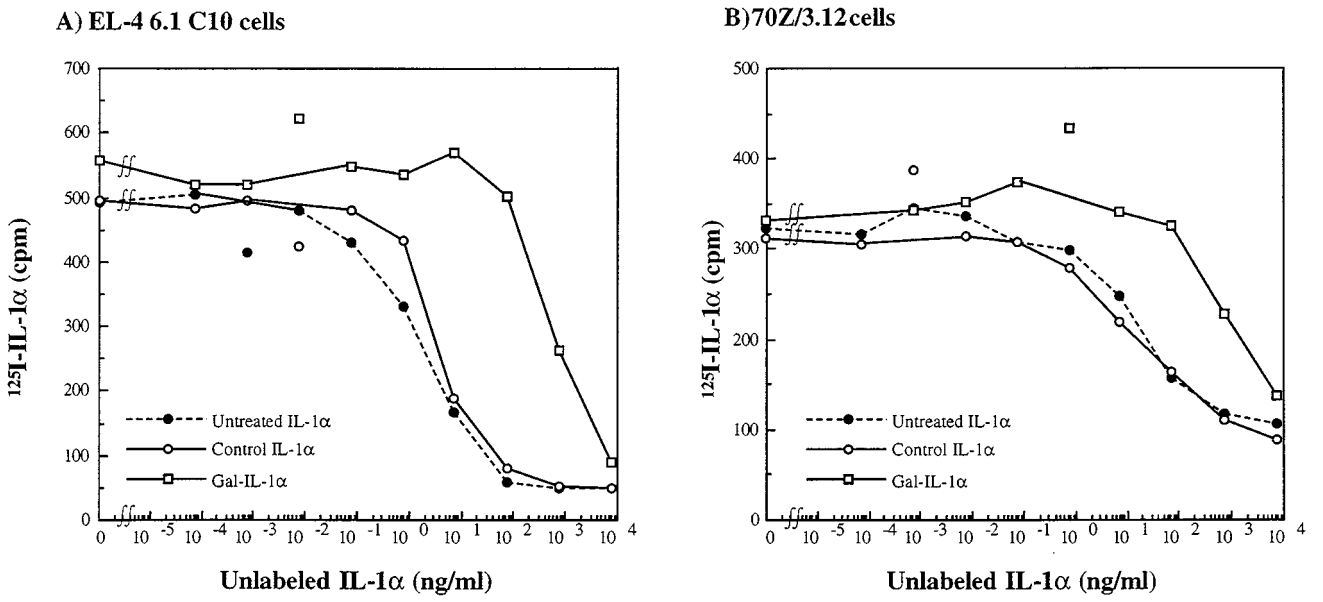

Figure 5. Inhibition of ^{125}I-IL-1*a* binding to EL-4 6.1 C10 cells (A), and 70Z/3.12 cells (B) by IL-1*a*. Mouse T cell line EL-4 6.1 C10 cell and mouse pre-B cell line 70Z/3.12 cells were incubated with ^{125}I-IL-1*a* in the presence of varying doses of unlabelled IL-1*a*s for 1 h at 4 °C. The free and bound radioactivity was separated by the binding oil column method.

Table 1. Comparison of biological activities *in vitro* of carbohydrate-introduced IL-1*a*s.

Coupled carbohydrate	*Man_2a (1,4)*	*Man_2a (1,6)*	*Gal*
Molecules of carbohydrates introduced into molar of IL-1*a*	4.7	5.2	9.1
T cell proliferative activity	1/10	1/30	1/30
Antiproliferative effect			
on M1	1/20	1/100	1/100
on A375	1/150	1/300	1/100
IL-6 induction	<1/1000	<1/1000	1/10
PGE_2 induction	1/100	1/700	<1/10000
Affinity to type I IL-IR	1/400	1/700	1/500
type II IL-IR	ND	1/600	1/100

*Activities compared to untreated IL-1*a*.
ND, not determined.

in Gal-IL-1α. Therefore, the reduction rate appeared to be dependent on the species of carbohydrates introduced into IL-1. The reduction rate was not correlated with the number of introduced carbohydrates, because Man_2α(1-4), Man_2α(1-6) and Gal coupled to IL-1 averaged at 4.7, 5.2 and 9.1 mol mol^{-1} respectively. Although we did not determine the region where carbohydrates were introduced, the coupling method is the same, namely amino residues of IL-1 react with the carbohydrates. Probably the combination of the region, species and the size of the introduced carbohydrates will be important.

IL-1 action is mediated through its specific receptor on the cell surface. There are two types of IL-1 receptor (IL-IR) cloned from many cell types. Type I IL-IR with a molecular weight (MW) of 80 kDa is mainly expressed on T cells and fiblobrast cells [23], and type II IL-IR with a MW of 60 kDa is mainly expressed on macrophages, bone marrow cells, and B cells [24]. Type I IL-IR functions to deliver IL-1 signals into cells. However, type II IL-IR is unable to transduce the IL-1 signal [25]. It is reported that in macrophages, bone marrow cells and B cells a small number of type I IL-IR receptors is expressed that transduce IL-1 signals. Type II IL-IR regulates IL-1 action by impairing IL-1 binding to type I IL-IR.

The most likely explanation of the reduced activity is due to the reduction in binding affinity to IL-IR. Gal-IL-1α exhibited reduced binding affinity to both type I and type II IL-IRs. The reduction to type I IL-IR was more marked. It is possible that carbohydrate introduction caused conformational changes that lead to the decreased binding affinity, or that carbohydrate interfered with IL-1 binding to IL-IR. However, the reduction in binding affinity alone cannot explain the decrease in biological activity, because T cell proliferation activity was reduced to only 1/30, while affinity to type I IL-IR decreased to 1/500. Therefore, the reduced affinity to IL-IR can only partially explain the reduced activity. It is possible that the varied reduction in the biological activity of Gal-IL-1α was due to the varied ratio of type I IL-IR/type II IL-IR expressed in each cell type. Or

other molecules associated with the IL-IR/IL-1 complex may be responsible for the varied reduction of biological activities depending on cell types. Recently IL-1R associated protein (IL-IR AcP) and IL-IR associated Ser/Thr kinase(IRAK) were cloned [26, 27].

It is interesting that by coupling different carbohydrates to a biologically active protein the nature of the alteration of biological activity differed. Therefore, this study suggests that a particular biological activity can be manipulated even *in vitro*.

Acknowledgements

We thank Dr H. Hayashi for valuable suggestions and Mr M. Hayashi for technical assistance. This work was supported in part by grants from the Ministry of Education, Science and Culture of Japan and The Research Foundation for Parmaceutical Science.

References

1 Dinarello CA (1996) *Blood* **87**: 2095–147.
2 Fujita T, Nishikawa M, Tamaki C, Takakura Y, Hashida M, Sezaki H (1992) *J Pharmacol Exp Ther* **263**: 971–78.
3 Fujita T, Furitsu H, Nishikawa M, Takakura Y, Sezaki H, Hashida M (1992) *Biochem Biophys Res Commun* **189**: 191–96.
4 Takei Y, Wada K, Chiba T, Hayashi H, Ishihara H, Onozaki K (1994) *Lymphokine Cytokine Res* **13**: 265–70.
5 Takei Y, Wada K, Chiba T, Hayashi H, Yamada M, Kuwashima J, Onozaki K (1995) *Lymphokine Cytokine Res* **15**: 713–19.
6 Takei Y, Yang D, Chiba T, Nabeshima S, Naruoka M, Wada K, Onozaki K (1996) *J Interferon Cytokine Res* **16**: 333–36.
7 Drickamer K, Mamon JF, Binns G, Leuhg JO (1984) *J Biol Chem* **259**: 770–78.
8 Holland EC, Leung JO, Drickamer K (1984) *Proc Natl Acad Sci USA* **81**: 7338–42.
9 Spiess M, Lodish HF (1985) *Proc Natl Acad Sci USA* **82**: 6465–6469.
10 Oda S, Sato M, Toyoshima S, Osawa T (1988) *J Biochem* (*Tokyo*) **104**: 600–5.
11 Oda S, Sato M, Toyoshima S, Osawa T (1989) *J Biochem* (*Tokyo*) **105**: 1040–43
12 Sato M, Kawakami K, Osawa T, Toyoshima S (1992) *J Biochem* (*Tokyo*) **111**: 331–36.
13 Hopkins SJ, Humphreys M (1989) *J Immunol Methods* **120**: 271–76.
14 Matsuda T, Hirano T, Kishimoto T (1988) *Eur J Immunol* **18**: 951–56.
15 Mosmann T (1983) *J Immunol Methods* **65**: 55–63.
16 Ruff MR, Gifford GE (1980) *J Immunol* **125**: 1671–77.
17 Watanabe S, Yoshida C, Kobayashi T, Okuyama H (1987) *Bio Pharm Bull* (in press).
18 Endo Y, Matsushima K, Oppenheim JJ (1986) *Immunobiol* **172**: 316–22.
19 Onozaki K, Tamatani T, Hashimoto T, Matsushima K (1987) *Cancer Res*., **47**: 2397–402.
20 Onozaki K, Matsushima K, Aggarwal BB, Oppenheim JJ (1995) *J Immunol* **135**: 3962–68.
21 Elias JA, Lentz V (1990) *J Immunol* **145**: 161–66.
22 Takii T, Akahoshi T, Kato K, Hayashi H, Marunouchi T, Onozaki K (1992) *Eur J Immunol* **22**: 1221–27.
23 Sims JE, Acres RB, Grubin CE, McMahan CJ, Wignall JM, March CJ, Dower SK (1989) *Proc Natl Acad Sci USA* **86**: 8946–50.
24 McMahan CJ, Slack JL, Masley B, Cosman D, Lupton SD, Brunton LL, Grubin CE, Wignall JM, Jenkins NA, Brannan Cl, Copeland NG, Huebner K, Croce CM, Cannizzarro LA, Benjamin D, Dower SK, Sproggs MK, Sims JE (1991) *EMBO J* **10**: 2821–32.
25 Colotta F, Dower SK, Sims JE, Mantovani A (1994) *Immunol Today* **15**: 562–66.
26 Greenfeder SA, Nunes P, Kwee L, Labow M, Chizzonite RA, Ju G (1995) *J Biol Chem* **270**: 13757–65.
27 Cao Z, Henzel WJ, Gao X (1996) *Sciesce* **271**: 1128–31.

Received 25 December 1996, Revised 17 February 1997, accepted 19 February 1997