

Production of Genetically Engineered Biotinylated Interleukin-2 and Its Application in a Rapid Nonradioactive Assay for T-Cell Activation

Robert A. Jordan,¹ Mark T. Preissler,² Jeffrey A. Banas,² and Edmund J. Gosselin^{2*}

*Transplantation Immunology Laboratory¹ and Center for Immunology & Microbial Disease,²
Albany Medical College, Albany, New York 12208-3479*

Received 7 December 2001/Returned for modification 6 November 2002/Accepted 17 January 2003

The development of reliable assay systems that can measure lymphocyte activation in vitro has been a major goal of immunodiagnosics. Traditionally, tritiated thymidine incorporation has been used to monitor T-cell activation. Other methods include enzyme-linked immunosorbent assay (ELISA), enzyme-linked immunospot assay, and colorimetric assays. We have established a lymphocyte activation assay that utilizes fluorescein isothiocyanate (FITC)-streptavidin bound to recombinant biotinylated human interleukin-2 (IL-2). Utilizing recombinant DNA technology, a unique monobiotinylated human IL-2 has been created and isolated using the Promega PinPoint vector system. ELISA has been used to demonstrate streptavidin binding and recognition by a human IL-2-specific antibody. IL-2 function has been demonstrated using a murine IL-2-dependent T-cell line, CTLL-2, responsive to human IL-2. Recombinant biotinylated human IL-2 conjugated to streptavidin-FITC or streptavidin-horseradish peroxidase has been used to monitor T-cell activation in the presence of antigen as well as mitogen. The sensitivity and convenience of this method make this lymphocyte activation assay an attractive alternative to tritiated thymidine incorporation as a method for monitoring T-cell activation. In addition, the availability of a recombinant biotinylated human IL-2 will permit the production of a uniform product suitable for diagnostic and clinical application.

Interleukin-2 (IL-2) is the principal cytokine responsible for progression of T lymphocytes from the G₁ to the S phase of the cell cycle. It is produced by CD4 T cells and to a lesser degree by CD8 T cells (9). On activation, T cells express high levels of the high-affinity form of the IL-2 receptor (IL-2R), to which IL-2 binds (12). IL-2, biotinylated by chemical means, has been used to detect activated T cells (3), isolate IL-2R (5), monitor IL-2R expression (3, 21, 24), and study IL-2R internalization (15). Studies also show that targeting IL-2 to tumor cells facilitates tumor elimination, presumably via enhanced T-cell activation (8, 10, 11, 14, 26). Therefore, the availability of a recombinant biotinylated human IL-2 (rbhIL-2) could not only provide a tool for monitoring T-cell activation but also facilitate the development of a therapeutic tool which utilizes single-chain variable-region antibody fragment-IL-2 fusion proteins to target IL-2 to tumors (8, 10, 11, 14) as well as antigen-presenting cells (APC) (23). In addition, large-scale production and purification of a uniformly biotinylated IL-2 product, required for diagnostic and clinical applications, would be facilitated. However, no genetically engineered biotinylated IL-2 is available at present.

The availability of the Promega PinPoint Xa protein purification system now makes the construction of rbhIL-2 possible. This system takes advantage of the fact that *Escherichia coli* produces a naturally biotinylated protein that is essential for fatty acid synthesis and growth of this organism (2, 18). The Promega PinPoint Xa vector contains a biotinylation tag similar to that present in the endogenous protein and is designed

for the production and purification of fusion proteins, which uniformly contain a single biotin. In chemical biotinylation, the number of biotins per protein molecule is variable. Also, in the Promega PinPoint vector system, the biotinylation tag can be easily removed by enzymatic cleavage should one wish to remove the biotinylation stub once the cytokine is purified. Furthermore, biotinylated fusion proteins produced in this fashion can be affinity purified using the SoftLink soft-release avidin resin, which, importantly, permits elution of the fusion protein under nondenaturing conditions.

In this paper the generation of an rbhIL-2 fusion protein is described, as well as its use to monitor human T-cell activation in vitro. This biotinylated fusion protein binds both streptavidin (SA) and the IL-2-specific antibody (Ab) and induces the proliferation of a murine IL-2-dependent T-cell line, CTLL-2. Furthermore, by utilizing fluorescein isothiocyanate (FITC)- or horseradish peroxidase (HRP)-labeled SA in combination with the rbhIL-2, antigen- and mitogen-induced T-lymphocyte activation can be monitored via flow cytometry or chemiluminescence assay, respectively.

MATERIALS AND METHODS

Generation of the rbhIL-2 construct. *E. coli* containing human IL-2 cDNA inserted into a cloning vector was obtained from the American Type Culture Collection (ATCC, Manassas, Va.). The cloning vector containing the IL-2 insert, PTCGF-11, was isolated by an alkaline lysis plasmid preparation and cleaned by the Qiagen method (Qiagen, Inc., Valencia, Calif.). PCR was used to create unique *Hind*III and *Kpn*I restriction enzyme sites (Promega Corp., Madison, Wis.) at the 5' and 3' ends of the IL-2 insert, respectively. The PCR product was digested with *Hind*III and *Kpn*I and cleaned by the Qiagen method. The PinPoint Xa3 vector (Promega) was digested with *Hind*III and *Kpn*I, and the released human IL-2 DNA was inserted into the PinPoint Xa3 expression vector. *E. coli* JM109 was then transformed with the PinPoint Xa3 vector containing the IL-2 DNA insert or the same vector lacking the IL-2 DNA insert. The product produced by the latter construct served as a negative control in many of the

* Corresponding author. Mailing address: Center for Immunology & Microbial Disease, Albany Medical College, MC 151, Albany, NY 12208-3479. Phone: (518) 262-5562. Fax: (518) 262-6161. E-mail: gossele@mail.amc.edu.

assays described here. 2YT (Becton Dickinson & Co., San Jose, Calif.) plates containing 100 μ g of ampicillin (Sigma Chemical Co., St. Louis, Mo.) per ml were used to select for transformants. Transformed *E. coli* JM109 was then cultured at 37°C with agitation on a Gyrotory shaker (New Brunswick Scientific Co., Inc., Edison, N.J.) in 2YT plates containing 100 μ g of ampicillin per ml and 8 μ M biotin (Sigma). After 2 h, the culture was induced to produce rbhIL-2 protein with 100 μ M isopropyl- β -D-thiogalactopyranoside (IPTG) (Sigma). After 4 h, the transformed *E. coli* JM109 cells were isolated from bulk culture and resuspended in lysis buffer containing 50 mM Tris (Fisher Biotech, Fair Lawn, N.J.), 50 mM NaCl (Sigma), and 0.02% sodium azide (Sigma), with one Complete mini protease inhibitor cocktail tablet (Roche, Indianapolis, Ind.).

Purification of the rbhIL-2 protein. The transformed *E. coli* JM109 cells were mechanically lysed with a French press (American Instrument Co., Inc., Silver Spring, Md.), and the rbhIL-2 was isolated on a SoftLink Avidin column as described by the manufacturer (Promega). The eluate containing the rbhIL-2 was concentrated using a Speed Vac concentrator (Savant, Hicksville, N.Y.) and dialyzed against phosphate-buffered saline (PBS) using a Slide-A-Lyzer cassette (15-kDa exclusion; Pierce Chemical Co., Rockford, Ill.). Purity was verified using Western blotting, and the protein concentration was determined using the bio-cinchoninic acid assay as specified by the manufacturer (Pierce).

SA binding and IL-2-specific Ab recognition of rbhIL-2. To illustrate SA binding by rbhIL-2 and its recognition by an IL-2-specific Ab, an enzyme-linked immunosorbent assay (ELISA) was performed. A high-binding 96-well ELISA plate (Corning Inc., Corning, N.Y.) was coated with 10 μ g of SA (Pierce) per ml in PBS and incubated at 4°C overnight. After the wells were washed three times with PBS containing 2 mg of bovine serum albumin (BSA) per ml and 0.02% sodium azide (PBS-BSA), they were blocked with PBS-BSA at room temperature for 1 h. The blocking solution was discarded, rbhIL-2 was added to appropriate wells, and the wells were incubated at room temperature for 90 min. The wells were then washed three times with PBS-BSA, and goat anti-human IL-2 (R&D Systems, Minneapolis, Minn.) was added for 4 h at room temperature. After the wells were washed three times with PBS-BSA, rabbit anti-goat IgG-alkaline phosphatase (AP) (Vector Laboratories, Inc., Burlingame, Calif.) was added at a 1:500 dilution in PBS-BSA, and the wells were incubated at room temperature for 90 min. The wells were then washed three times with PBS-BSA, and 1 mg of AP substrate (Sigma) per ml diluted in ELISA buffer (0.05 Na₂CO₃, 10 mM MgCl [pH 9.8]) was added. The samples were incubated at room temperature for 1 h, and absorbance was read on a microplate reader (Molecular Devices, Sunnyvale, Calif.) at 405 nm.

Stimulation of an IL-2-dependent T-cell line by using rbhIL-2. To demonstrate rbhIL-2 function, the IL-2-dependent murine T-cell line CTLL-2 (ATCC), which responds to recombinant human IL-2 (rhIL-2), was utilized. The CTLL-2 cells were maintained at 37°C and 5% CO₂ for 2 days in 25-cm² flasks (Becton Dickinson) containing RPMI 1640 (GibcoBRL), 10% fetal bovine serum (Sigma), 10 mM HEPES (Sigma), 2 mM sodium pyruvate (Sigma), 2 mM glutamine (Sigma), 2.5 g of glucose (Sigma) per liter, and 1 U of rhIL-2 (R&D Systems) per ml. The cells were then washed three times with medium and resuspended, and 50 μ l of cells/well was plated in a 96-well plate at 5×10^3 cells/well. The plates were then incubated at 37°C and 5% CO₂ for 2 days in the presence of rbhIL-2 or rhIL-2 at different concentrations. Subsequently, the CTLL-2 cells were pulsed with 1 μ Ci of tritiated thymidine (³H]TdR) (ICN, Aurora, Ohio) 24 h before being harvested. The cells were harvested using a Harvester 96 (Tomtec, Hamden, Conn.), and ³H]TdR incorporation was measured using a 1450 microbeta liquid scintillation counter (EG&G Wallac, Gaithersburg, Md.).

rbhIL-2 binding to activated T cells. To demonstrate binding of rbhIL-2 to antigen- and mitogen-activated T cells, human peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood as previously described (7). With regard to the use of human subjects, this research has complied with all relevant federal guidelines and institutional policies. PBMC were resuspended in AIM V (Gibco-BRL) at 2.5×10^6 cells/ml. Then 50- μ l volumes of cells were added to individual wells of a 96-well plate containing 5.0 μ g of phytohemagglutinin mitogen (PHA) (Sigma) per ml. The plates were then incubated at 37°C and 5% CO₂ for 3 days. One day prior to completion of this incubation, 60 μ g of rbhIL-2 per ml was combined in equal volume with 120 μ g of SA-FITC (Vector) per ml in PBS-BSA and incubated at 4°C overnight on a Vari-Mix rocker. Following the 3-day incubation of cells, stimulated PBMC were washed three times in AIM V and resuspended at 12.5×10^6 cells/ml in AIM V. Then 50- μ l volumes of cells were added to wells of a 96-well plate, followed by 50 μ l of rbhIL-2-SA-FITC conjugate, and the plates were incubated at 4°C for 2 h with rocking. Biotin-labeled anti-human CD25 (IL-2R) monoclonal Ab (MAb) (Becton Dickinson) bound to SA-FITC was used as a positive control for T-cell activation (IL-2R upregulation) (1). SA-FITC was used as a negative control for rbhIL-2 binding. The cells were then washed three times with PBS-BSA and

resuspended in 2% methanol-free formalin in PBS (Eastman Kodak, Rochester, N.Y.). PBMC were analyzed by flow cytometry, and mean fluorescence intensity (MFI) was determined using Cell Quest (Becton Dickinson).

Evaluation of lymphocyte activation by using rbhIL-2-SA-FITC conjugates and flow cytometry. PBMC were resuspended in AIM V with different concentration of tetanus toxoid antigen (TT) (Accurate Chemical & Scientific Corp., Westbury, N.Y.) or PHA and incubated in a 96-well plate as described above. rbhIL-2-SA-FITC conjugate was then combined with stimulated PBMC as described above and incubated at 4°C for 1 h with rocking. The cells were then washed, fixed, and analyzed by flow cytometry.

Cell specificity of rbhIL-2 binding. To demonstrate that the majority of lymphocytes detected in this assay are activated T cells, a lymphocyte activation assay was performed using PBMC incubated for 1 day in AIM V as described above with 1.0 μ g of PHA per ml. In this case, however, equal volumes of rbhIL-2 (15 μ g/ml) and SA-FITC (60 μ g/ml) were combined and incubated at 4°C overnight on a Vari-Mix rocker to obtain the rbhIL-2-SA-FITC conjugate. To identify T and B lymphocytes, mouse immunoglobulin G1 (IgG1) anti-human CD3 peridinin chlorophyll protein (PerCP) (Becton Dickinson) and mouse IgG1 anti-human CD19 phycoerythrin (PE) (Becton Dickinson) were also added to the cells in combination with rbhIL-2-SA-FITC. Mouse IgG1 PE (Becton Dickinson) and mouse IgG1 PerCP (Becton Dickinson) were used as negative controls. The cells were washed, fixed, and analyzed using flow cytometry.

Evaluation of lymphocyte activation using rbhIL-2-SA-HRP and chemiluminescence. PBMC were resuspended in AIM V at 2.5×10^6 cells/ml with different concentrations of TT or PHA and incubated for 1 day as described above. Equal volumes of rbhIL-2 (15 μ g/ml) and SA-HRP (Pierce) (10 μ g/ml) were combined and incubated at 4°C overnight on a Vari-Mix rocker. The rbhIL-2-SA-HRP conjugate was combined with stimulated PBMC and incubated at room temperature for 1 h with rocking, as was done with the rbhIL-2-FITC conjugate. The chemiluminescence assay was performed as described by the manufacturer (Boehringer Mannheim, Indianapolis, Ind.). The cells were washed three times with PBS and resuspended in 100 μ l of chemiluminescence working solution. Chemiluminescence was measured using a 1450 microbeta liquid scintillation counter.

Comparison of the rbhIL-2-SA-HRP assay to lymphocyte activation measured by [³H]TdR incorporation. PBMC were added to plates followed by different concentrations of PHA or TT and incubated for 3 days as described above for the chemiluminescence analysis. To monitor T-cell activation using [³H]TdR incorporation, PBMC were pulsed with 1 μ Ci of [³H]TdR overnight before being harvested. The cells were harvested, and [³H]TdR incorporation was measured as described above when using CTLL-2 cells to detect rbhIL-2-induced T-cell proliferation.

RESULTS

Production of rbhIL-2. By utilizing recombinant DNA technology, rbhIL-2 was created and isolated using the Promega PinPoint Xa3 vector system, as described in Materials and Methods. Western blot analysis with SA-HRP revealed a banding pattern of 28.9 kDa, consistent with the presence of an rbhIL-2 protein (data not shown).

SA binding and Ab recognition of rbhIL-2 by ELISA. ELISA, utilizing SA-coated wells and goat anti-human IL-2, was used to demonstrate the binding of rbhIL-2 to SA and its recognition by Ab specific for human IL-2. When added to SA-coated wells, the rbhIL-2 bound to SA and was detected by the addition of goat anti-human IL-2 followed by rabbit anti-goat Ig-AP. When the PinPoint vector control or nonbiotinylated rhIL-2 was used in place of rbhIL-2, binding of goat anti-human IL-2 was not detected (Fig. 1).

rbhIL-2 stimulates the proliferation of an IL-2-dependent T-cell line. To illustrate the function of rbhIL-2, the murine (human IL-2-responsive) T-cell line CTLL-2 was used. rbhIL-2 induced CTLL-2 proliferation in a dose-responsive manner, as did nonbiotinylated rhIL-2. However, rhIL-2 was 20- to 30-fold more active than was rbhIL-2. The PinPoint vector control, which did not contain the rbhIL-2 protein, did not induce CTLL-2 proliferation (Fig. 2).

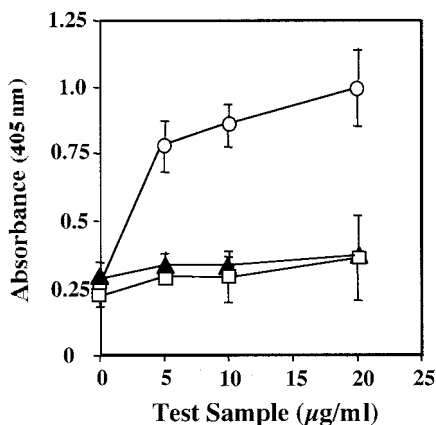


FIG. 1. rbhIL-2 detection by ELISA. PinPoint vector control (open squares), nonbiotinylated rhIL-2 (solid triangles), and rbhIL-2 (open circles) were incubated in wells coated with SA, and goat anti-human IL-2 and rabbit anti-goat IgG-AP were added. Samples were developed and read as described in Materials and Methods. Data represent the mean and standard deviation for triplicate samples.

rbhIL-2 binds to PHA-activated T cells. PHA-stimulated human PBMC were incubated with rbhIL-2-SA-FITC to demonstrate the binding of rbhIL-2 to activated T lymphocytes. rbhIL-2 did bind to PHA-activated PBMC, although at a level 25% lower than that of anti-CD25-FITC. Despite this, rbhIL-2-SA-FITC binding (MFI = 145) was significantly higher than that of SA-FITC or the PinPoint vector control (MFI = 10) (Fig. 3).

Detection of lymphocyte activation by using rbhIL-2-SA-FITC conjugates. To illustrate that rbhIL-2-SA-FITC conjugates can be used to distinguish activated from nonactivated lymphocytes, PBMC incubated in the presence or absence of PHA were incubated with rbhIL-2-SA-FITC. Approximately 74% of the cells bound rbhIL-2-SA-FITC following stimula-

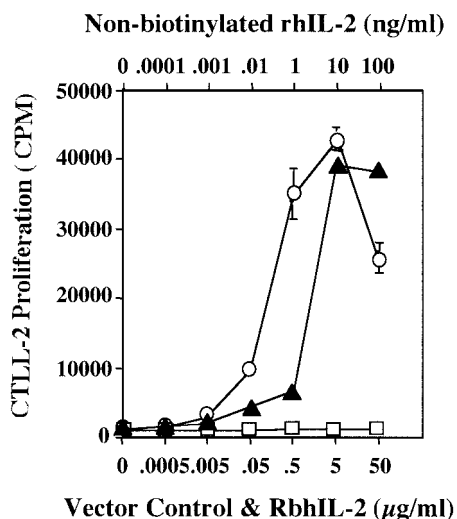


FIG. 2. CTLL-2 proliferation in response to rbhIL-2. PinPoint vector control (open squares), nonbiotinylated rhIL-2 (solid triangles), and rbhIL-2 (open circles) were used to stimulate CTLL-2 cells as described in Materials and Methods. Data represent the mean and standard deviation for triplicate samples.

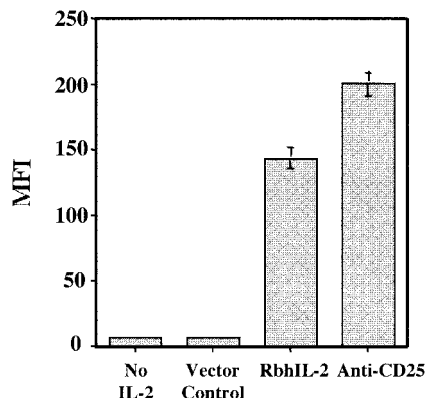


FIG. 3. rbhIL-2 binds to PHA-activated lymphocytes. PBMC were stimulated in the presence of PHA. rbhIL-2 or anti-CD25 combined with SA-FITC was then added, and the fluorescence (MFI) was measured by flow cytometry as described in Materials and Methods. Data represent the mean and standard deviation for triplicate samples.

tion with PHA, whereas approximately 5% of the cells bound rbhIL-2-SA-FITC in the absence of PHA stimulation (Fig. 4).

The incubation time required for the successful detection of lymphocyte activation and the ability to detect differences in the level of cell stimulation were also evaluated. Cell activation could be detected over a wide range of PHA and TT concentrations following a single day of incubation. There was little apparent advantage to increasing the incubation time to 2 or 3 days (Fig. 5).

T-cell specificity of rbhIL-2 binding. B cells are known to express IL-2R (4). To demonstrate that the majority of lymphocytes detected with rbhIL-2-SA-FITC conjugate are in fact T cells, PBMC incubated with 1.0 µg of PHA or TT per ml for 1 day were then stained with anti-CD3-PerCP, anti-CD19-PE, and rbhIL-2-SA-FITC. Approximately 21% of the cells stimulated with PHA were positive for both rbhIL-2-SA-FITC and anti-CD3-PerCP, whereas only 2.4% were positive for both rbhIL-2-SA-FITC and anti-CD19-PE. When stimulated with TT, approximately 4.1% of the cells were positive for both rbhIL-2-SA-FITC and anti-CD3-PerCP whereas only 0.5% were positive for both rbhIL-2-SA-FITC and anti-CD19-PE (Table 1).

T-lymphocyte activation monitored using rbhIL-2-SA-HRP conjugates and chemiluminescence. To illustrate the versatility with which different detection mechanisms can be applied when using rbhIL-2, the above strategy was applied in combination with rbhIL-2-SA-HRP and chemiluminescence detection methods. Similar to the results obtained when using rbhIL-2-SA-FITC conjugate and flow cytometry, as the concentration of PHA or TT increased, chemiluminescence (rbhIL-2-SAHRP binding) also increased in a dose-responsive manner (Fig. 6).

Comparison of the chemiluminescence (rbhIL-2-SA-HRP) assay to T-lymphocyte activation measured by [³H]TdR incorporation. To compare the ability of rbhIL-2-SA-HRP conjugate and chemiluminescence to detect T-cell activation with the ability of [³H]TdR incorporation to do so, TT- or PHA-stimulated PBMC were used as described above, except that T-cell activation was monitored via [³H]TdR incorporation. For PHA stimulation, titration curves using [³H]TdR incorpo-

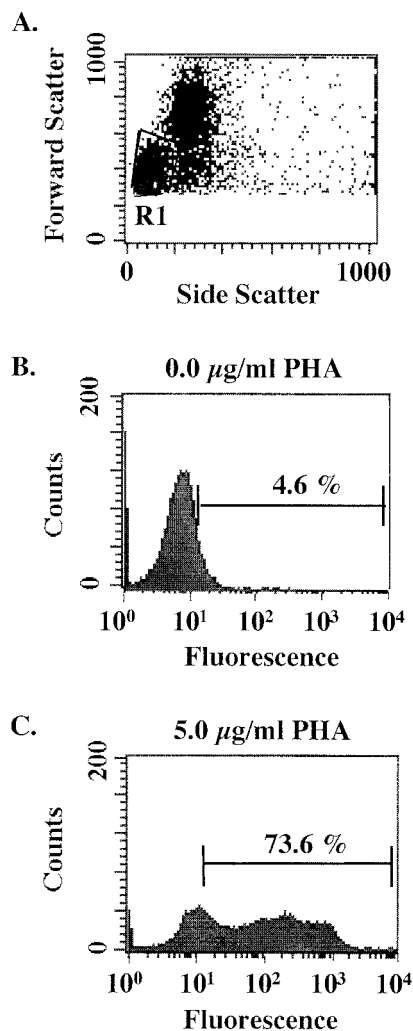


FIG. 4. rbhIL-2 binding is specific for activated lymphocytes. PBMC were incubated in the presence or absence of PHA and subsequently labeled with rbhIL-2-SA-FITC conjugates as described in Materials and Methods. (A) Dot plot indicating the lymphocyte population gated on for analysis of rbhIL-2-SA-FITC binding. (B) Histogram representing gated lymphocytes and rbhIL-2-SA-FITC binding after incubation in the absence of PHA. (C) Histogram representing gated lymphocytes and rbhIL-2-SA-FITC binding after incubation in the presence of PHA.

ration were similar to those generated via the chemiluminescence assay (Fig. 6A and B). For TT stimulation and the chemiluminescence assay, however, a 50% maximal response was detected at 0.04 μg of TT per ml whereas the same value was approximately 0.4 $\mu\text{g}/\text{ml}$ when using [^3H]TdR incorporation (Fig. 6C and D). It should be noted, however, that in the latter case, a maximal (saturable) response to TT was not actually obtained. Thus, although we have traditionally observed saturation of the TT response at between 5 and 10 μg of TT per ml when using [^3H]TdR incorporation, the actual dose of TT required to achieve a 50% maximal response may be higher.

DISCUSSION

The availability of highly purified recombinant cytokines has facilitated advances in immunological research and the devel-

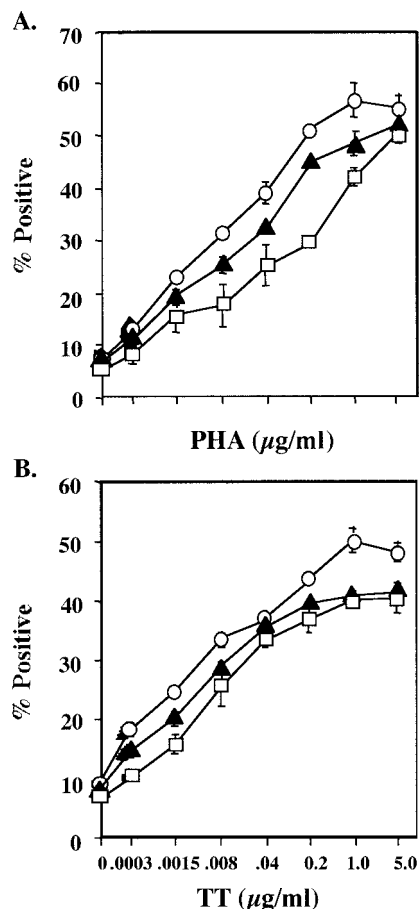


FIG. 5. rbhIL-2-SA-FITC binds to PHA- and TT-activated lymphocytes after 1 day of incubation over a wide range of PHA and TT concentrations. PBMC were incubated with different concentrations of PHA or TT for 1 day (open squares), 2 days (solid triangles), or 3 days (open circles) and were then labeled with rbhIL-2-SA-FITC, as described in the text. Data represent the mean and standard deviation for triplicate samples.

opment of cytokine-based immunotherapies. Several prokaryotic and eukaryotic systems have been used for the production of recombinant cytokines. Alternative approaches to cytokine production using retroviral, adenoviral, and direct DNA gene transfer also have met with success. Genetic manipulation of autologous or allogeneic cells transduced to secrete cytokines also was highly effective in several murine models (19, 25). No

TABLE 1. Specificity of rbhIL-2-SA-FITC conjugate binding^a

| Stimulant | Ab specificity | % of cells positive for ^b : | |
|-----------|----------------|--|-----------------|
| | | rbhIL-2-SA-FITC | SA-FITC |
| PHA | CD3 (T) | 20.97 \pm 1.75 | 0.06 \pm 0.02 |
| | CD19 (B) | 2.39 \pm 0.14 | 0.05 \pm 0.00 |
| TT | CD3 (T) | 4.09 \pm 0.17 | 0.00 \pm 0.00 |
| | CD19 (B) | 0.48 \pm 0.20 | 0.00 \pm 0.00 |

^a The analysis represents the examination of the lymphocyte population only, as determined by forward and side scatter.

^b Data represent the percentage of cells positive for binding of rbhIL-2-SA-FITC or SA-FITC and Ab against the indicated markers.

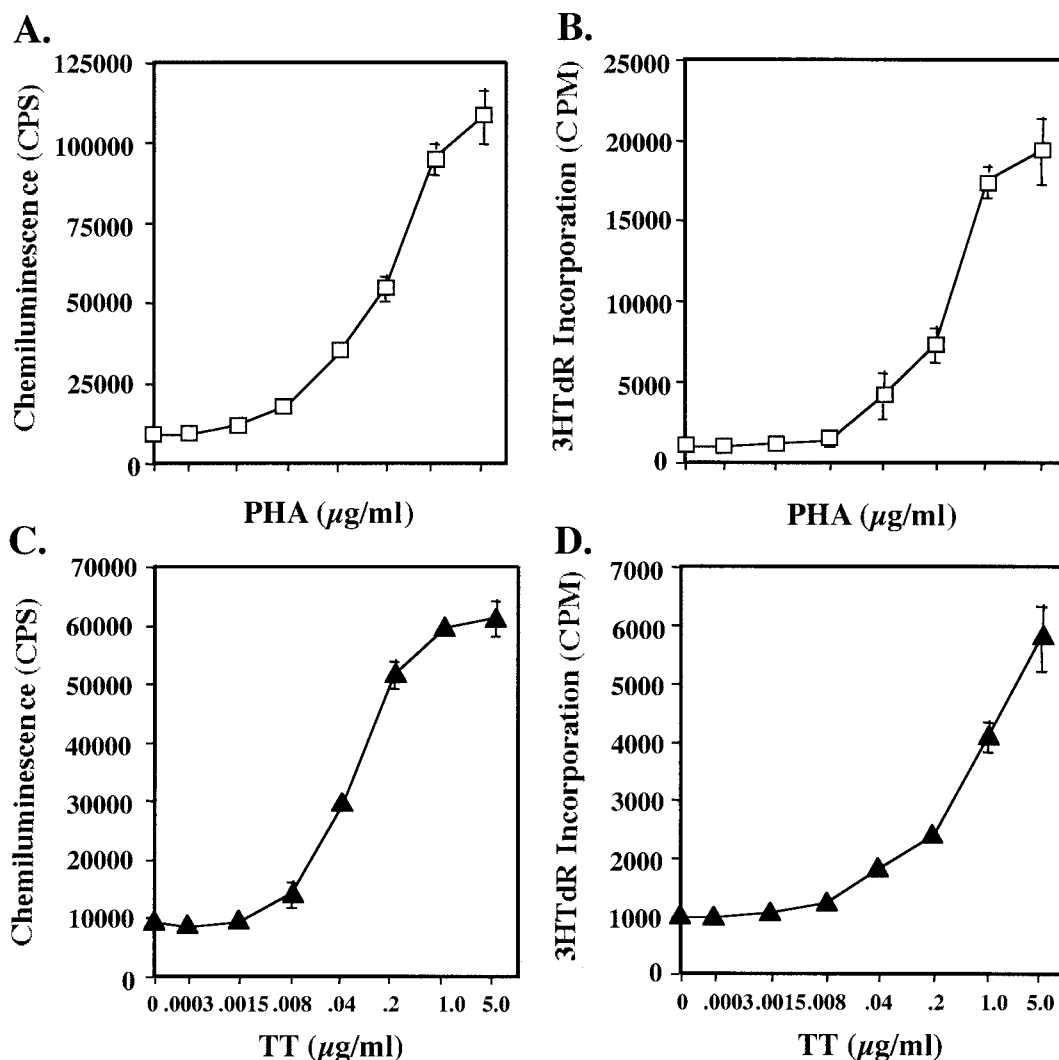


FIG. 6. Analysis of T-cell activation by using rbhIL-2 and chemiluminescence versus ^3H TdR incorporation. PBMC were incubated in the presence of PHA (open squares) (A and B) or TT (solid triangles) (C and D) for 1 day for chemiluminescence analysis (A and C), and 3 days for ^3H TdR incorporation (B and D). Incubations and assays were carried out as described in Materials and Methods. Data represent the mean and standard deviation for triplicate samples.

genetically engineered biotinylated cytokines are commercially available at present.

The PinPoint vector system allows the production of unique, monobiotinylated proteins which can be affinity purified using SoftLink soft-release avidin resin. In addition, the biotinylation tag can be easily removed enzymatically to produce nonbiotinylated product, should this be required. Therefore, we attempted to use this system to generate a monobiotinylated human IL-2. One potential caveat was the presence of an endogenously biotinylated protein normally present in *E. coli*, which may coisolate with rbhIL-2. While we did not observe significant levels of this protein in our rbhIL-2 preparation, potential solutions to this problem include dialysis of the rbhIL-2 preparation by using a size exclusion filter through which the endogenous protein can pass but rbhIL-2 cannot. Alternatively, molecular genetic techniques could be used to tag the endogenously biotinylated protein and facilitate its removal from rbhIL-2 preparations. Another caveat, which we

did observe, was reduced activity of rbhIL-2 compared to nonbiotinylated rhIL-2. One possible explanation for this observation could be steric interference, or structural alterations, due to the presence of the biotinylation stub.

Having produced a genetically engineered rbhIL-2, we determined whether it could be used to monitor T-cell activation. Traditionally, T-cell activation has been monitored by ^3H TdR incorporation, a process that is closely related to underlying changes in the T-cell number. Other methods to monitor in vitro T-lymphocyte activation include detection of T-cell cytokines such as IL-2 and gamma interferon by ELISA (16), measurement of CD8 T-cell activation by granzyme B enzyme-linked immunospot assay (17), use of the IL-2-dependent cell line CTLL-2, MTT reduction (22), and detection of the lymphoid activation marker CD69 by flow cytometry (20). Additional assays include light absorbency to measure lymphocyte proliferation (6) and a combination of intracellular cytokine expression, cytokine secretion, and cytokine receptor ex-

pression (1). However, most of these assays require substantial time and the use of radioactive isotopes and/or are expensive to run.

We provide an alternative method to monitor T-cell activation by using FITC-SA or HRP-SA conjugated to rbhIL-2. This assay can be completed in 1 day and does not require the use of radioactive isotopes. This method is comparable in sensitivity to the traditional [³H]TdR incorporation assay when stimulating cells with PHA and is up to 10-fold more sensitive when analyzing TT-stimulated cells by chemiluminescence techniques (Fig. 6). In addition, the time required to complete the assay is one-quarter to one-sixth of that required to measure [³H]TdR incorporation. It remains to be determined whether the use of a biotinylated anti-CD25 (IL-2R α) MAb would be an equally effective tool for monitoring T-cell activation by the described strategy. However, rbhIL-2 would be expected to bind only IL-2R capable of binding IL-2 itself (3, 21). IL2R binding by rbhIL-2 is indicated by the fact that purified rbhIL-2 stimulates the proliferation of an IL-2-dependent T-cell line whereas similarly purified material from bacteria transfected with a biotinylation vector lacking the IL-2 insert does not (Fig. 2). In addition, rbhIL-2 binds primarily to T cells (Table 1), the binding correlates with CD25 expression (Fig. 3), and it increases with the level of T-cell activation, as measured by [³H]TdR incorporation (Fig. 6). In addition, bhIL-2 would not be expected to bind to Fc receptors, a potential complication when using intact anti-CD25 MAb. Furthermore, therapeutic use of anti-CD25 MAb would probably be problematical, since host Ab would be generated to the variable regions and any mouse components that are present. This is important since rbhIL-2 has the potential for application in vaccines and immunotherapeutics (8, 10, 11, 13). For example, our laboratory has recently developed a targeting strategy which has the ability to target biotinylated proteins to APC (23). We are currently exploring its use in similarly targeting cytokines to APC in order to influence the degree and direction of the immune response. Furthermore, by changing the specificity of the targeting component, it may also be possible to target biotinylated cytokines to tumors, a strategy previously shown to be effective for the elimination of cancer cells (8, 10, 11, 14).

In summary, an rbhIL-2 has been generated which has potential application not only in immunodiagnosics but in clinical immunotherapeutics as well.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants AI46968, AI40442, and AI35327 from the National Institutes of Health.

We thank the members of the Flow Cytometry Core Facility at Albany Medical College for their assistance and for the use of the equipment and facilities.

REFERENCES

- Collins, D. P., B. J. Luebering, and D. M. Shaut. 1998. T-lymphocyte functionality assessed by analysis of cytokine receptor expression, intracellular cytokine expression, and femtomolar detection of cytokine secretion by quantitative flow cytometry. *Cytometry* **33**:249–255.
- Cronan, J. E., Jr. 1990. Biotinylation of proteins in vivo, a post-translational modification to label, purify, and study proteins. *J. Biol. Chem.* **265**:10327–10333.
- De Jong, M. O., H. Rozemuller, J. G. Bauman, and J. W. Visser. 1995. Biotinylation of interleukin-2 (IL-2) for flow cytometric analysis of IL-2 receptor expression. Comparison of different methods. *J. Immunol. Methods* **184**:101–112.
- de Toter, D., P. Francia di Celle, A. Cignetti, and R. Foa. 1995. The IL-2 receptor complex: expression and function on normal and leukemic B cells. *Leukemia* **9**:1425–1431.
- Foxwell, B. M., D. Taylor, B. Greiner, M. J. Mihatsch, V. Olivieri, and B. Ryffel. 1988. Biotinylated recombinant interleukin-2. A tool for research on the interleukin-2 receptor. *J. Immunol. Methods* **113**:221–229.
- Gao, Z. H., W. A. Briggs, N. R. Rose, and J. F. Burdick. 1998. Use of a simple light absorbance assay to measure lymphocyte proliferation. *J. Immunoassay* **19**:129–143.
- Gosselin, E. J., K. Wardwell, D. R. Gosselin, N. Alter, J. L. Fisher, and P. M. Guyre. 1992. Enhanced antigen presentation using human Fc gamma receptor (monocyte/macrophage)-specific immunogens. *J. Immunol.* **149**:3477–3481.
- Holden, S. A., Y. Lan, A. M. Pardo, J. S. Wesolowski, and S. D. Gillies. 2001. Augmentation of antitumor activity of an antibody-interleukin 2 immunocytokine with chemotherapeutic agents. *Clin. Cancer Res.* **7**:2862–2869.
- Jadus, M. R. 1999. Clinical uses of cytokines and their receptors, p. 311–330. In J. H. Nichols (ed.), *Therapeutic drug monitoring and toxicology*, vol. 20. American Association of Clinical Chemistry, Inc., Washington, D.C.
- Lode, H. N., and R. A. Reisfeld. 2000. Targeted cytokines for cancer immunotherapy. *Immunol. Res.* **21**:279–288.
- Matsumoto, H., S. Liao, F. Arakawa, A. Ueno, H. Abe, A. Awasthi, and M. Kuroki. 2002. Targeting of interleukin-2 to human MK-1-expressing carcinoma by fusion with a single-chain Fv of anti-MK-1 antibody. *Anticancer Res.* **22**:2001–2007.
- Nelson, B. H., and D. M. Willerford. 1998. Biology of the interleukin-2 receptor. *Adv. Immunol.* **70**:1–77.
- Nichols, J., F. Foss, T. M. Kuzel, C. F. LeMaistre, L. Platanius, M. J. Ratain, A. Rook, M. Saleh, and G. Schwartz. 1997. Interleukin-2 fusion protein: an investigational therapy for interleukin-2 receptor expressing malignancies. *Eur. J. Cancer Suppl.* **1**:S34–S36.
- Penichet, M. L., and S. L. Morrison. 2001. Antibody-cytokine fusion proteins for the therapy of cancer. *J. Immunol. Methods* **248**:91–101.
- Peters, D. K., and D. H. Norback. 1990. Binding and internalization of biotinylated interleukin-2 in human lymphocytes. *Blood* **76**:97–104.
- Petrovsky, N., and L. C. Harrison. 1995. Cytokine-based human whole blood assay for the detection of antigen-reactive T cells. *J. Immunol. Methods* **186**:37–46.
- Rininsland, F. H., T. Helms, R. J. Asaad, B. O. Boehm, and M. Tary-Lehmann. 2000. Granzyme B ELISPOT assay for *ex vivo* measurements of T cell immunity. *J. Immunol. Methods* **240**:143–155.
- Samols, D., C. G. Thornton, V. L. Murtif, G. K. Kumar, F. C. Haase, and H. G. Wood. 1988. Evolutionary conservation among biotin enzymes. *J. Biol. Chem.* **263**:6461–6464.
- Schoenhaut, D. S., A. O. Chua, A. G. Wolitzky, P. M. Quinn, C. M. Dwyer, W. McComas, P. C. Familletti, M. K. Gately, and U. Gubler. 1992. Cloning and expression of murine IL-12. *J. Immunol.* **148**:3433–3450.
- Simms, P. E., and T. M. Ellis. 1996. Utility of flow cytometric detection of CD69 expression as a rapid method for determining poly- and oligoclonal lymphocyte activation. *Clin. Diagn. Lab. Immunol.* **3**:301–304.
- Taki, S., T. Shimamura, M. Abe, T. Shirai, and Y. Takahara. 1989. Biotinylation of human interleukin-2 for flow cytometry analysis of interleukin-2 receptors. *J. Immunol. Methods* **122**:33–41.
- VanBuskirk, A. M., P. W. Adams, and C. G. Orosz. 1995. Nonradioactive alternative to clinical mixed lymphocyte reaction. *Hum. Immunol.* **43**:38–44.
- Walsh, M. C., J. A. Banas, S. P. Mudzinski, M. T. Preissler, R. F. Graziano, and E. J. Gosselin. 2003. A two component modular approach for enhancing T cell activation utilizing a unique anti-Fc γ R1-streptavidin construct and microspheres coated with biotinylated antigen. *Biomol. Eng.* **20**:21–33.
- Wang, H., and S. K. Beckner. 1992. A colorimetric method for detection of specific ligand binding. *Anal. Biochem.* **204**:59–64.
- Yoshimoto, T., K. Kojima, T. Funakoshi, Y. Endo, T. Fujita, and H. Nariuchi. 1996. Molecular cloning and characterization of murine IL-12 genes. *J. Immunol.* **156**:1082–1088.
- Zhang, G., W. Li, L. Holle, N. Chen, and W. Y. Chen. 2002. A novel design of targeted endocrine and cytokine therapy for breast cancer. *Clin. Cancer Res.* **8**:1196–1205.