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Biotinylation of interleukin-2 (IL-2) for flow cytometric analysis of IL-2 receptor expression

Comparison of different methods

Marg O. De Jong [a,*], Henk Rozemuller [a], Jan G.J. Bauman [b], Jan W.M. Visser [c]

[a] *Department of Hematology, Erasmus University Rotterdam, Rotterdam, Netherlands*
[b] *E.C. Slater Institute, University of Amsterdam, Amsterdam, Netherlands*
[c] *The Laboratory of Stem Cells, Lindsley F. Kimball Research Institute, New York Blood Center, New York, USA*

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Abstract

The main prerequisites for the use of biotinylated ligands to study the expression of growth factor receptors on heterogeneous cell populations, such as peripheral blood or bone marrow, by flow cytometric methods, are that the biotinylated ligand retains its binding ability and that binding of the biotinylated ligand to the receptor does not inhibit the subsequent interaction of biotin with fluorescently tagged avidin or streptavidin. Using interleukin-2 (IL-2), we compared the usefulness of various biotinylation reagents, NHS-biotin, S-NHS-biotin, S-NHS-LC-biotin, DBB and photobiotin, and developed optimal biotinylation conditions for the preparation of biologically active biotin-labeled IL-2 and the detection of IL-2 receptor expressing cells by flow cytometry. As determined by spot blot analysis, biotinylation of IL-2 was most efficient at the highest biotin-to-protein (B:P) ratio used. At a B:P ratio of 100, most of the biological activity of IL-2 was retained when S-NHS-LC-biotin was used. In contrast, most of the biological activity of IL-2 samples that were labeled with NHS-biotin or photobiotin was lost under these conditions. Biotin-labeled IL-2 preparations were tested in order to detect IL-2 receptors on IL-2 dependent CTLL-2 cells by flow cytometry after sequential staining with the biotinylated IL-2 and fluorescence tagged streptavidin. A high B:P ratio generally resulted in a high specific fluorescence intensity of the cells, particularly when S-NHS-LC-biotin was used as the biotinylation reagent. Biotin-IL-2 could also be used to detect IL-2 receptors expressed by lymphocytes in peripheral blood and bone marrow. Comparison of staining of lymphocytes with biotinylated IL-2 and an antibody against the IL-2 receptor α chain demonstrated that only a subset of the cells that showed a strong fluorescence signal after staining with biotinylated IL-2 expressed high numbers of the IL-2 receptor α chain. This is in agreement with the expression of functional IL-2 receptors on resting T cells and NK cells which do not express the α chain.

Abbreviations: B:P ratio, biotin:protein ratio; FACS, fluorescence activated cell sorter; GF, growth factor; IL-2, interleukin-2; NHS-biotin, *N*-hydroxy succinimide ester of biotin; S-NHS-biotin, *N*-hydroxysulfo-succinimide ester of biotin; S-NHS-LC-biotin, sulfosuccinimidyl-6-(biotinamido) hexanoate; DBB, *p*-diazobenzoyl biocytin precursor; photobiotin, photoactivatible biotin; PBL, peripheral blood lymphocytes; PHA, phytohemagglutinin.

* Corresponding author. At: Department of Hematology, Room Ee1387A, Erasmus University, P.O. Box 1738, 3000 DR Rotterdam, Netherlands. Tel.: +31.10.4087876; Fax: +31.10.4362315.

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After stimulation with PHA, virtually all lymphocytes expressed the α chain, whereas only part of these cells showed a strong fluorescence signal after staining with biotin-IL-2, while the rest of the cells had very low numbers of IL-2 binding sites. Our results demonstrate that, in addition to staining individual receptor subunits with antibodies, staining with biotinylated IL-2 is a useful indicator of functional IL-2 receptor expression.

Keywords: Biotin; Growth factor; Receptor; IL-2; Flow cytometry

1. Introduction

Growth factors control the growth, differentiation and maturation of hemopoietic cells as well as, in many cases, the functional activity of mature end cells, by binding to specific cell surface receptors. To examine the target cell specificity of growth factors (GF) and the presence of GF receptors, in vitro culture systems are used to study biological responses (Sonoda et al., 1988; Warren et al., 1989; Ishida et al., 1991), as well as direct methods to study receptor expression. Direct receptor labeling studies can be performed using radiolabeled GF (Foxwell et al., 1988a; Nicola et al., 1988; Park et al., 1989) with the advantage that only functionally intact GF receptors are labeled. However, because ^{125}I-labeled GF are usually quantified as mean number of receptors per cell, these studies are of limited use for heterogeneous cell populations. Receptor expression on subsets of cells in heterogeneous populations can be studied using a fluorescence activated cell sorter (FACS) when cells are labeled with antibodies against the receptors (Weil-Hillman et al., 1990), and double-stained for other markers. However, many receptors consist of several different subunits, and the expression of more than one subunit is often required to generate functional, high affinity receptors (Takeshita et al., 1992b; Ishii et al., 1994; Nakamura et al., 1994; Nelson et al., 1994). In addition, some receptors share subunits, e.g. the high affinity receptors for human IL-3, GM-CSF and IL-5 (Kitamura et al., 1991; Tavernier et al., 1991), and the high affinity receptors for IL-2, IL-4 and IL-7 (Kawahara et al., 1994; Kondo et al., 1993; Noguchi et al., 1993; Russell et al., 1993). Since antibodies usually recognize only one of the constituents of a receptor complex, antibody binding may not always be indicative of expression of functional receptors.

Instead of antibodies or radiolabeled GF, biotinylated GF may be used (Foxwell et al., 1988b; Yamasaki et al., 1988; Newman et al., 1989; Peters and Norback, 1990; Wognum et al., 1990) to combine the advantages of both other methods. Biotin is a small molecule of 244 Daltons, which can easily be coupled to proteins such as GF. The strong interaction between biotin and the egg white protein avidin, or the bacterial protein streptavidin, can be used to detect binding of biotinylated GF to target cells (Wilchek and Bayer, 1988). By such a method, functionally intact receptors are labeled and the cells can be analyzed by flow cytometry and sorted using a FACS. The efficiency of this approach depends on the efficiency of the biotinylation reaction and on its effect on the binding affinity and, thus, on the biological activity.

Many different kinds of biotinyl derivatives are available. For biotinylation of proteins, the *N*-hydroxy succinimide ester of biotin (NHS-biotin) or its water soluble analog *N*-hydroxysulfo-succinimide biotin (S-NHS-biotin), both of which bind primarily to lysine residues under alkaline conditions, are possibly the most often used (Newman et al., 1989; Pieri and Barritault, 1991). Variants with an extended spacer arm (S-NHS-long chain-, S-NHS-LC-biotin) can be used to reduce the effect of steric hindrance (Hnatowich et al., 1987). If a sufficient number of lysine residues is not available, or when labeling of lysine causes changes of the biological activity of the protein, other classes of reactive biotin derivatives can be used to biotinylate other functional groups. Tyrosines or histidines can be labeled with *p*-diazobenzoyl biocytin (DBB) (Wilchek et al., 1986). Sulfhydryls can be biotinylated with *N*-iodoace-

tyl-*N'*-biotinyl-hexanediamine, or with *N*-[6-(biotinamido)hexyl]-3-(2-pyridyldithio) propionamide (iodoacetyl-biotin and biotin-HPDP, respectively). An advantage of biotin-HPDP is, that the S-S bridge which is formed by the biotinylation reaction can be cleaved to remove the biotin (Shimkus et al., 1985), which may be useful to recover biotinylated proteins from avidin affinity columns. Cleaving off biotins from proteins is also possible when sugar residues on glycoproteins are biotiny-

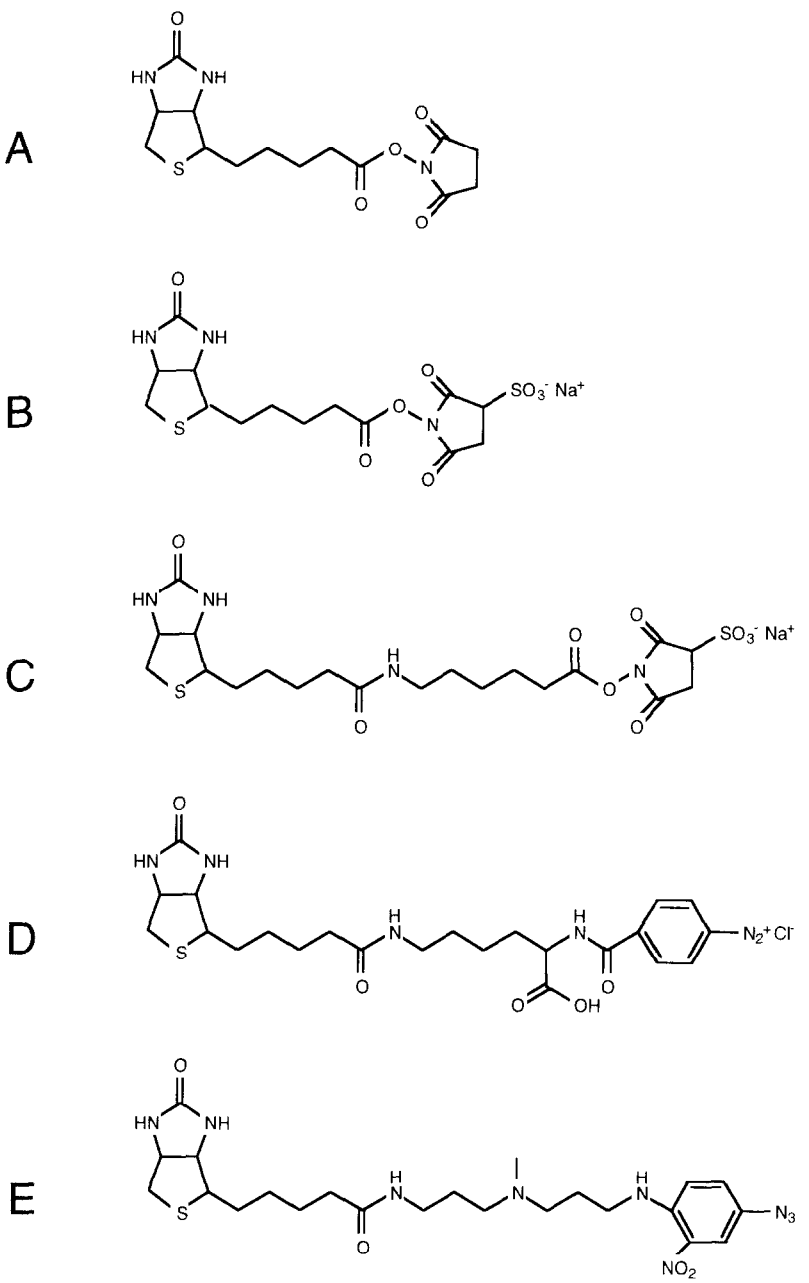

Fig. 1. Structure of the different biotinyl derivatives that were used in this study. *A*: NHS-biotin; *B*: S-NHS-biotin; *C*: S-NHS-LC-biotin; *D*: DBB; and *E*: photobiotin.

lated using biotin hydrazide (O'Shannessy et al., 1984; Wognum et al., 1990). Another biotinylation method uses photobiotin, a photo-activatible analog of biotin (Forster et al., 1985; Lacey and Grant, 1987) which forms stable (presumably covalent) linkages when illuminated.

In this study, we have compared the effect of conjugation with different biotin derivatives on the biological activity as well as on the receptor binding ability and resulting labeling intensity of IL-2 and examined the application of biotinylated IL-2 to detect receptor expression on subsets of peripheral blood and bone marrow cells.

2. Materials and methods

2.1. Biotinylation

E. coli derived human recombinant IL-2 (specific activity 2.8×10^6 U/mg; Biogen, Geneva, Switzerland) was biotinylated using different biotin derivatives (Fig. 1). NHS-biotin, the water soluble S-NHS-biotin and its extended spacer arm analog S-NHS-LC-biotin (all from Pierce, Rockford IL, USA) were dissolved in dimethyl sulfoxide (DMSO) or in 0.1 M carbonate-bicarbonate buffer pH 8.5. DBB precursor (Calbiochem, San Diego, CA, USA) was dissolved in DMSO, and prepared according to the manufacturer's instructions. IL-2 was diluted in 0.1 M carbonate-bicarbonate buffer pH 8.5 (for the different NHS-biotins) or in 0.05 M borate or carbonate buffer pH 9.0 (for DBB). Biotin was added to the GF solution to obtain a molar biotin:protein (B:P) ratio of 100, 20, or 4 during the reaction. As controls, samples were mock-biotinylated (at B:P 0) by just adding DMSO or buffer without biotin. The biotinylation reactions were allowed to take place for 1.5 h (DBB) or 3 h (NHS-biotin) at room temperature, or for 3 h on ice (S-NHS-biotins). Photoactivatible biotin (photobiotin, stock solution of 1 mg/ml; Vector Laboratories, Burlingame CA, USA) was used, according to the manufacturer's instructions at B:P reaction ratios of 10:1, 1:1 and 1:10 (w/w; corresponding to molar B:P ratios of approximately 250, 25 and 2.5, respectively). These reaction mixtures and a control without photobiotin were illuminated with UV light for 30 min on ice using a mercury arc lamp. The reaction was stopped by adding an equal volume of 0.1 M Tris pH 9.0. The remaining free biotin molecules in the samples were removed either by size exclusion chromatography, using a PD-10 column containing Sephadex G-25 (Pharmacia, Uppsala, Sweden), or by extensive dialysis at 4° C. More protein material was lost during size exclusion chromatography than during dialysis (up to 50% and 15%, respectively). Dialysis of small volumes was achieved by floating a low-protein-binding filter unit with the sample (10000 NMWL filter unit, cat. no. UFC3 LGL00, Millipore, Bedford MA, USA) on PBS containing 0.04% Tween 20 and 0.02% (w/v) sodium azide. The biotinylated GF were stored at 4° C in the presence of 0.02% (w/v) sodium azide. The protein concentration after biotinylation was determined with the BCA micro protein assay (Pierce). The presence of biotinylated IL-2 was determined by spotting 1 μl of stepwise dilutions (1/2) of the samples directly on a nitrocellulose membrane, followed by immunoenzymatic staining of the membrane with streptavidin-alkaline phosphatase and 4-nitro blue tetrazolium chloride 5-bromo-4-chloro-3-indolyl phosphate (NBT BCIP) development.

2.2. Cell line and biological activity assays

Cells from the IL-2 dependent mouse cytotoxic T cell line CTLL-2 were grown in RPMI 1640 supplemented with 10% (v/v) heat inactivated fetal calf serum, 0.1 mM β-mercaptoethanol, 2 mM L-glutamine, and 20 U/ml of IL-2. Cultures were maintained at 37° C in a humidified atmosphere of 5% CO_2 in air. To determine the biological activity of the biotinylated GF, 10^4 cells per well of 96 well microtiter plates were cultured in 200 μl RPMI medium containing serial dilutions of either biotin-IL-2 or unmodified IL-2. After 24 h, 0.25 μCi [^{3}H]thymidine was added to each well. The cells were harvested after 16–18 h of thymidine incorporation, and the radioactivity was measured in a liquid scintillation counter. Alternatively, in a non-radioactive assay the number of nucleated cells per well was

determined by staining the nuclei with propidium iodide (PI) and measuring the fluorescence intensity. For this purpose, the cells were cultured for 40–48 h, after which 0.5% (v/v) Triton X-100 and 0.002% (w/v) PI were added to the wells. After approximately 15 min the microtiter plates were read in a Leitz Diavert inverted microfluorometer using green excitation by a halogen lamp.

2.3. Mouse bone marrow cells

Bone marrow from 7-week-old BCBA (C57Bl/LiARij × CBA/BrARij)F1 mice was harvested by flushing femora and tibiae with 1 ml of ice-cold Hanks' balanced salt solution (Laboratoires Eurobio, Paris, France) buffered at pH 6.9 with 10 mM Hepes (Merck) (HH, osmolarity 300–305 mosmol/kg). A single cell suspension was obtained by filtration through a six-layer nylon sieve.

2.4. Human peripheral blood cells

Peripheral blood cells from normal human volunteers were cultured in a humidified atmosphere of 5% CO_2 in air at 37° C in RPMI 1640 supplemented with 10% (v/v) heat inactivated fetal calf serum, 0.1 mM β-mercaptoethanol, 2 mM L-glutamine and 90 mg/ml phytohemagglutinin (PHA, 1% of stock solution, Wellcome Foundation, Dartford, England).

2.5. Immunocytochemical staining and flow cytometry

Cells were stained in 50 μl PBS/serum/azide (PSA, human cells) or HH/serum/azide (HSA, mouse cells): PBS or HH, containing 5% (v/v) fetal or newborn calf serum and 0.02% (w/v) sodium azide. Cells (10^6 per sample) were incubated with biotin-IL-2 (14–140 nM) in a volume of 50 μl for 60 min at room temperature. Similar results were obtained by incubation on ice. The samples were then stained for 15–30 min on ice, using either avidin-FITC (1:300 v/v, Vector Laboratories, Burlingame CA, USA), streptavidin-BODIPY (1:100 v/v, Molecular Probes), or streptavidin-phycoerythrin (streptavidin-PE, 1:5 v/v, Becton Dickinson, San Jose, CA, USA; or 1:200 v/v, Molecular Probes, Eugene OR, USA). After each incubation the samples were washed in PSA or HSA. Low intensity fluorescence signals were amplified by incubating the cells for 15–30 min on ice with alternate layers of biotinylated goat anti-(strept)avidin (both 1:100 v/v, Vector) and fluorescence labeled (strept)avidin. Control cells were incubated only with the fluorescence labeled (strept)avidin and the amplifying layers of biotinylated anti-(strept)avidin and fluorescence labeled (strept)avidin. Specificity of binding of the different biotin-IL-2 samples was determined by incubating the cells with biotin-IL-2 in the presence of a 100× molar excess of unbiotinylated IL-2.

For staining with anti-IL-2 receptor α chain antibody, mouse cells were incubated with the antibody 5A2 (Moreau et al., 1987) (undiluted culture supernatant of hybridoma cells, kindly provided by Dr. Thèze, Département d'Immunologie, Institut Pasteur, Paris, France) for 30 min on ice. Human cells were incubated with the rat anti-TAC antibody 36A1.2 (Olive et al., 1986), followed by staining with FITC-conjugated goat anti-rat antibodies (GARa-FITC; 1:100 v/v, Tago, Burlingame, CA, USA). In double-staining experiments, these antibodies were added during amplification of the streptavidin-PE signal: the anti-TAC antibody was added to the cells together with biotinylated goat anti-streptavidin, followed by incubation with GARa-FITC and streptavidin-PE. In some experiments a combination of streptavidin-BODIPY and GARa-PE (1:200 v/v, Tago) was used.

To analyze the expression of IL-2 receptors on subsets of human peripheral blood lymphocytes, cells were double-stained with mouse anti-CD4 antibodies (1:20 v/v, Becton Dickinson) and either anti-TAC or biotin-IL-2, combined with PE-conjugated goat anti-mouse antibodies (GAM-PE; 1:100 v/v, Sigma Chemical Company, St. Louis, MO, USA) and either GARa-FITC or streptavidin-BODIPY.

Samples were analyzed using a flow cytometer (RELACS: Rijswijk Experimental Light Activated Cell Sorter, ITRI-TNO, Rijswijk, Netherlands). Cells were illuminated with the 488 nm

Table 1
Spot-blot analysis of the different biotin-IL-2 samples

	Minimal detectable amount (ng) of biotin-IL-2 per μl [a]							
	NHS	S-NHS		S-NHS-LC		DBB		Photo
	DMSO	DMSO	Buffer	DMSO	Buffer			
B:P [b] 0	100	> 100	100	> 100	> 100	> 100	B:P 0	> 100
B:P 4	5	20	50	10	10	90	B:P 2.5	> 100
B:P 20	1	5	10	1	1	10	B:P 25	40
B:P 100	< 1	< 1	< 1	< 1	< 1	< 1	B:P 250	< 1

[a] The minimal detectable amount (ng) of biotin-IL-2 per μl was determined by spotting 1 μl of stepwise dilutions of the biotin-IL-2 samples on nitrocellulose, followed by immunoenzymatic staining with streptavidin-alkaline phosphatase and NBT BCIP development.
[b] B:P = biotin:protein ratio.

line of an argon ion laser. Green FITC or BODIPY fluorescence was measured through a 530 nm/30 nm bandpass filter (Becton Dickinson, Mountain View, CA, USA). Orange PE fluorescence was measured through a 577 nm/10 nm bandpass filter.

3. Results

3.1. Biotinylation of IL-2

IL-2 was biotinylated using either NHS-biotin, S-NHS-biotin, S-NHS-LC-biotin, DBB or photobiotin (Fig. 1). The degree of biotinylation of the different samples was studied by spotting 1 μl of stepwise dilutions of biotin-IL-2 on a nitrocellulose filter and comparing the intensity of the spots following incubation with streptavidin-alkaline phosphatase and NBT BCIP development. The various biotin-IL-2 samples contained different amounts of biotin, as shown by the amounts minimally needed to give a visible stain on the nitrocellulose membrane (Table 1). As expected, the highest degree of biotinylation was achieved at the highest B:P reaction ratios, whereas the B:P 0 controls were not visible on the membranes. The water-soluble S-NHS-LC-biotin appeared to label with similar efficiency after dissolving in DMSO or in buffer. S-NHS-biotin labeled slightly more efficiently after dissolving in DMSO than in buffer. NHS-biotin and S-NHS-LC-biotin gave the best biotinylation efficiency, followed by S-NHS-biotin and DBB, which were 4–10-fold less efficient. The lowest degree of biotinylation occurred when IL-2 was labeled with the photobiotin reagent (Table 1).

Table 2
Biological activity of the different biotin-IL-2 samples

	Recovery (%) of biological activity [a]							
	NHS	S-NHS		S-NHS-LC		DBB		Photo
	DMSO	DMSO	Buffer	DMSO	Buffer			
B:P [b] 0	100	100	100	100	100	100	B:P 0	100
B:P 4	100	100	100	100	100	100	B:P 2.5	100
B:P 20	33	90	85	90	100	80	B:P 25	33
B:P 100	5	33	72	40	80	20	B:P 250	7

[a] IL-2 dependent CTLL-2 cells were grown in the presence of different amounts of biotin-IL-2. The amount of biotin-IL-2 needed per well for 50% maximal stimulation of the cells was calculated for the different samples. Results are expressed as % biological activity, as compared to unbiotinylated IL-2 (100%).
[b] B:P = biotin:protein ratio.

3.2. Biological activity of biotinylated IL-2 preparations

Using the IL-2 dependent mouse cytotoxic T cell line CTLL-2, the B:P ratio was found to affect the biological activity of biotin-IL-2 (Table 2). At the lowest B:P ratio used in the biotinylation reaction, biological activity was not lost as a result of biotinylation. Higher B:P ratios had a marked negative effect on the biological activity of some biotinyl derivatives (Table 2). Loss of biological activity was highest when IL-2 was biotinylated with NHS-biotin or photobiotin. When used at B:P 100, biotinylation with DBB also led to a large loss of biological activity. The use of the water-soluble NHS-biotins dissolved in DMSO resulted in two-fold larger losses of biological activity than when these reagents were dissolved in buffer. The highest recovery of biological activity ($\geq$ 70–80% at B:P 100) was achieved with S-NHS-LC-biotin and S-NHS-biotin (Table 2).

3.3. Cell staining

Immunocytochemical staining properties of the different biotin-IL-2 samples were compared using flow cytometry. Fig. 2 shows an example of the fluorescence histograms, resulting from the incubation of CTLL-2 cells with biotin-IL-2 made by biotinylation with S-NHS-LC-biotin at different B:P ratios. The mean fluorescence intensities of cells, labeled with the different biotin-IL-2 preparations are presented in Table 3. IL-2 labeled with the NHS-biotins gave the highest fluorescence intensities: with the extra long spacer arm between the protein and the actual biotin molecule (S-NHS-LC-biotin) the fluorescence signal was even higher than with the normal spacer arm (NHS-biotin and S-NHS-biotin). Dissolving the water soluble S-NHS-biotin or S-NHS-LC-biotin in DMSO before biotinylating the IL-2 resulted in higher fluorescence intensities of stained cells than dissolving in buffer. In comparison with the various NHS-biotins, biotinylation with DBB resulted in a lower fluorescence signal. The fluorescence signal resulting from photobiotin-IL-2 was almost indistinguishable from the background fluorescence of the cells (Table 3). For most biotinyl derivatives, the preparations

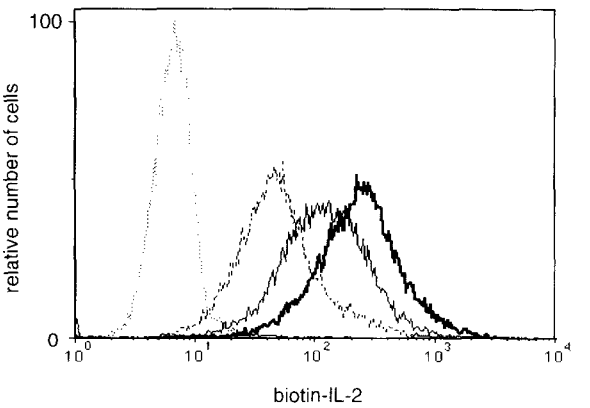

Fig. 2. Fluorescence histograms of CTLL-2 cells, incubated with different biotin-IL-2 samples and streptavidin-PE. The fluorescence signal was amplified by two cycles of sequential incubations with biotinylated anti-streptavidin antibodies and streptavidin-PE. The cells were labeled with biotin-IL-2, biotinylated with S-NHS-LC-biotin that was dissolved in DMSO; from right to left are B:P 100, B:P 20 and B:P 4, and the B:P 0 control.

Table 3
Fluorescence intensity of CTLL-2 cells, stained with the different biotin-IL-2 samples

	Mean fluorescence intensity (arbitrary units) [a]							
	NHS	S-NHS		S-NHS-LC		DBB		Photo
	DMSO	DMSO	Buffer	DMSO	Buffer			
B:P [b] 0	7	7	8	7	8	7	B:P 0	7
B:P 4	41	20	9	73	28	13	B:P 2.5	18
B:P 20	157	48	32	164	116	59	B:P 25	9
B:P 100	100	164	122	316	288	99	B:P 250	24

[a] CTLL-2 cells were incubated with the different biotin-IL-2 samples and streptavidin-PE, amplified with biotin-anti-streptavidin and streptavidin-PE.
[b] B:P = biotin:protein ratio.

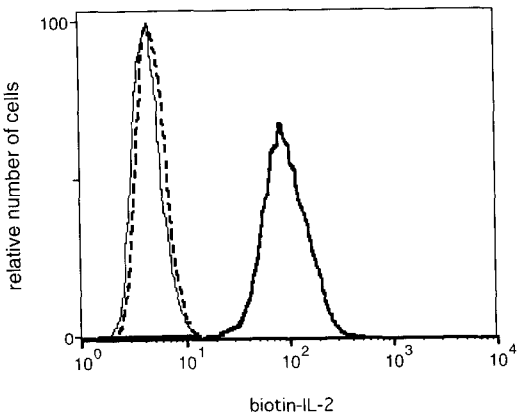

Fig. 3. Fluorescence histograms of CTLL-2 cells, incubated with biotin-IL-2 (thick solid line), in the presence of a 100× molar excess of unbiotinylated IL-2 (broken line), and fluorescence labeled streptavidin, or stained only with fluorescence labeled streptavidin without biotin-IL-2 (thin solid line). The fluorescence signal was amplified using biotinylated anti-streptavidin antibodies and a second incubation with fluorescence labeled streptavidin.

with the highest B:P ratios resulted in the highest fluorescence intensities. Only with the standard NHS-biotin was an impaired staining of the B:P 100 sample (relative to the B:P 20) consistently observed (Table 3). For all biotin-IL-2 samples, the fluorescence signal of cells incubated in the presence of a 100× molar excess of unbiotinylated IL-2 was similar to that of control cells incubated without biotin-IL-2, which indicated specific binding of biotin-IL-2 to the cells. This is illustrated in Fig. 3 for CTLL-2 cells, incubated with biotin-IL-2 prepared with NHS-biotin.

When IL-2 receptors on lymphocytes from peripheral blood or bone marrow were stained, a difference was found between incubation with biotin-IL-2 and with antibodies against the α chain of the IL-2 receptor. The majority of unstimulated lymphocytes from mouse bone marrow as well as human peripheral blood were biotin-IL-2 positive (Fig. 4A and 4C, respectively), whereas a much smaller fraction expressed the IL-2 receptor α chain at a level above background fluorescence (Fig. 4B and 4D). Double-staining experiments revealed that the α chain positive cells were all biotin-IL-2 positive (data not shown). After stimulation of human peripheral blood lymphocytes (PBL) with PHA virtually

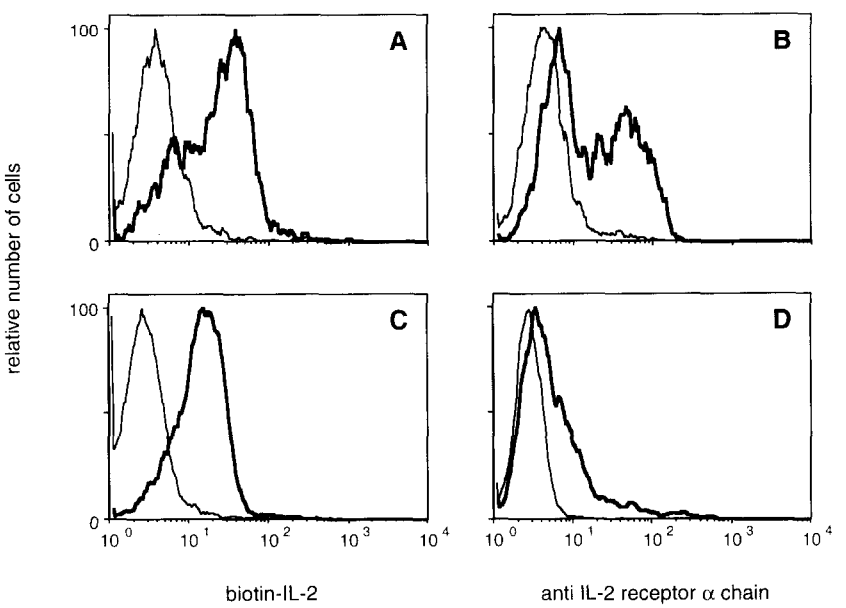

Fig. 4. Fluorescence histograms of mouse bone marrow lymphocytes (*A* and *B*) and human peripheral blood lymphocytes (*C* and *D*), stained with biotin-IL-2 and streptavidin-PE, and amplified (*A* and *C*), or stained with rat anti-IL-2 receptor α chain antibodies and FITC conjugated goat anti-rat antibodies (*B* and *D*). Thick lines represent stained cells, thin lines unstained control cells.

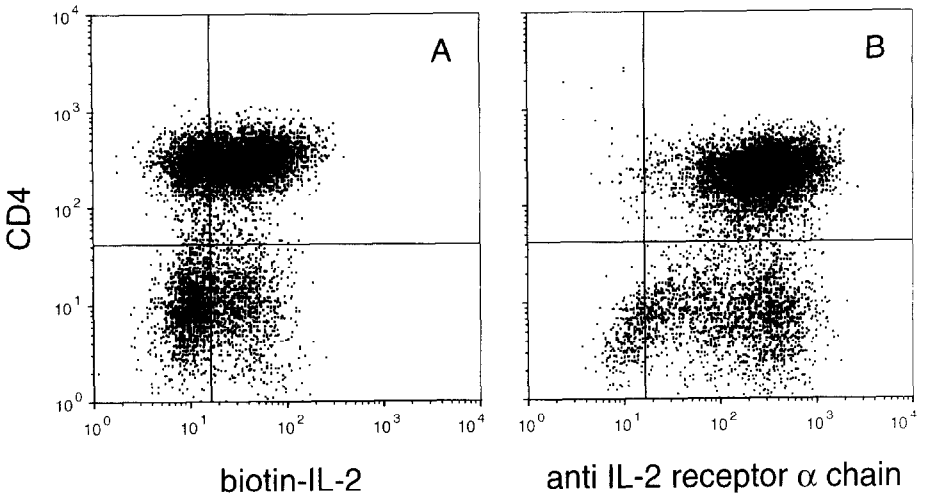

Fig. 5. Dot plots of human peripheral blood lymphocytes that were stimulated with PHA for 3 days, and stained with mouse anti-CD4 antibodies in the first step, in combination with either biotin-IL-2 (*A*) or rat anti-IL-2 receptor α chain antibodies (*B*). In the second step, cells were stained with PE-conjugated goat anti-mouse antibodies in combination with either streptavidin-BODIPY (*A*) or FITC-conjugated goat anti-rat antibodies (*B*). Quadrants were set on the basis of background fluorescence of unstained cells to discriminate between brightly stained cells and cells with low or no fluorescence signal.

all cells became α chain-positive (Fig. 5B), whereas a much smaller fraction of the cells stained strongly with biotin-IL-2 (Fig. 5A). Most of the cells that showed a strong fluorescence signal after staining with biotin-IL-2 or anti-TAC were CD4 positive. The proportion of CD4 negative cells that expressed high levels of IL-2 receptor was much lower. The remaining cells were either IL-2 receptor negative or expressed IL-2 receptors at low levels (Fig. 5).

4. Discussion

This study has evaluated the biotinylation of IL-2 using five different biotin derivatives. The data presented extends previous observations concerning the use of GF labeled with (mostly NHS-)biotin (Foxwell et al., 1988b; Yamasaki et al., 1988; Taki et al., 1989; Peters and Norback, 1990; Pieri and Barritault, 1991; De Jong et al., 1992; Wognum et al., 1993), by comparing the results of labeling with NHS-biotin, S-NHS-biotin, S-NHS-LC-biotin, DBB and photobiotin. The highest biotinylation efficiency, retention of biological activity and the highest specific staining of IL-2 receptor expressing cells was achieved using S-NHS-LC-biotin. The results with S-NHS-LC-biotin could be explained by a more efficient biotinylation, because the extended spacer arm permits the biotin to reach the lysine groups, or by a more efficient interaction of the biotinylated IL-2 with the streptavidin conjugates.

Most biotin-IL-2 samples showed reduced activity in stimulating the growth of the IL-2 dependent cell line CTLL-2 when high B:P ratios were used. We cannot exclude the possibility that part of the biological activity of the biotin-IL-2 samples is due to residual unmodified IL-2 molecules. Attempts to separate biotin-IL-2 from IL-2 on an avidin-agarose column were not successful because IL-2 bound nonspecifically to the column. The decreased biological activity of samples with a high B:P ratio is probably the result of impaired receptor binding, caused by conformational changes of the biotin-IL-2 or by steric hindrance when the biotin molecules are close to the receptor binding site(s) of IL-2. It is also possible that these biotinylated IL-2 molecules can bind to the cells but fail to stimulate cell proliferation. The IL-2 receptor consists of different subunits (Takeshita et al., 1992a), with different IL-2 bind-

ing characteristics (Lowenthal and Greene, 1987). A low affinity IL-2 receptor contains only the 55 kDa α chain (Leonard et al., 1984; Nikaido et al., 1984). A heterodimeric IL-2 receptor with intermediate affinity for IL-2 consists of the 70–75 kDa β chain (Sharon et al., 1986; Baccarini et al., 1989) and the 64 kDa γ chain (Takeshita et al., 1992a,b). A 'pseudo-high' affinity IL-2 receptor is formed by interaction of α and β chains (Nakarai et al., 1994), which can only be internalized when combined with the γ subunit (Takeshita et al., 1992a), thereby forming the trimeric high affinity IL-2 receptor which has a higher association rate and a lower dissociation rate than the $\alpha\beta$ complex (Matsuoka et al., 1993). Heterodimerization of the β and γ chain is required for signalling (Nakamura et al., 1994; Nelson et al., 1994). The decreased biological activity of samples with a high B:P ratio might thus be caused by steric hindrance, which impedes receptor binding and/or the formation of receptor complexes that are required for cell signalling or internalization.

Despite the partial loss of biological activity, the biotinylated IL-2 samples prepared at higher B:P ratios generally yielded better fluorescence signals. It is possible that at higher B:P ratios, a higher proportion of biotinylated IL-2 molecules have sufficient numbers of biotin molecules facing away from the receptor binding sites to permit binding of (strept)avidin molecules to receptor bound biotin-IL-2. Only the samples with high B:P ratios that lost almost all biological activity (NHS-biotin and photobiotin) showed low fluorescence intensities.

The weak performance of DBB compared to the different NHS-biotins may be explained from the amino acid composition of IL-2: NHS-biotins mainly react with lysine residues, of which there are 11 in IL-2, while there are only three histidines and four tyrosines (Taniguchi et al., 1983) which are necessary for DBB binding. Direct measurement of the number of biotin molecules present on the IL-2, e.g. with 2-(4'-hydroxy-phenylazo)benzoic acid (HABA) (Baxter, 1964), was not attempted, because the procedure requires a large amount of biotinylated protein.

Our experiments show that biotinylated IL-2 can be used for cell staining experiments. When IL-2 receptors on unstimulated lymphocytes from mouse bone marrow or human peripheral blood were labeled, only part of the biotin-IL-2 positive cells expressed high levels of the IL-2 receptor α chain (Fig. 4). This is in agreement with the finding that only intermediate affinity IL-2 receptors are present on the surface of resting T cells and NK cells (Dukovich et al., 1987), while the IL-2 receptor α chain is induced only after activation of the cells (Hamblin, 1985; Lai et al., 1991). After stimulation of PBL with PHA, virtually all cells stained brightly with anti-TAC, particularly in the CD4 positive subset (Fig. 5B). However, only about two thirds of these cells showed a high fluorescence signal after incubation with biotin-IL-2 (Fig. 5A). The cells with a low binding capacity for biotin-IL-2 may have a limiting number of IL-2 receptor β and/or γ chains to form high affinity receptors with the α chains. Biotin-IL-2 binding thus appears to be a better marker for the expression of functional IL-2 receptor complexes than binding of anti-TAC. A possible advantage of staining with biotinylated GF for in vivo studies with isolated receptor expressing cells is that avidin-tagged cells are not selectively removed by the immune system of the recipient animals. In contrast, antibody stained cells may be eliminated by phagocytosis induced by the F_c parts of the antibodies or by complement-mediated lysis (Bauman et al., 1985). As shown in previous studies for other cytokines (Wognum et al., 1990; De Jong et al., 1992; Wognum et al., 1993), flow cytometric analysis of receptor expression using biotinylated GF is very sensitive: cells expressing as few as 100 cell surface receptors can be detected. Therefore, biotinylated GF provide a useful tool for investigating the distribution of functional GF receptors, e.g. on subsets of early hemopoietic cells in bone marrow.

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