Activity of Recombinant Mitogillin and Mitogillin Immunoconjugates*

Marc Better$$, Susan L. Bernhard†, Shau-Ping Lei†, Dianne M. Fishwild‡, and Stephen F. Carroll§

From the XOMA Corporation, ‡Santa Monica, California 90404 and §Berkeley, California 94710

A synthetic gene for the *Aspergillus* protein toxin mitogillin has been synthesized and expressed in *Escherichia coli*. The recombinant mitogillin is a potent inhibitor of protein synthesis *in vitro* with an IC_{50} of 9.7 μM. Immunconjugates of recombinant mitogillin derivatized with S-acetylmercaptosuccinic anhydride and 5-methyl-2-iminothiolane modified H65 antibody kill T cell lines and peripheral blood mononuclear cells expressing the human CD5 surface antigen. Native mitogillin contains 4 cysteine residues which form two disulfide pairs (Fernandez-Luna, J. L., Lopez-Otin, C., Soriano, F., and Mendez, E. (1985) *Biochemistry* 24, 861–867). Three derivatives of mitogillin have been assembled which substitute alanine residues for cysteine residues 5, 147, or 5 and 147. Each of these molecules retains the ability to inhibit protein synthesis *in vitro* with at most a 2-fold reduction in activity. The derivative mitogillinC147A can be conjugated to 5-methyl-2-iminothiolane-modified H65 antibody directly without pretreatment with S-acetylmercaptosuccinic anhydride, and the immunconjugate is active against HSB2 cells. Genetic manipulation of toxin genes to expose an accessible cysteine residue into a recombinant product can thus be used to generate immuno-toxins without initial derivatization by nonspecific cross-linking reagents.

Mitogillin is a cytotoxic protein produced by *Aspergillus restrictus* which inhibits protein synthesis *in vitro* and *in vivo*. Like the related *Aspergillus* proteins α-sarcin and restrictocin, mitogillin is a ribonuclease, cleaving the 28 S rRNA of eukaryotic ribosomes thereby inactivating translation by inhibiting elongation factor 1-catalyzed binding of amino acyl-tRNA to ribosomes (1, 2). Mitogillin can also cleave *Escherichia coli* 25 S rRNA (3), *Zea mays* chloroplast 23 S rRNA (4), and yeast 25 S rRNA (5), all at homologous positions. These cleavage events are site-specific and catalytic.

The activities of mitogillin, α-sarcin, and restrictocin have been studied extensively. Restrictocin differs from mitogillin by only one amino acid (6, 7), and α-sarcin shares 90% amino acid homology (8). The first of this group to be discovered, α-sarcin, was originally identified as an anti-tumor agent (9), and all three proteins exhibit specific cytotoxicity to tumor cell lines *in vitro*. Cell lines infected with a variety of viruses are also particularly sensitive to the cytotoxic effects of this class of ribonuclease-inactivating proteins (10). Although initial interest in these *Aspergillus* proteins was related to their potential as chemotherapeutic agents, there has been recent interest in their use as immunocytotoxins with monoclonal antibodies. Chemical coupling of restrictocin to antibodies which recognize cancer cell targets has been described, and specific cytotoxicity has been observed (11, 12).

Mitogillin is an attractive candidate for linkage to monoclonal antibodies because of its potent inhibition of protein synthesis and relatively small size, with an M_{r} of about 16,900. The gene encoding mitogillin has not been cloned, and the DNA sequence of the native gene is therefore unknown. However, Fernandez-Luna et al. (8) have reported the complete amino acid sequence of the protein. With this information, we have synthesized a recombinant version of mitogillin and expressed this material in *E. coli*. Mitogillin immunocjugates were formed with cross-linking agents which introduce reactive sulfhydryl moieties into both antibody and recombinant mitogillin as each lacks a free cysteine. Derivatives of mitogillin with unpaired cysteine residues were also made which facilitate conjugation to antibody molecules without prior nonspecific derivatization with cross-linking reagents. The properties of these recombinant mitogillin and immunocjugates are described herein.

**MATERIALS AND METHODS**

**Bacterial Strains and Plasmids**—The *E. coli* host for production of mitogillin is a derivative of W3110, obtained from the *E. coli* Genetic Stock Center, Yale University, New Haven, CT. The plasmid used for expression of mitogillin is an inducible secretion vector in which gene expression is under the control of the Salmonella typhimurium araB promoter (10). Secretion of protein through the cytoplasmic membrane is conferred by the *Eruinia carotovora* pelB leader sequence (14). This expression system has been described previously for expression of antibody fragments in *E. coli* (15).

**Mammalian Cells**—The HSB2 human T cell line is available from the American Type Culture Collection (Rockville, MD): K562 is a multipotent hematopoietic malignant cell line, ATCC CCL243. Human peripheral blood mononuclear cells (PBMC) obtained from healthy adults were isolated over ficoll-paque as described (16).

**Assembly of Recombinant Mitogillin**—The mitogillin gene was assembled as described in Fig. 1 from oligonucleotides synthesized with a Cyclone model 8400 DNA Synthesizer, Milligen/Biosearch. The

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The abbreviations used are: PBMC, peripheral blood mononuclear cells; SAMSA, S-acetylmercaptosuccinic anhydride; M2IT, 5-methyl-2-iminothiolane; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; DTNB, 5,5'-dithiobis(nitrobenzoic acid); H65-(MS)-mitogillin, an H65-mitogillin conjugate with an M2IT-SAMSA linker; H65-(MS)-mitogillin, a chimeric H65-mitogillin conjugate with an M2IT-SAMSA linker; H65-(MS)-mitogillin, an H65-mitogillin conjugate with an M2IT linker; PHA, phytohemagglutinin; bp, base pair(s).

* The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EMBL Data Bank with accession number(s) M94249.

§ To whom correspondence should be addressed: XOMA Corporation, 1545 17th St., Santa Monica, CA 90404.
Taq polymerase and the PCR GeneAmp Kit, Perkin-Elmer Cetus Instruments.

Sites engineered into Mito-1 and Mito-6 (SphI and XhoI) and cloned into a vector with compatible restriction sites. Candidate genes were then assembled by mixing 10 µl of the Mito-1,2,3,4 reaction with 10 µl of the Mito-3,4,5,6 reaction and bringing the volume to 20 µl.

The synthetic mitogillin gene was cut with restriction enzymes at sites engineered into Mito-1 and Mito-6 (SphI and XhoI) and cloned into a vector with compatible restriction sites. Candidate genes were sequenced with Sequenase (U.S. Biochemical Corp.) to assure that no base changes had occurred during PCR amplification. The mitogillin gene was excised as an FspI to XhoI fragment and positioned downstream of the pelB leader sequence in pING1500 (15), and cloned pING3520 was identified. The pelB-mitogillin gene was then cloned downstream of the araB promoter into the final expression vector pING3522. Upon arabinose induction, this vector can direct the expression of mitogillin which is secreted from E. coli.

Design and Assembly of Recombinant Mitogillin with Altered Cysteine Residues—Cysteine residues at positions 5 and/or 147 were changed to alanine by site-directed mutagenesis using PCR as described by Ho et al. (17). To change the Cys5 to Ala, pING3522 DNA was amplified with oligonucleotide Mito-11 and araB2, and separately with Mito-10 and Mit3'-5'. Typical reaction conditions were 100 pmol of each primer, a 200 µM concentration of each dNTP, 0.05 µg of template DNA, 2.5 units of Taq polymerase, 50 mM KCl, 10 mM Tris pH 8.3, and 1.5 mM MgCl2. These reactions amplified 240- and 250-bp DNA fragments, respectively. The products of these reactions were mixed and amplified with the primers araB2 and Mit3'-5'. This product was cut with FspI and EcoRI, and the 410-bp piece was purified. This was ligated into the vector along with the 179-bp EcoRI to XhoI fragment, which contains the 5' end of mitogillin, to reassemble the altered mitogillin gene.

The Cys residue 147 was changed to alanine similarly by amplifying pING3522 with primers Mito-12 and HindIII-2, and Mito-13 and Mito-250. These reactions amplified 125- and 210-bp fragments, respectively. These fragments were mixed, amplified with Mito-250 and HindIII-2, cut with EcoRI and XhoI, and the 179-bp fragment was purified. This was closed back into the original vector and verified by DNA sequence analysis.

A mitogillin variant with both Cys to Ala changes was assembled from the individually altered plasmids.

Purpose of Recombinant Mitogillin—Each mitogillin plasmid was transformed into E. coli. Mitogillin was produced by induced cultures grown either in a shake flask in tryptone broth or in a Chemap 1-liter fermenter. Mitogillin was purified from the concentrated culture supernatant in 10 liters of minimal medium supplemented with 0.7% glycerol. For shake flask production, cells were grown to an A600 of 0.4 and induced with 0.1% arabinose for 16 h. In the fermenter, the cells were induced by adding arabinose to 0.05% when an A600 = 50 was reached, and the cells were incubated for approximately 16 h following induction.

Mitogillin was purified from the culture supernatant of a 1-liter induced shake flask culture by Blue Toyopearl (Toyosoda) column chromatography. The pH of the culture supernatant was adjusted to 6.5 with 0.2 M sodium phosphate buffer and diluted with water to a conductivity of 1.0 millisiemens/cm. The sample was filtered through a 0.45-µm filter, and 800 ml was loaded onto a 2-ml Blue Toyopearl column (equilibrated with 10 mM sodium phosphate, pH 6.5) at 1 ml/min. After sample loading, the column was washed with 30 ml of 10 mM sodium phosphate buffer, pH 6.5, and the bound mitogillin eluted with 20 ml of 0.5 M NaCl, 10 mM sodium phosphate, pH 6.5. The sample was concentrated and analyzed by SDS-PAGE (see Fig. 3). The mitogillin consisted of 56% 36-kDa and 44% 19-kDa proteins under nonreducing gel conditions. Upon reduction, only a 19-kDa protein existed which is monomeric mitogillin. It is estimated that at least 1.0 µg/ml mitogillin was produced in the shake flask supernatant.

Each of the four forms of recombinant mitogillin was purified from the concentrated culture supernatant of a 10-l bacterial fermentation broth. The concentrated fermentation broth was exchanged into 10 mM sodium phosphate, pH 7.5, using a DC10 with S300 column (Amicon), and loaded onto a CM-52 (Whatman) column preequilibrated with 10 mM sodium phosphate, pH 7.5. Protein was eluted from this column with a 0–300 mM NaCl linear gradient in 10 mM sodium phosphate buffer, pH 7.5; mitogillin eluted between 150 and 240 mM NaCl.

Mitogillin Enzyme-linked Immunosorbent Assay Procedure—Rabbits were immunized with mitogillin to generate specific antibody, which was affinity purified from serum. Stock solutions of 1 mg/ml in PBS (10 mM sodium phosphate, 0.15 M NaCl) pH 7.4, were stored at -20 °C. A portion of this material was biotinylated (NHS-LC-Biotin, Pierce) and was stored at 0.56 mg/ml in 1% BSA-PBS, pH 7.4, at -20 °C. A mitogillin standard (18 mg/ml in 0.5% BSA, 50% glycerol, PBS, pH 7.4) was prepared from purified mitogillin.

A sandwich enzyme-linked immunosorbent assay was used for detection of mitogillin in culture supernatants. Plates were coated with affinity-purified rabbit anti-mitogillin antibody at a final concentration of 4°C. Wells were then blocked with 1% BSA-PBS for 30 min at 37°C, and sample dilutions were added. After three washes, biotinylated affinity-purified rabbit anti-mitogillin antibody was added for 1 h at
Recombinant Mitogillin and Mitogillin Immunoconjugates

**TABLE I**

Oligonucleotides used to construct mitogillin

| Oligonucleotides used to construct mitogillin |
|---------------------------------------------|
| Mito-1: 5'-GAT TGC ATG CGC AAC ATG GAC CT-3' |
| Mito-2: 5'-TCC ATT CGT GAA CCA GTG AGG ATA CGA ACT TCC AGT-3' |
| Mito-3: 5'-CTG GTC CAC GAA TGT ATA CGA TGG AAA CGG TAT TTG-3' |
| Mito-4: 5'-ATC GAA TTT GTA GTC ATG TCC ATC TGG AAA TCG GAG-3' |
| Mito-5: 5'-CAT GAC TAC AAA TTC TAT CAG AAA CTT AAC AAG GAG-3' |
| Mito-6: 5'-ACA TGA GCT CGA CTA CCT TCC TCA ATG AGA ACA-3' |
| Mito-7: 5'-GAT TGC ATG CGC AAC ATG GAC CT-3' |
| Mito-8: 5'-ACA TGA TGG CGA GCA CTG CTA CTG TGC ATG AGA TTC-3' |
| Mito-10: 5'-GAC CGC TAT CAA CCA GCA ACT TAA-3' |
| Mito-11: 5'-GCT GGT TGA TAG CGG TCC ATG TGT C-3' |
| Mito-12: 5'-CTC AGC TTC TCA TGG AAG GGA GAG-3' |
| Mito-13: 5'-TTC AAT GAG CGA GTC GGA GTG GAT C-3' |
| Mito-250: 5'-CAT TCG CAG AAA GGT ATG-3' |
| Mit3'-5': 5'-CCA TCT GGA AAT GTC GG-3' |
| HindII-2: 5'-CTG TAG CAA TTT AAC TGT GAT-3' |
| araB2: 5'-GCG ACT CTC TAC GTG TTC-3' |

**Preparation**

**H65 and eH65—**H65 is a murine IgG1/α monoclonal antibody which recognizes the human CD5 antigen (20-22). A chimera antibody (mouse variable regions, human constant regions) version of H65 antibody was constructed as described (23). Chimeric H65 antibody (eH65) was expressed in mammalian cells and exhibited binding characteristics identical to the murine H65 antibody.²

**Preparation of H65-RTA—**H65-RTA is an immunoconjugate composed of the anti-human CD5 murine monoclonal antibody H65 linked through N-succinimidyl 3-(2-pyridylthiolyloxy)propionate (Pierce) to RTA. The details of its preparation have been described (20). H65-RTA is cytotoxic to cells expressing the human CD5 antigen.

**Preparation of Murine and Chimeric H65-(M2IT)(SAMSA)-mitogillin, and IND2-MS-mitogillin—**To prepare this disulfide-linked immunoconjugate, both antibody and toxin were modified at lysine residues to introduce reactive sulfhydryl groups. H65 or eH65 antibody was modified with 5-methyl-2-iminothiolane (M2IT) in the presence of 5,5'-dithiobis(nitrobenzoic acid), DTNB, as described (24). Recombinant mitogillin was treated for 30 min at room temperature with equimolar amounts of S-acetylmercaptoacetic anhydride (SAMSA, Sigma) (25), and then desalted on Trisacryl GF05 (IBF Biotechnics). For conjugation, a 5-fold molar excess of mitogillin-SAMSA was combined with derivatized H65 in the presence of hydroxyamine (60 mM, pH 7.5) and incubated at room temperature for 3 h (or 18 h at 4°C) prior to column purification. A mixture of immunoconjugate and free antibody was separated from unconjugated toxin on an Ultragel AcA44 column (IBF Biotechnics) equilibrated with 10 mM Tris-Cl, 150 mM NaCl, pH 7. Free antibody was separated from pure immunoconjugate by loading onto a Blue Toyopearl column equilibrated with 10 mM Tris-Cl, 20 mM NaCl, pH 7.5, and eluting with 10 mM Tris-Cl, 0.25 mM NaCl, pH 7.5. Samples of the final product were examined by 5% nonreducing SDS-PAGE, Coomassie stained, and scanned with a Shimadzu laser densitometer to quantify the number of toxin molecules/antibody.

IND2-MS-Mitogillin was prepared by the method described above. IND2 is an isotype-matched negative control antibody specific for a melanoma antigen and does not bind to human T cells.

**Preparation of H65-(M2IT)-mitogillin<sub>araB2</sub>—**H65 antibody was modified with a 12-fold molar excess of M2IT in the presence of 2 mM DTNB. The mitogillin<sub>araB2</sub> was treated briefly with DTI to expose a reactive sulfhydryl group at position 5 and then desalted (the presence of a free sulfhydryl was verified by reaction with DTNB). H65 derivatized with M2IT was incubated with reduced mitogillin<sub>araB2</sub> at a molar ratio of 1:5 at room temperature for 3 h and then 16 h at 4°C. The conjugate was purified on AcA44 followed by Blue Toyopearl columns. Samples of the final product were run on 5% nonreducing SDS-PAGE, Coomassie stained, and scanned with a Shimadzu laser densitometer to quantitate the number of toxin molecules/antibody.

² D. M. Fishwild, unpublished observations.
Whole Cell Cytotoxicity Assays—These assays measure the inhibition of macromolecular synthesis in intact T cells by immunoconjugates. H65-RTA and immunoconjugate samples were diluted with RPMI without leucine at half-log concentrations ranging from 2,000 to 0.632 ng/ml. All dilutions were added in triplicate to microtiter plates containing 1 × 10^6 HS52 cells. These cells were incubated 20 h at 37 °C and then pulsed with [3H]leucine for 4 h before harvesting. Samples were counted on the Inotech Trace 96. By comparison with an untreated sample, IC_{50} can be calculated. The extent of kill was calculated by dividing the [3H]leucine uptake (cpm) at the highest concentration of immunoconjugate tested (usually 1 μg/ml) by that uptake in the absence of any added immunoconjugate, subtracting that result from 1, and multiplying by 100%. An identical assay with K562 cells which do not express the CD5 antigen was done as a negative control.

The whole cell cytotoxicity assay with human PBMC was performed in a similar manner (16). PBMC (1 × 10^6/well) were treated in triplicate with various concentrations of immunoconjugates in microtiter plates and stimulated with 3 μg/ml leukoagglutinin (PHA, Pharmacia LKB Biotechnology Inc.). Cultures were incubated for 90 h at 37 °C in a 10% CO2 incubator with humidified air. Tritiated thymidine ([3H]Tdr, DuPont-New England Nuclear, 1 μCi/well) was added 16 h prior to harvest. Uptake of [3H]TDR was quantitated with the Inotech Trace 96 Detection System. The IC_{50} was determined from the immunoconjugate concentration resulting in a 50% inhibition of the maximum (no immunoconjugate) [3H]TDR uptake as follows:

\[
\text{50\% inhibition} = \frac{\text{cpm (PHA, no immunoconjugate)} - \text{cpm (no PHA)}}{2}
\]

The extent of kill was calculated as described above, substituting [3H]Tdr uptake for [3H]leucine uptake.

RESULTS

Design and Assembly of Recombinant Mitogillin—Based on the reported amino acid sequence of mitogillin, oligonucleotides were synthesized and used to assemble a gene which encodes the mitogillin amino acid sequence. These oligonucleotides ranged in length from 92 to 98 bp and were assembled into a complete gene and cloned into an expression vector for regulated expression of recombinant product. The procedure used to assemble mitogillin is outlined in Fig. 1, and the complete DNA sequence of the synthetic mitogillin gene is shown in Fig. 2. Mitogillin expression is under the control of the araBAD promoter which is tightly regulated by the inducer arabinose. Mitogillin could be detected in the culture supernatants of induced E. coli cultures with specific antibodies and appears as a band of 32 kDa on Coomassie-stained gels. Without reduction, both mitogillin A and mitogillin A have 30-35% available free SH as determined by Ellman's reagent.

Three additional mitogillin genes were constructed which contained alanine substitutions for cysteine residues at positions 5, 147, or 5 and 147. These residues form an intrachain disulfide bond, are near the N and C termini of the protein and may be accessible to the surface of the molecule. Each of these mitogillin proteins was produced in the same E. coli expression system and could be purified directly from induced culture supernatants.

Characterization of the Recombinant Mitogillin Proteins—Fig. 3, A and B, shows the typical SDS-PAGE band patterns seen for each of the recombinant mitogillin proteins. All four recombinant forms were efficiently produced in E. coli, but the yield of wild-type mitogillin is about four times that of the modified molecules (data not shown). Wild-type recombinant mitogillin may associate in E. coli supernatants to form an apparent dimer which can be reduced to monomer of M, 19,000, although the fraction of dimer found in the fermentation broth is variable (compare lanes 1 and 3, Fig. 3A).

Mitogillin A was subjected to a number of proteolytic treatments which are variable (compare lanes 1 and 3, Fig. 3A). Mitogillin A and mitogillin A were shown to form a stable disulfide bond, as shown by SDS-PAGE analysis of four recombinant proteins of mitogillin purified from fermentation broth. Lane 1, mitogillin A; lane 2, mitogillin A; lane 3, mitogillin A; lane 4, mitogillin A. This 12.5% gel was run under nonreducing conditions.

In Vitro Activity of Recombinant Mitogillin—Recombinant mitogillin and its analogs were assayed for inhibition of protein synthesis in a rabbit reticulocyte assay (RLA). As shown
Recombinant Mitogillin and Mitogillin Immunoconjugates

in Table II, the IC_{50} was 9.7 pm. This value for mitogillin is similar to that reported for restrictocin of 10 pm (12). The three derivatives of mitogillin in which 1 or 2 of the 4 cysteine residues were converted to alanines were also tested in this assay. Each of these altered forms of mitogillin retained the ability to inhibit protein synthesis in vitro. The IC_{50} for each of the single alanine derivatives was 10.2 pm (mitogillin_{C147A}) and 13.7 pm (mitogillin_{CSA}), whereas the IC_{50} for mitogillin_{CSAC147A} was 18.7 pm. The IC_{50} of RTA_{30} was determined for comparison, and the relative potency of the four mitogillin species to RTA_{30} is shown in Table II as a ratio. Recombinant mitogillin and mitogillin_{CSAC147A} are roughly 40% as active as RTA_{30}, whereas the activity of mitogillin_{CSA} is slightly lower. Mitogillin_{CSAC147A} is approximately 20% as active as RTA_{30}.

Characterization of Recombinant Mitogillin Immunoconjugates—Recombinant mitogillin, mitogillin_{CSA}, and mitogillin_{CSAC147A} were conjugated with H65, a murine antibody which recognizes the human CD5 antigen. Mitogillin was also conjugated to chimeric H65 antibody. Antibody lysine residues were derivatized to produce an activated thiol for conjugation, using M2IT in the presence of DTNB. Recombinant mitogillin was derivatized with SAMSA to provide a reactive thiol, whereas mitogillin_{CSA} and mitogillin_{CSAC147A} were conjugated directly to the activated antibody through the unique unpaired cysteine at Cys^{41} or Cys^{8}, respectively. (The available cysteine at position 5 in mitogillin_{CSAC147A} was confirmed by protein microsequencing after in situ alkylation with 4-vinylpyridine (26).) Conjugation efficiency (shown in all lanes in Fig. 4) was 60-70% for H65-MS-mitogillin, chH65-MS-mitogillin, and H65-M-mitogillin_{CSA}, but only 8% for H65-M-mitogillin_{CSAC147A}. The purity and average toxin to antibody ratio of each final product (except H65-M-mitogillin_{CSA}) were determined by densitometry of Coomassie-stained SDS-PAGE. Because of poor conjugation efficiency, the mitogillin_{CSA} conjugate was lost during purification. The remaining conjugates were all greater than 98% pure after column chromatography (shown in all c lanes in Fig. 4). The toxin to antibody ratios were 1.3 for H65-MS-mitogillin and cH65-MS-mitogillin, and 1.6 for H65-M-mitogillin_{CSAC147A}.

Activity of Recombinant Mitogillin Immunoconjugates—The purified immunom conjugates were tested for specific cytotoxicity on the human T cell line HSB2 and on PBMC. A typical PBMC assay with H65-MS-mitogillin, chH65-MS-mitogillin, IND2-MS-mitogillin, and H65-RTA is shown in Fig. 5. The figure illustrates the concentration-dependent cytotoxicity, the IC_{50} values, and the extent of kill achieved with these conjugates. The negative control IND2-MS-mitogillin was nontoxic. The results of cytotoxicity assays for all anti-CD5 mitogillin conjugates tested are summarized in Table III. On HSB2 cells, the IC_{50} values of chH65- and H65-MS-mitogillin (~200 pm toxin) were 90-96% of that measured for the H65-RTA standard, which is a well characterized immunon conjugate (16, 20-22). The extent of kill for the two mitogillin conjugates was comparable (70-75%) to that of H65-RTA (78%). The cytotoxicity of the H65-MS-mitogillin_{CSAC147A} conjugate was 3-fold lower (640 pm, toxin) and the extent of kill was not as great (55%) on the T cell line. Against PBMC, H65-MS-mitogillin (IC_{50} = 97.8 pm toxin) was twice as cytotoxic as H65-RTA, whereas the chimeric H65-MS-mitogillin conjugate had an intermediate IC_{50} (158 pm toxin). The extent of kill for all immunoc conjugates was similar (approximately 75%). H65 conjugates were not cytotoxic to the antigen-negative cell line K562 (data not shown).

Cytotoxic proteins are produced by prokaryotes and eukaryotes, plants and animals, presumably conferring some selec-

Fig. 4. SDS-PAGE analysis of mitogillin immunom conjugates. Mitogillin immunon conjugates with murine H65 (H65) or chimeric H65 (cH65) were prepared as described under “Materials and Methods.” Each reaction product was examined on a 5% nonreducing gel. The a lanes show antibody alone; b lanes show crude immunon conjugate reaction mix; and c lanes show purified immunon conjugate products. Panel 1, H65-MS-mitogillin; panel 2, H65-MS-mitogillin; panel 3, H65-M-mitogillin_{CSAC147A}; and panel 4, H65-M-mitogillin_{CSA}. The purified immunom conjugate product of H65-M-mitogillin_{CSA} is not shown (see text).

Fig. 5. Cytotoxicity of H65-mitogillin conjugates and H65-RTA against PBMC. Whole cell kill assays were performed as described under “Materials and Methods.” Results are plotted as percent of ([^3]H)TdR incorporation versus immunon conjugate concentration. The results from a single experiment are shown. The extent of kill, the percent decrease in DNA synthesis at the highest conjugate concentration tested, was 75-79% in this assay for all samples. IND2-MS-mitogillin (●) is a negative control (IC_{50} > 1000 ng/ml). □, H65-RTA (IC_{50} 19.8 ng/ml); ▲, H65-MS-mitogillin (IC_{50} 7.1 ng/ml); ■, cH65-MS-mitogillin (IC_{50} 15.0 ng/ml); -----, 50% of maximum response.

| Toxin name | IC_{50}^a | Relative potency RTA/toxin |
|------------|-----------|---------------------------|
| Mitogillin  | 9.7       | 0.40                      |
| Mitogillin_{CSA} | 13.7  | 0.28                      |
| Mitogillin_{CSAC147A} | 10.2  | 0.38                      |
| Mitogillin_{CSAC147A} | 18.7  | 0.21                      |
| RTA 30      | 3.9       | 1.00                      |

^a The IC_{50} values reported are averages of at least three assays. The IC_{50} for mitogillin reflects an average of five assays, and the value for RTA 30 is an average of 20 assays.
Recombinant Mitogillin and Mitogillin Immunonoconjugates

| Conjugate name  | HSB2 cells | PBMC |
|-----------------|------------|------|
|                | IC50 | IC50 | % Kill | IC50 | IC50 | % Kill |
| ng/ml | pm*T | ng/ml | pm*T |
| H65-MS-mitogillin | 26.2 | 198 | 70 | 13.0 | 97.8 | 75 |
| H65-M-mitogillin| 70.5 | 640 | 55 | NA | NA | NA |
| H65-MS-mitogillin| 27.9 | 210 | 75 | 20.9 | 158 | 78 |
| H65-RTA | 22.5 | 190 | 78 | 22.0 | 210 | 77 |

IC50 is expressed here as mol of mitogillin or RTA toxin. The average number of toxin molecules/antibody was calculated by densitometry of SDS-PAGE in Fig. 4 and used to determine the molecular weight of the immunonoconjugate. This factor multiplied times the (pm) IC50 gives the IC50 as molar toxin (pm*T). The IC50 values are averages of at least three assays.

Acknowlegdements—We are grateful to Connie Galicia and Ann Orme for preparation of immunonoconjugates; Hsueh-Mei Wu, Eddie Bautista, and Maria Molina for in vitro assays; Keith Duncan for microsequencing analysis; and Maria Fang and Marcelo Ortigao for excellent technical assistance. We also thank Julie Lane and Lynn Grinn for critically reading the manuscript.

REFERENCES
1. Endo, Y., and Wool, I. G. (1982) J. Biol. Chem. 257, 9043-9060
2. Fando, J. L., Alaba, I., Escarinas, C., Fernandez-Luna, J. L., Mendez, E., and Salinas, M. (1985) Eur. J. Biochem. 149, 29-34
3. Brosius, J., Dull, T. J., and Noller, H. F. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 201-204
4. Edwards, R. K., and Rosenfeld, H. (1981) Nucleic Acids Res. 9, 2853-2869
5. Veldman, R. J., Koolwijk, J., and de Rege, Y. C. H. F. (1981) Nucleic Acids Res. 9, 6935-6952
6. Opent-Off, D. B., Fernandez-Luna, J. L., Soriano, F., and Mendez, E. (1984) Eur. J. Biochem. 143, 621-654
7. Fernandez-Luna, J. L., Lopez-Off, C., Soriano, F., and Mendez, E. (1985) Biochemistry 24, 861-867
8. Oakley, S. H., and Gonzalez, G. L. (1985) Appl. Microbiol. 13, 714-721
9. Fernandez-Pontes, C., and Carrasco, L. (1980) Cell 20, 769-775
10. Orlandi, R., Canevari, S., Conde, F. P., Leoni, F., Mezzanzanica, D.,...
Recombinant Mitogillin and Mitogillin Immunoconjugates

Ripamonti, M., and Colnaghi, M. I. (1988) Cancer Immunol. Immunother. 26, 114–120

12. Conde, F. P., Orlando, R., Caneveri, S., Mezzanzanica, D., Ripamonti, M., Monoz, S. M., Jorge, P., and Colnaghi, M. I. (1988) Eur. J. Biochem. 178, 786–803

13. Johnston, S., Lee, J. H., and Ray, D. S. (1985) Gene (Amst.) 34, 137–145

14. Lei, S.-P., Lin, H. C., Wang, S. S., Callaway, J., and Wilcox, G. (1987) J. Bacteriol. 169, 4379–4383

15. Better, M., Chang, C. P., Robinson, R. R., and Horwitz, A. H. (1988) Science 240, 1041–1043

16. Fishwild, D. M., Steakiewicz, M. O., Wu, H.-M., and Carroll, S. F. (1991) Clin. Exp. Immunol. 86, 506–513

17. Ho, S. N., Hunt, H. D., Horton, R. M., Pulkin, J. K., and Pease, L. R. (1988) Gene (Amst.) 77, 51–59

18. Press, O. W., Vitetta, E. S., and Martin, P. J. (1986) Immunol. Lett. 14, 37–41

19. Trown, P. W., Reardon, D. T., Carroll, S. F., Stoudemire, J. B., and Kawahata, R. T. (1991) Cancer Res. 51, 4219–4225

20. Kernan, N. A., Knowles, R. W., Burza, M. J., Breznmeyer, H. E., Lu, L., Lee, H. M., Kawahata, R. T., Scannon, P. J., and Dupont, B. (1984) J. Immunol. 133, 137–146

21. Kernan, N. A., Byers, V., Scannon, P. J., Mischak, R. P., Brochstein, J., Flomenberg, N., Dupont, B., and O'Reilly, J. (1988) JAMA 259, 3154–3157

22. Byers, V., Henabee, C. F., Kernan, N., Blazer, B. R., Gingrich, R., Phillips, G. L., LeMaistre, C. F., Gilliland, G., Antin, J. H., Vogelsang, G., Martin, P., Tutschka, P. J., Trown, P., Ackerman, S. K., O'Reilly, R. J., Scannon, P. J. (1990) Blood 75, 1426–1432

23. Robinson, R. R., Charzic, J. Jr., Chang, C. P., Horwitz, A. H., and Better, M. (1991) Hum. Antibod. Hybridomas 2, 84–93

24. Goff, D. A., and Cassell, S. F. (1990) Bioconjugate Chem. 1, 381–386

25. Weston, P. D., Devreis, J. A., and Wrigglesworth, R. (1980) Biochem. Biophys. Acta 612, 40–49

26. Andrews, P. C., and Dixon, J. E. (1987) Anal. Biochem. 161, 524–528

27. Roga, V., Hedernain, L. P., and Olson, B. H. (1971) Cancer Chemother. Rep. 55, 101–113

28. Arruda, L. K., Patte-Mills, T. A. E., Fox, J. W., and Chapman, M. D. (1980) J. Exp. Med. 152, 1529–1532