Structure-Based Design of a Bispecific Receptor Mimic That Inhibits T Cell Responses to a Superantigen†

Nancy M. Lehnert,‡ David L. Allen,‡ Beth L. Allen,‡ Paolo Catasti,‡ Patrick R. Shiflett,‡ Michael Chen,§ Bruce E. Lehnert,‡ and Goutam Gupta*,‡

Bioscience Division, Mail Stop M888, Los Alamos National Laboratory, Los Alamos, New Mexico 87545, and Texas BioGene, Inc., 1303 Columbia Drive, Suite 201, Richardson, Texas 75081

Received September 14, 2000; Revised Manuscript Received December 15, 2000

ABSTRACT: Key surface proteins of pathogens and their toxins bind to the host cell receptors in a manner that is quite different from the way the natural ligands bind to the same receptors and direct normal cellular responses. Here we describe a novel strategy for "non-antibody-based" pathogen countermeasure by targeting the very same "alternative mode of host receptor binding" that the pathogen proteins exploit to cause infection and disease. We have chosen the *Staphylococcus* enterotoxin B (SEB) superantigen as a model pathogen protein to illustrate the principle and application of our strategy. SEB bypasses the normal route of antigen processing by binding as an intact protein to the complex formed by the MHC class II receptor on the antigen-presenting cell and the T cell receptor. This alternative mode of binding causes massive IL-2 release and T cell proliferation. A normally processed antigen requires all the domains of the receptor complex for its binding, whereas SEB requires only the α1 subunit (DRα) of the MHC class II receptor and the variable β subunit (TCRVβ) of the T cell receptor. This prompted us to design a bispecific chimera, DRα−linker−TCRVβ, that acts as a receptor mimic and prevents the interaction of SEB with its host cell receptors. We have adopted $(GSTAPPA)_2$ as the linker sequence because it supports synergistic binding of DRα and TCRVβ to SEB and thereby makes DRα−$(GSTAPPA)_2$−TCRVβ as effective an SEB binder as the native MHC class II−T cell receptor complex. Finally, we show that DRα−$(GSTAPPA)_2$−TCRVβ inhibits SEB-induced IL-2 release and T cell proliferation at nanomolar concentrations.

Protein toxins are important mediators as well as causative agents of bacterial pathogenesis. As a defense mechanism, bacteria release their toxins to help support their growth and proliferation in host environments that tend to threaten their survival. Upon being released, the toxins target the host cells by binding to surface receptor domains other than those bound by the natural ligand (*1*, *2*). Such binding allows the bacterial toxin to subvert the normal function of the host cell receptor and to trigger a cascade of intra- and extracellular effects leading to cytotoxicity. Although the use of antitoxin antibodies is an obvious countermeasure against bacterial infection, we propose an alternative approach that may be as effective as antitoxin antibodies. This approach involves the use of receptor mimics, or decoy molecules, that prevent the first step in toxin pathogenesis, namely, the binding of bacterial toxins to their specific receptors on the surface of human host cells. We hypothesize that the structural requirements for toxin−receptor binding can be exploited in the structure-based design of a toxin-specific ligand that can compete with cell surface receptors. The strategy for the construction of such a receptor mimic is to incorporate the structural elements of the receptor required only for toxin binding and not those required for the binding of the natural ligand. Thus, the designed receptor mimics are expected to inhibit only the binding of toxins to the host cell receptors and not the binding of the natural ligands to the same host cell receptors.

We have tested our hypothesis using *Staphylococcus* enterotoxin B (SEB), a superantigen, as a model toxin. SEB is a 28 kDa protein produced by the Gram-positive bacterium *Staphylococcus aureus*. It is a member of the *Staphylococcus* superantigen family that is responsible for about a quarter of the reported cases of food poisoning in the United States (*3*). Like other members of the family (SEA, SEC1−3, SED, and SEE), SEB is presented to the host immune system in a manner that is quite different from the way normal antigens are presented (*2*, *3*). A normal antigen is internalized, processed into small peptide fragments, and presented by the major histocompatibility complex (MHC) class II receptor on the surface of the antigen-presenting cells (APCs). The MHC class II receptor is an αβ heterodimer with α1/α2 and β1/β2 subdomains (*4*). The antigen-bound MHC class II receptor forms a molecular synapse with the T cell receptor (TCR) on the T cell. The TCR is also an αβ heterodimer with each chain comprised of immunoglobulin-like variable Vα/β and constant Cα/β domains (*5*). The processed antigen docks snugly in the cavity formed by the appropriate folding and contact of all the α/β subdomains of the MHC class

† This work was supported by DARPA Grant RK78 (Structural Biology of Bacterial Superantigens) and DOE-LDRD Grant X1AC (Host-Pathogen Interactions).

* Corresponding author. Telephone: (505) 665-2587. Fax: (505) 665-3493. E-mail: gxg@lanl.gov.

‡ Los Alamos National Laboratory.

§ Texas BioGene, Inc.

10.1021/bi002172e CCC: $20.00
Published on Web 03/15/2001

II−TCR synapse. A normal antigen typically induces proliferation of only 1 out of 1000 T cells via the clonal expansion of antigen specific cells (*2*). Unlike normal antigens, SEB binds as an intact protein to the MHC class II−TCR complex and bypasses the normal route of antigen processing. Also, SEB binds to the exterior of the MHC class II−TCR complex by making contacts only with the α1 subdomain (or DRα) of the MHC class II receptor and the Vβ subdomain (or TCRVβ) of the TCR (*6*, *7*). Since this external mode of SEB binding is possible for a large number of MHC class II alleles and a sizable subset of human TCRVβ alleles, SEB displays extremely potent immune stimulatory activities, including excessive release of various cytokines, including interleukin 2 (IL-2), and massive (i.e., 1 out of 10) proliferation of CD4+ and CD8+ T cells. IL-2 release occurs within hours of SEB exposure, whereas T cell proliferation peaks at 2−3 days (*8*). In vivo studies demonstrate that physiological effects of SEB, such as weight loss in mice, can be correlated with excessive cytokine release (*9*).

Since DRα and TCRVβ are the only two receptor-binding domains required for SEB pathogenesis, we envisioned that a DRα−linker−TCRVβ chimera should be an efficient receptor mimic of the cell surface-bound MHC class II−TCR complex. Like the native MHC class II−TCR complex, such a chimera should also target two nonoverlapping sites on SEB. As a result, this bispecific chimera should act as a decoy and prevent SEB from binding to the target APC and T cell, and thereby inhibit SEB-induced IL-2 production and T cell proliferation. The length and amino acid sequence of the linker should be designed to allow the simultaneous binding of these two subdomains to SEB and not to sterically hinder the native folding of the individual DRα and TCRVβ. The DRα−linker−TCRVβ chimera (a bispecific ligand) should also show a higher affinity for SEB binding than the individual DRα and TCRVβ ligands because the presence of two different binding modules in the same molecule should facilitate the synergy of the two binding events. That is, the covalent linkage should provide spatial proximity of each of the ligands to the SEB. Thus, the binding of DRα to SEB should increase the local concentration of TCRVβ and its level of binding to SEB and vice versa.

In this paper, we provide experimental proof in support of our hypothesis. First, we describe the structural basis of the design of a human DRα−linker−TCRVβ chimera. Second, we demonstrate that the chimera can be cloned and overexpressed in *Escherichia coli* and the purified protein can be appropriately folded. Third, we show that DRα−linker−TCRVβ chimera specifically binds to SEB and the linker supports synergistic binding of the individual DRα and TCRVβ domains. Finally, we show that the chimera inhibits SEB-induced IL-2 release and T cell proliferation. SEB at 0.5 nM can target human blood cells and cause excessive IL-2 release and massive T cell proliferation. We show that the chimera at SEB-equivalent concentrations can inhibit the cytotoxic effects of SEB on human blood. However, in this range of concentrations (i.e., 1−10 nM) and for the duration of the assays (i.e., 4−72 h), the chimera causes no damage or cytotoxicity to human blood cells.

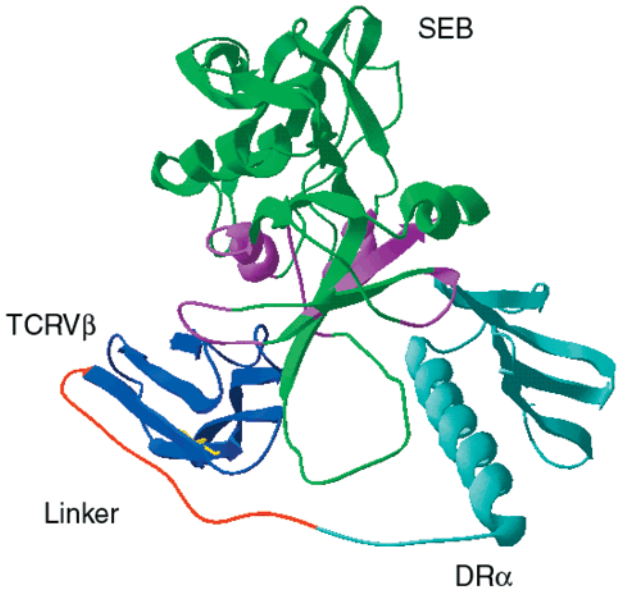

FIGURE 1: Representative low-energy model of the DRα−$(GSTAPPA)_2$−TCRVβ chimera obtained using a restrained simulated annealing approach. Different structural elements are color coded: DRα, cyan; TCRVβ, blue; linker, red; and SEB, green and magenta. The flexible linker for the most part is extended except for the C-terminal end where it loops to form a covalent bond with the N-terminus of TCRVβ. A part of the linker β strand associates with the "b" β strand of TCRVβ in an antiparallel orientation. DRα and TCRVβ make contacts on the same side of SEB but with different surface epitopes (shown in magenta). The PDB file of the SEB−chimera complex containing the appropriate numbering scheme and designation of secondary structural elements can be obtained from the authors.

EXPERIMENTAL PROCEDURES

Molecular Modeling

We performed the following steps to arrive at the final computer design of the chimera. First, we obtained the sequences of DRα and TCRVβ that were most likely to retain the native folds which are shown as cyan and blue modules, respectively, in Figure 1. Finally, we chose the sequence of the linker (shown in red in Figure 1) that supported simultaneous and synergistic binding of the two individual modules of the chimera to SEB.

Design of DRα and TCRVβ. We computed the folding energy of a chosen set of 15 DRα sequences (*4*) as the energy difference between the fully extended ($\varphi = -155°$, $\psi = 150°$) DRα conformation and the native folding of DRα as present in the MHC class II−SEB complex (*6*). A subset of the five most stably folded DRα sequences were further tested for optimum contacts with SEB, as judged by the level of surface exposure of the key DRα residues.

IKEEHVIIQAEFYLNPDQSGEFMFDFDGDEIFHVDMAKKETVWRLEEFGRFASFEAQGALANIAVDKANLEIMTKRSNYTPITN appeared to be the candidate human DRα sequence most likely to show stable folding and favorable SEB binding. Folding energies were also computed for a chosen set of 15 TCRVβ sequences (*5*). A subset of most stably folded TCRVβ sequences were further tested for the optimum distribution of the surface loops, CDR1, CDR2, HV4, and CDR3, that are involved in SEB binding (*7*). We deleted the last β hairpin loop from the TCRVβ sequence since the CDR3 loop does not show any contact with SEB

in the TCR−SEB complex. In the chosen human (HVβ3) TCRVβ sequence, VFLEC̲VQDNDHENMFWYRQDPGLGLRLIYFSYDVKNKEKGDIPEGYSVSREKKERFSLILESASTNQTSMYLC̲A, the two Cs (underlined) were retained to ensure native folding through disulfide bonding between the "b" and "f" strands.

Design of the Linker. The designed DRα and TCRVβ sequences were covalently joined by the linker sequence, $(GSTAPPA)_2$, to obtain the chimera. A restrained molecular dynamics (MD) simulated annealing was performed for the chimera−SEB complex such that the backbone (φ and ψ) conformations of DRα, TCRVβ, and SEB and the SEB−DRα and SEB−TCRVβ contacts were kept close to those of their native MHC class II−SEB and TCR−SEB complexes (*6*, *7*). Snapshots from the 300K MD trajectory were energy-minimized to obtain a set of representative models of the chimera−SEB complex in which the linker adopted appropriate conformations to allow simultaneous binding of DRα and TCRVβ.

Protein Overexpression and Purification

The variable β (Hβ3) region of the T cell receptor was synthesized as multiple oligonucleotide fragments and ligated stepwise to complete the modified receptor sequence. The MHC class II DRα was amplified by PCR from chromosomal DNA in consecutive enrichment reactions to include nonvector restriction sites. Both DRα and TCRVβ were cloned into the *Xho*I−*Bgl*II site of pET 32B and expressed in AD 494 cells as thioredoxin−His fusion proteins. The presence of an N-terminal enterokinase domain between the fusion domain and the DRα and TCRVβ fragments allowed the release of mature proteins by enterokinase cleavage. For the chimera, the designed DRα and TCRVβ fragments were ligated to the linker DNA sequence encoding $(GSTAPPA)_2$ and subsequently cloned into the *Xho*I−*Bgl*II site of pET 32B. The chimera was then subcloned into AD 494 cells and expressed as a thioredoxin−His fusion protein containing an N-terminal enterokinase domain. AD 494 cells containing the chimera construct were grown to an OD_{560} of 0.7 at 37 °C. Cultures were induced with 2 mM IPTG, and the temperature was shifted to 30 °C for an additional 3 h. Cells were collected by centrifugation and pellets resuspended in 20 mM phosphate buffer (pH 7.5) to a concentration of 3 mL/L of starting culture. Cells were disrupted by a French press, and the soluble fraction was removed. Insoluble pellets were redissolved in a 6 M urea solution [5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl (pH 8.0), and 6 M urea]. Cells were again centrifuged, and the supernatant was loaded on charged Ni^{2+} columns and eluted according to manufacturer's directions. Chimeras were then digested by enterokinase and re-eluted from Ni^{2+} columns to separate the thioredoxin−His component from the chimera. Proteins were run on 16% SDS PA, electroeluted into dialysis tubing, concentrated, and dialyzed versus 50 mM NaCl and 5 mM phosphate buffer (pH 7.5). To delete the enterokinase−electroelution steps of the chimera, we proceeded to sublcone the chimeric sequence into a pRSET (Invitrogen, Carlsbad, CA) vector. We estimated that the loss of the large thioredoxin fusion would not adversely affect the expression of the chimera. In addition, we determined that the few extraneous N/C residues would possibly allow more efficient conjugation of the chimera to the sensor platform or to an enterokinase-specific mAb. The sequence of the chimera was amplified by PCR from the original pET 32B clone as a *Pst*I−*Hin*dIII fragment and subcloned into the same sites of pRSET C. Clones were screened with a restriction profile and confirmed by DNA sequence analysis. The chimera−pRSETC construct was transformed into BL21(de3)pLysS *E. coli* cells for expression. Single colonies were inoculated into 1 L of an LB/Amp^{100}/$chloramphenicol^{50}$ mixture and incubated at 37 °C overnight without shaking. Cultures were then induced with 2 mM IPTG to an OD_{600} of 0.5 and shaken for 4 h at 30 °C. Cells were pelleted and resuspended in pH 8.0 sonication buffer [50 mM NaH_2PO_4, 10 mM Tris (pH 8.0), 100 mM NaCl, and 1.0% Tween 20] with 20 μg/mL PMSF. Cells were incubated on ice with lysozyme (100 μg/mL) and sonicated in a Branson model 450 sonifier with the micro tip limit set at 7 and a constant duty cycle of 2 × 15 s. The soluble supernatant was removed and the insoluble pellet washed in 1× PBS, and resolubilized in 6 M urea buffer [6 M urea, 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 5 mM imidazole]. Cells were resonicated to reduce the viscosity, and cell debris was pelleted by centrifugation. The supernatant was incubated with Talon metal affinity resin (Clontech, Palo Alto, CA) according to the manufacturer's directions and eluted in 50 mM NaH_2PO_4, 6 M urea, 20 mM PIPES, and 500 mM NaCl (pH 6.2). The eluted protein was dialyzed overnight at room temperature in 2 M urea, 50 mM Tris-HCl (pH 8.0), 1 mM $CaCl_2$, and 0.1% Tween 20. Dialysis was repeated in two changes of identical buffer without urea. Glycerol was then added to the protein to a final concentration of 15% with the addition of BSA to a final concentration of 0.1 μg/μL, aliquoted, and stored at −20 °C. In addition to the N-terminal His tag and the enterokinase cleavage sequence (i.e., MRGSHHHHHHG-MASMTGGQQMGRDLYDDDDKDRWIRPRDLQ), the pRSETC chimera contains the C-terminal extra (KLD-PAANKARKEAELAAATAEQ) sequence. Before all BIAcore and cell biology experiments, the proteins were run through size exclusion columns to remove trace amounts of aggregates or degradation products. A 50 kDa cutoff for aggregates and a 5−10 kDa cutoff for degradation products were used.

Binding Measurements

Interactions between SEB and the chimera were studied by surface plasmon resonance on a BIAcore 2000 instrument (Pharmacia Biosensor AB). SEB at two concentrations was covalently attached to CM-5 chips using an amine coupling kit (Pharmacia Biosensor AB). A negative control for each surface chip was prepared by activating and deactivating the surface of flow cell 1. The response level of flow cell 1 was subtracted from flow cells 2−4 during the run (BIAcore control Software, version 3.0.2). In all experiments, HBS-EP buffer was used as the running as well analyte dilution buffer. Analytes were injected for 1−6 min followed by a 1−5 min dissociation period during which HBS-EP buffer was passed over the flow cells. Kinetic analyses were performed using BIAEvaluation version 3.0.2.

IL-2 Release and T Cell Proliferation Assays

Nanomolar concentrations of SEB (Toxin Technologies, Sarasota, FL) were preincubated with increasing molar

concentrations of TCRVβ, DRα, and the chimera for 1 h at 37 °C. After overnight incubation at 4 °C to allow further binding between the mimics and SEB, human PBMC isolated by density gradient centrifugation from whole blood or a mixture of autologous human PBMC and dendritic cells (Clonetics, San Diego, CA) at a 20:1 ratio was added to the cultures at a concentration of 8×10^5 cells/mL and the mixture incubated for 16 h at 37° C in 5% CO_2 and RPMI 1640 culture media (Gibco BRL, Rockville, MD) containing 10% fetal bovine serum (Hyclone, Logan, UT), or in LGM-3 culture media (Clonetics) containing 10% autologous plasma. Supernatants from the IL-2 assays were harvested 9 or 16 h later and assessed by the ELISA (Pharmingen, San Diego, CA) for IL-2 concentrations. The cells in the proliferation cultures were harvested at 3 days, and the human T cells were stained with FITC-conjugated anti-CD3 antibody (Pharmingen); an isotonic propidium iodide DNA staining solution was used to flow cytometrically measure DNA content to index proliferation. The cells were analyzed by a FACS Caliber flow cytometer (Becton Dickinson, Franklin Lakes, NJ), and the percentages of cells in the G_1, S, and G_2M phases of the cell cycle were calculated from the DNA histograms with Multicycle (Phoenix Flow Systems, San Diego, CA) or ModFit (Verity Software House, Inc.) cell cycle software. Cell viability experiments using dye (trypan blue) infusion and flow cytometry were performed to test whether treatment of various receptor mimics alone for 4−72 h at inhibitory (1−30 nM) doses caused any damage to human blood cells. Also, cytokine release and T cell proliferation assays were conducted to examine whether treatment of various receptor mimics alone for 4−72 h at inhibitory (1−30 nM) doses caused any cytotoxic effect in human blood cells.

RESULTS

Design of a Bispecific Receptor Mimic

Single-crystal structures of the MHC class II−SEB and TCR−SEB complexes reveal specific DRα−SEB and TCRVβ−SEB contacts (*6*, *7*). We have performed molecular modeling to design (i) an 84-amino acid polypeptide fragment that retains the native folding of DRα and (ii) a 74-amino acid polypeptide that retains the native folding of TCRVβ. The linker of the chimeric molecule, DRα−linker−TCRVβ, is 14 amino acids long and contains two copies of the sequence GSTAPPA.

Choice of the Linker. GSTAPPA is a part of the repeat sequence (TSAPDTRPAPGSTAPPAHVTSA) of a tandem human repeat protein, mucin, that is present on epithelial cells. Our previous work (*10*, *11*) on structural and immunogenic properties of human mucins prompted us to choose this peptide as the linker that was appropriate for synergistic binding of DRα and TCRVβ in the chimera. In normal human cells, mucins are O-glycosylated at T/S residues, whereas in breast cancer cells, they are not O-glycosylated. However, only the APDTR sequence of the mutated mucin repeat becomes antigenic in breast cancer patients. We have explained this phenomenon by demonstrating that $(TSAPDTRPAPGSTAPPAHVTSA)_{3,6}$ structures fold in such a way that APDTR is always exposed as a protruding loop and the rest of the repeat sequence, including GSTAPPA, acts as a flexible linker (*10*, *11*). In addition, we have shown that the replacement of APDTR with a conformationally isomorphous HIV-1 V3 loop (GPGRA) in the repeat sequence results in an HIV-1 antigen (rather than a mucin antigen). In the new construct, the grafted HIV-1 GPGRA sequence is still presented as the surface-exposed loop and the rest of repeat sequence acts as the flexible linker (*11*). Therefore, $(GSTAPPA)_2$ was chosen as the linker because it is flexible, adaptive, and potentially nonantigenic. Figure 1 shows a model of the DRα−$(GSTAPPA)_2$−TCRVβ chimera binding to SEB. Note that the two regions of SEB (shown in magenta) that make contacts with DRα and TCRVβ are nonoverlapping and fall on different halves of SEB. We have performed simulated annealing to adjust the structure of the flexible linker such that both DRα and TCRVβ maintain the same pairwise contacts with SEB as observed in the single-crystal structures of the MHC class II−SEB and TCR−SEB complexes (*6*, *7*).

Overexpression and Purification of Receptor Mimics

The rationally designed DRα, TCRVβ, and DRα−$(GSTAPPA)_2$−TCRVβ chimera were cloned in pET 32B and expressed in *E. coli* AD 494 cells as fusion proteins containing thioredoxin, a His_6 tag, and an enterokinase cleavage site on the N-termini of the desired proteins. The His tag was utilized to purify the fusion proteins on charged Ni^{2+} columns, and the chimera was removed from the thioredoxin−His fusion component by enterokinase cleavage. This form is termed the pET 32B. The chimera was also produced in a thioredoxin-free background through expression in the pRSETC vector in a BL21(de3)pLysS *E. coli* strain. The expressed protein, which contains 22 additional C-terminal amino acid residues, was again purified by metal affinity chromatography. This form is termed the pRSETC. The gel purity of DRα, TCRVβ, and the chimera is documented in section A of the Supporting Information. DRα, TCRVβ, and the chimera were refolded in the renaturing and nonreducing buffer to ensure correct folding and the formation of disulfide bridges in TCRVβ, and the chimera (see Materials and Methods). Circular dichroism spectra provided qualitative proof that DRα, TCRVβ, and chimeras were all folded. Each receptor mimic, as expected from the model in Figure 1, exhibited predominantly β strand structure. Spectral deconvolution readily revealed about 15% α helix in DR. In fact, one- and two-dimensional NMR spectroscopy of DRα confirmed the presence of a C-terminal α helix as shown in Figure 1. Since they tended to aggregate at NMR concentrations (i.e., millimolar or higher), similar NMR experiments could not be carried out for TCRVβ and the chimera.

Assessment of SEB Binding

The binding of the pRSETC chimera, DRα−$(GSTAPPA)_2$−TCRVβ, was assessed by surface plasmon resonance on a BIAcore 2000 instrument. In our hands, the pET 32B chimera failed to produce reliable SEB binding in BIAcore experiments probably due to the occlusions of the majority of the SEB binding sites after amine linkage. However, due to the presence of the His tag and enterokinase site on the N-terminus and 22 additional amino acids on the C-terminus, the pRSETC chimera offers a greater number of possible amine linkage sites without interfering with the SEB binding

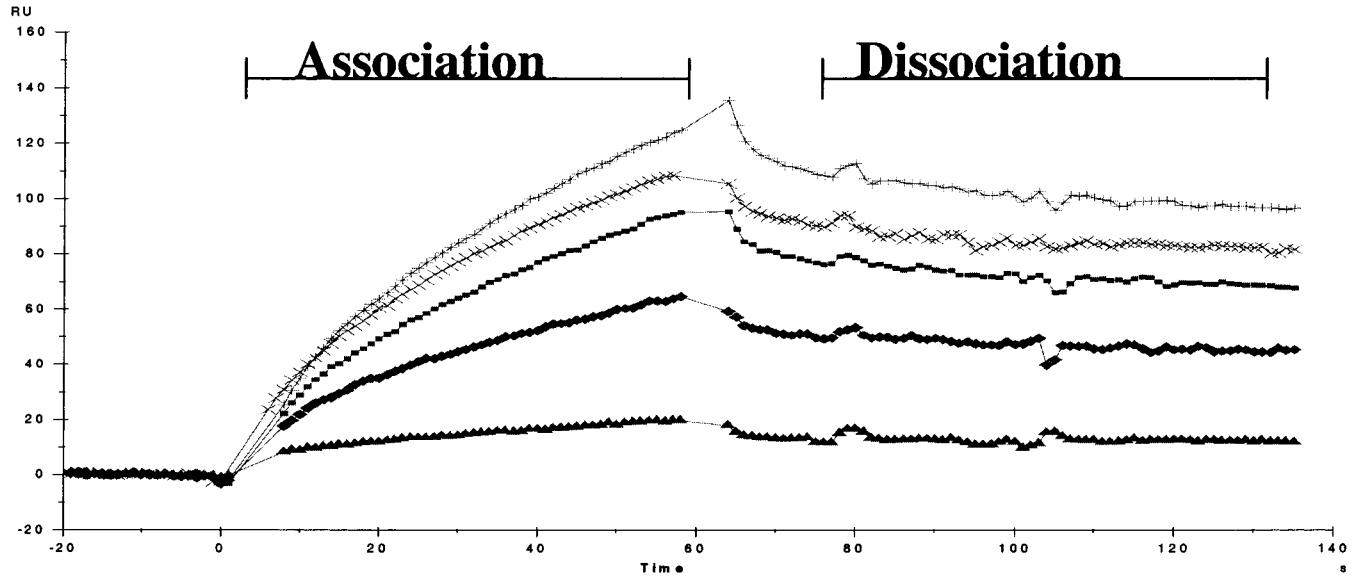

FIGURE 2: Kinetic analysis of the binding of SEB to the DRα−(GSTAPPA)$_2$−TCRVβ chimera (the prSETC chimera) coupled to the surface of a CM-5 chip with an R_{max} of 127 response units. SEB (5−92 μM) was prepared and injected over the chimera surface at a flow rate of 10 μL/min. The resulting sensograms were fitted to a 1:1 Langmuir model using BIAEvaluation software (see section B of the Supporting Information). The specific binding of the chimera to SEB was confirmed by the observation of no binding in a control experiment in which SEB was coupled to CM-5 and was also run in the flow as an analyte.

sites. Indeed, we were able to obtain binding data when the pRSETC chimera was immobilized on the dextran matrix of a sensor chip through amine coupling. The equilibrium dissociation constant, K_D, was measured by injecting varying concentrations of SEB over the immobilized chimera; see section B of the Supporting Information. To eliminate the possibility that the binding of chimera merely reflects nonspecific binding of an immunoglobulin-like fold to SEB, we have studied by BIAcore the binding of the enterokinase-specific but SEB-irrelevant mAb (Invitrogen, catalog no. R910-25). While it binds to our chimera−pRSETC product that bears an N-terminal entrokinase tag (DLYDDDDK), this antibody shows no detectable binding to SEB; see section C of the Supporting Information. As shown in Figure 2, a K_D of 5.8 μM was obtained after correction for nonspecific binding. Similar BIAcore experiments have been reported (*12*, *13*) in which the receptors are used as immobilized ligands and SEB is used as an analyte. It appears that the TCRVβ chain containing both the Vβ and Cβ subdomains binds SEB with a K_D of 140 μM, whereas the human MHC class II receptor fails to bind SEB unless loaded with the antigenic hemagglutinin (HA) peptide, in which case the K_D has been reported to be 14 μM (*12*−*14*). We also show that the individual DRα and TCRVβ subdomains constructed by us lack appreciable binding to SEB; see section D of the Supporting Information. Thus, the linker in the chimeric ligand supports synergy of the two binding events. The dissociation constant, K_D, is not available for SEB, but the K_D for SEA binding to the appropriate MHC class II−TCR complex is ~2 μM which is similar to the K_D (5.8 μM) for SEB binding to our chimera (*14*).

Inhibition of IL-2 Release and T Cell Proliferation

IL-2 release and T cell proliferation assays were performed to test the SEB inhibitory efficacies of the His-tagged (pRSETC) and non-His-tagged (pET 32B) chimeras in human blood. Both chimeras showed nearly identical inhibitory properties. Typically, IL-2 release was monitored after SEB treatment for 4−16 h and T cell proliferation was monitored after SEB treatment for 72 h. Treatment of various receptor mimics alone for 4−72 h at inhibitory (1−30 nM) doses caused neither any cytotoxic effect such as cytokine release and T cell proliferation nor any damage to human blood cells. An irrelevant 10 kDa protein produced in the same expression system was also used as a negative control. IL-2 release was monitored by the enzyme-linked immunosorbent assay (ELISA) using whole blood from healthy human donors and a 20:1 mixture of human peripheral blood mononuclear cells (PBMCs) and dendritic cells. Figure 3A shows the percent inhibition of the SEB-induced IL-2 release by DRα, TCRVβ, DRα and TCRVβ, and the DRα−(GSTAPPA)$_2$−TCRVβ chimera as monitored by the ELISA using whole blood from a healthy donor after treatment with 0.5 nM SEB for 9 h. Note that the individual DRα and TCRVβ subdomains inhibited the IL-2 release by 50% at ≥30 times the SEB concentration. However, the chimera, DRα−(GSTAPPA)$_2$−TCRVβ, inhibited the IL-2 release by 50% at 3−10 times the SEB concentration, thereby indicating that it is a substantially better inhibitor than the individual DRα and TCRVβ fragments. The cells in Figure 3A were also tested for the percent inhibition of the SEB-induced T cell proliferation by DRα, TCRVβ, DRα and TCRVβ, and the DRα−(GSTAPPA)$_2$−TCRVβ chimera. Cell proliferation was monitored by a flow cytometric assay in which the DNA content for the proliferative (S, G_2, and M) phases of the cell cycle was measured after treatment with SEB and ligand for 72 h. As shown in Figure 3B, the DRα−(GSTAPPA)$_2$−TCRVβ chimera is a better inhibitor of T cell growth than DRα, TCRVβ, or both. The DRα−(GSTAPPA)$_2$−TCRVβ chimera inhibited T cell proliferation by 50% at a concentration of 0.5 nM, which was the same as the SEB dose. DRα and TCRVβ at the same concentration showed a 35% inhibition of proliferation, whereas DRα or TCRVβ alone showed an only ~15% inhibition. Figure 3C shows the

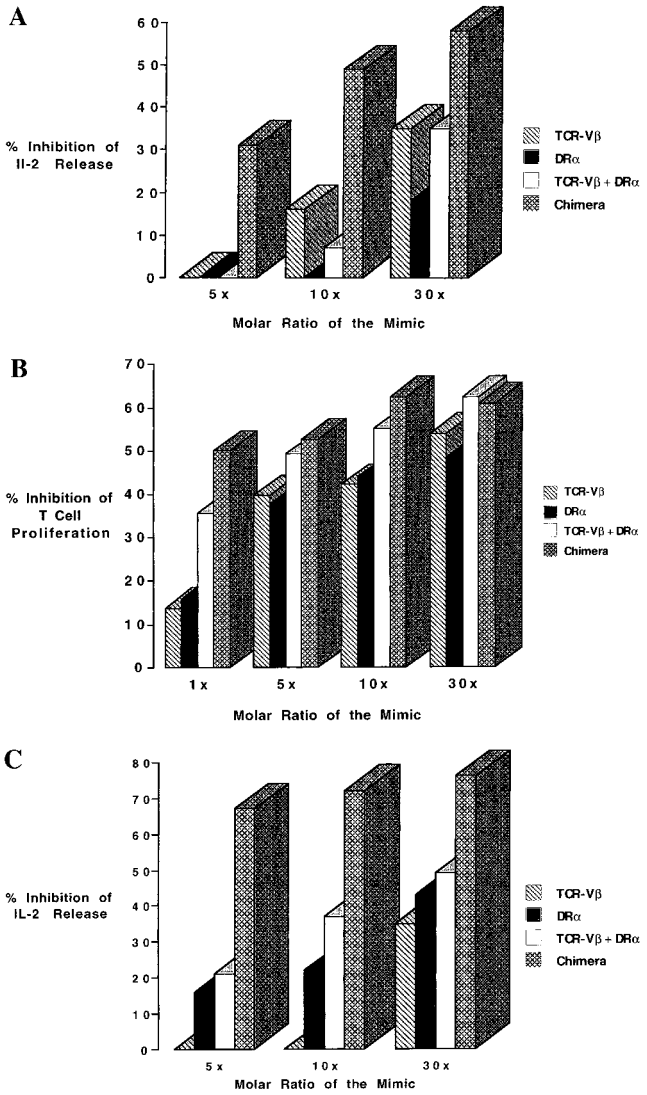

FIGURE 3: (A) PBMCs isolated from whole human blood by density gradient centrifugation were cultured for 9 h after they had been treated with 0.5 nM SEB alone and 0.5 nM SEB with increasing molar concentrations of TCRVβ, DRα, or the pET 32b chimera. IL-2 concentrations in the culture supernatants were measured by the ELISA, and the percent inhibition of IL-2 release by a given mimic was computed relative to the amount (picograms per milliliter) of IL-2 released by the cells treated with SEB alone. These data are representative results of three replicate experiments. (B) PBMCs isolated from whole human blood by density gradient centrifugation were cultured for 3 days after they had been treated with 0.5 nM SEB alone and 0.5 nM SEB with increasing molar concentrations of TCRVβ, DRα, or the chimera. The DNA contents of the cultured cells were measured by flow cytometry, and the percentages of cells in the proliferative stages of the cell cycle, S and G_2M, were determined. The percent inhibition of proliferation caused by a given mimic was computed relative to the extent of T cell proliferation by the cells treated with SEB alone. These data are representative results of three replicate experiments. (C) A mixture of human PBMC and dendritic cells at a 20:1 ratio was cultured for 16 h with 0.35 nM SEB that had been preincubated with increasing molar concentrations of the mimics, TCRVβ, DRα, or the pRSETC chimera. IL-2 concentrations of the culture supernatants were measured by the ELISA, and the percent inhibition of IL-2 release by a given mimic was computed relative to the amount (picograms per milliliter) of IL-2 released by the cells treated with SEB alone. These experiments were performed in triplicate.

percent inhibition of SEB-induced IL-2 release by DRα, TCRVβ, DRα and TCRVβ, and the DR$\alpha-(GSTAPPA)_2-$TCRVβ chimera as monitored by the ELISA using a 20:1 mixture of PBMCs and dendritic cells from a healthy donor after treatment with 0.33 nM SEB for 16 h. The inhibition of Il-2 release by a 20:1 mixture of PBMCs and dendritic cells also revealed that the DR$\alpha-(GSTAPPA)_2-$TCRVβ chimera is a better inhibitor than DRα and TCRVβ together and is far superior to the inhibitory actions of DRα or TCRVβ alone.

DISCUSSION

Binding data in Figure 2 and IL-2 release and T cell proliferation data in Figure 3 demonstrate that the receptor mimic, DR$\alpha-(GSTAPPA)_2-$TCRVβ, inhibits SEB binding to the MHC class II receptor−TCR synapse and blocks SEB-induced IL-2 release and T cell proliferation. Note that nanomolar concentrations of SEB induce cytotoxicity in human cells. Since the dissociation constants for superantigen binding to the MHC class II−TCR complex and to our chimera are very similar (*14*), it is logical that the chimera concentration required for competitive inhibition is also in the nanomolar range. An important aspect of the inhibitory mechanism is the simultaneous and synergistic binding of DRα and TCRVβ to SEB that is facilitated by the appropriate design of the linker in the bispecific chimera. The bispecific receptor mimic offers a special advantage because it specifically blocks the two functional sites on SEB that are absolutely required for its pathogenesis. We have recently obtained a monoclonal antibody that binds to SEB with an affinity of 10 pM (gift from J. Aldrich of the Naval Research Laboratory, Bethasda, MD). However, the mAb only weakly prevents the binding of DRα to SEB and does in no way interfere with the binding of TCRVβ. As a result, this antibody acts as a poor inhibitor of SEB-induced IL-2 release and T cell proliferation (see section E of the Supporting Information).

Since other *Staphylococcus* enterotoxin superantigens, such as SEA, SEC, SED, etc., also target the MHC class II−TCR complex, a similar design of an appropriate bispecific chimera against other superantigens is a simple and straightforward extension of the current work. The use of bispecific ligands can readily be extended to bacterial toxins that cause intracellular toxicity after receptor-mediated entry into host cells. For example, the receptor-mediated binding of the *Bacillus anthracis* protective antigen (PA), an 83 kDa protein toxin, to a whole variety of host cells, including macrophages, and the subsequent cleavage by furin protease define the first step in pathogenesis (*15*). A bispecific chimera that simultaneously targets the furin cleavage and receptor binding sites will be expected to be an excellent inhibitor of PA pathogenesis. A bispecific chimera will also be useful in blocking interactions between receptors and pathogen surface proteins, e.g., the binding of the HIV-1 surface glycoprotein, gp120, to its primary receptor CD4 and coreceptor CCR5/CXCR4 on macrophages or T cells (*16*, *17*). A bispecific chimera constructed by the two gp120-binding domains taken from CD4 and CCR5/CXCR4 should block the interaction of gp120 with its cellular receptor and coreceptor.

In conclusion, bispecific receptor mimics will be a viable non-antibody-based pathogen countermeasure especially against the pathogen proteins that target the immune cells

and subvert their ability to mount antibody-directed immunity.

SUPPORTING INFORMATION AVAILABLE

(A) Gel purity data of TCRVβ, DRα, and the chimera and a description of the circular dichroism experiments that were performed to qualitatively prove that TCRVβ, DRα, and the chimera were correctly folded, (B−D) BIAcore data for various controls, which in essence support our claim that the chimera binds specifically to SEB with a micromolar dissociation constant, and (E) experimental evidence that specific blocking of the functional sites (and not just simple binding) is crucial for the inhibition of SEB pathogenesis. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Eble, J. A., Wecherpfennig, K. W., Gauthier, L., Dersch, P., Krukonis, E., Iseberg, R. R., and Helmer, M. E. (1998) *Biochemistry 37*, 10945−10955.
2. Krakauer, T. (1999) *Immunol. Res. 20*, 163−173.
3. Marrack, P., and Kappler, J. (1990) *Science 248*, 705−711.
4. Bontrop, E. E., Otting, N., Slierendregt, B. L., and Lanchbury, J. S. (1995) *Immunol. Rev. 143*, 33−62.
5. Novotny, J., Tonegawa, S., Saito, H., Kranz, D. M., and Eisen, H. N. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 742.
6. Jardetzky, T. S., Brown, J. H., Gorga, J. C., Stern, L. J., Urban, L. J., Chi, C., Stauffacher, C., Strominger, J. L., and Wiley, D. C. (1994) *Nature 386*, 711−718.
7. Li, H., Llera, A., Tsuchiya, D., Leder, L., Ysern, X., Schlievert, P. M., Karjalainen, K., and Mariuzza, R. A. (1998) *Immunity 9*, 807−816.
8. Blackman, M. A., and Woodland, D. L. (1995) *Life Sci. 57*, 1717−1735.
9. Marrack, P., Blackman, M. A., Kushner, E., and Kappler, J. (1990) *J. Exp. Med. 171*, 455−464.
10. Fontenot, J. D., Mariappan, S. V., Catasti, P., Domenech, N., Finn, O. J., and Gupta, G. (1995) *J. Biomol. Struct. Dyn. 13*, 245−260.
11. Fontenot, J. D., Gatewood, J. M., Mariappan, S. V., Pau, C. P., Parekh, B. S., George, J. R., and Gupta, G. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 315−319.
12. Leder, L., Llera, A., Lavoie, P. M., Lebedeva, M. I., Li, H., Sekaly, R. P., Bohach, G. A., Gahr, P. J., Schlievert, P. M., Karjalainen, K., and Mariuzza, R. A. (1998) *J. Exp. Med. 187*, 823−833.
13. Malchiodi, E. L., Eisenstein, E., Fields, B. A., Ohlendorf, D. H., Schlievert, P. M., Karjalainen, K., and Mariuzza, R. A. (1995) *J. Exp. Med. 182*, 1833−1845.
14. Redpath, S., Alam, S. M., Lin, C. M., O'Rourke, A. M., and Gascoigne, N. R. (1999) *J. Immunol. 163*, 6−10.
15. Duesbery, N. S., and Vande Woude, G. F. (1999) Anthrax toxins, *Cell. Mol. Life Sci. 55*, 1599−1609.
16. Bour, S., Geleziunas, R., and Wainberg, M. A. (1995) *Microbiol. Rev. 59*, 63−93.
17. Berger, E. A., Murphy, P. M., and Farber, J. M. (1999) *Annu. Rev. Immunol. 17*, 657−700.

BI002172E