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# Distinct binding sites on HLA-DR for invariant chain and staphylococcal enterotoxins

**(major histocompatibility complex/superantigens)**

DAVID R. KARP*†, ROBERT N. JENKINS*, AND ERIC O. LONG‡

*Simmons Arthritis Research Center, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75235-8884; and ‡Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, Rockville, MD 20852

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**ABSTRACT** **During biosynthesis, class II molecules of the major histocompatibility complex exist as complexes of the polymorphic $\alpha$ and $\beta$ chains in association with trimers of the invariant chain (Ii). The nonpolymorphic Ii contains sequences necessary for proper targeting of class II to endosomal compartments, where Ii is degraded. Ii also prevents the premature association of antigenic peptides with class II molecules. It is not known whether the effect of Ii on peptide binding extends to other ligands of class II, specifically exogenous superantigens. Cells expressing a mutant Ii molecule stably associated with HLA-DR at the cell surface were tested for their ability to interact with staphylococcal toxins. Most toxins (staphylococcal enterotoxins A–E and exfoliative toxin) were found to bind to cells expressing this $\alpha\beta$Ii complex with levels comparable to cells expressing only $\alpha\beta$ chains at the cell surface. Cells expressing surface $\alpha\beta$Ii complexes stimulated polyclonal populations of peripheral blood T cells in association with these toxins. Binding of toxic shock syndrome toxin (TSST) and subsequent stimulation of T cells were reduced by the presence of the Ii. This reduction was not due to an alteration in the repertoire of T cells responding to TSST in the presence of Ii. Data from experiments with a T-cell clone suggest that interactions between class II molecules and T-cell antigen receptors occur during staphylococcal enterotoxin-mediated stimulation and that surface Ii does not interfere with such interactions.**

Class II molecules encoded by the major histocompatibility complex (MHC) bind antigenic peptides and present them to $CD4^+$ T cells (reviewed in ref. 1). Unlike class I molecules, which have a strict requirement for interaction with peptide for proper folding and efficient transit out of the endoplasmic reticulum (2, 35), class II molecules do not encounter peptides until a much later stage in their biosynthesis. In general, peptides bind to class II in an acidic, endosomal compartment (3). Prior to that time, the class II $\alpha$ and $\beta$ chains are associated with a third, nonpolymorphic protein, the invariant chain (Ii) (4). The $\alpha\beta$Ii complex binds antigenic peptides poorly *in vitro* (5, 6). Presentation of peptide antigen by antigen-presenting cells expressing the surface $\alpha\beta$Ii complex is reduced (7). In addition, Ii is extremely sensitive to the proteases found in endosomes (8, 9). Therefore, Ii may serve to prevent the class II molecule from binding self peptides until it reaches the endosome. Whether this is accomplished by direct competition with the putative peptide binding site or by altering the class II conformation is unknown. Ii also serves as an intracellular chaperone for the $\alpha$ and $\beta$ chains (10, 36). The localization of newly synthesized class II in endosomes is dependent on the presence of Ii (11, 12). This endosomal targeting or retention signal has been localized in the amino-terminal (cytoplasmic) portion of Ii (11, 13).

Evidence from the use of class I/class II hybrids suggests that the interaction between class II and Ii is controlled by the membrane-distal portion of the class II molecule (14). However, no specific portion of class II has been identified as the Ii binding site. Based on limited amino acid sequence homology between Ii and certain staphylococcal enterotoxins, it has been suggested that both the toxins and Ii share the same class II-binding domain (15). These toxins belong to a group of proteins known as microbial superantigens (16). They are capable of moderate- to high-affinity binding to many human and mouse class II molecules and cause polyclonal T-cell activation based on T-cell antigen receptor (TCR) $\beta$-chain variable gene segment ($V_\beta$) usage. The binding sites for several staphylococcal toxins have been identified. The structurally similar toxins staphylococcal enterotoxins A and E (SEA and SEE) bind to a conserved region of the class II $\beta$ chain including the histidine residue at position 81 (17, 18). Toxic shock syndrome toxin (TSST) requires sequences in the $\alpha$1 domain for high-affinity binding to human class II (19) and in both the $\alpha$1 and $\beta$1 domains of mouse class II (20). Finally, the residues necessary for binding of antigenic peptides to class II have been shown to be distinct from those involved in toxin binding (21).

We have recently developed a series of transfected human fibroblast cell lines expressing surface $\alpha\beta$Ii complexes (7). These cells were transfected with Ii cDNA coding for molecules lacking the amino-terminal 15 or 20 residues. All the detectable HLA-DR $\alpha$ and $\beta$ chains expressed on the surface of these cells were present as part of an $\alpha\beta$Ii trimer. The ability of these cells to present antigenic peptide was reduced by a factor of $\approx$50. These transfected cells expressing surface $\alpha\beta$Ii complexes provided the opportunity to directly test whether the binding sites for Ii and for superantigens were similar or distinct. Here we report on the ability of cells expressing class II $\alpha\beta$Ii complexes to bind staphylococcal superantigens and stimulate T cells.

## MATERIALS AND METHODS

**Cells.** The 4N5 cell line is a cloned, HLA-DR1-expressing transfectant of the class II- and Ii-negative human fibroblastic cell line M1 (22). The 4N5-Ii, 4N5-IiΔ15, and 4N5-IiΔ20 cell lines are stable transfectants of 4N5 expressing the full-length (p33) human Ii or an Ii lacking the amino-terminal 15 (Δ15) or 20 (Δ20) amino acids (7). Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 2 mM glutamine, 10 mM Hepes (pH 7.4), and antibiotics. Transfectants were kept in selective medium with either G418 (0.5 mg/ml, dry weight), for 4N5, or G418 plus

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Abbreviations: Ii, invariant chain; MHC, major histocompatibility complex; SEA to SEE, staphylococcal enterotoxins A to E; TCR, T-cell antigen receptor; TSST, toxic shock syndrome toxin.

†To whom reprint requests should be addressed.

mycophenolic acid (10 μg/ml) and xanthine (0.1 mg/ml), for all others.

**Toxin Binding.** The staphylococcal toxins SEA, SEB, $SEC_3$, SED, SEE, TSST, and exfoliative toxin were obtained from Toxin Technology (Sarasota, FL). Binding of the various toxins to cells expressing DR1 or DR1 plus Ii was assayed as described (18). Simultaneous measurement of HLA-DR expression was determined by incubating the cells with the monoclonal antibody L243 followed by fluoresceinated goat anti-mouse IgG. The cells were also stained for the surface expression of wild-type or truncated human Ii. The cells were first stained with the monoclonal antibody POP.I4.3 (IgM) (23), followed by rabbit anti-mouse μ chain, and then fluoresceinated goat anti-rabbit IgG. Microcytofluorimetry was performed on a FACScan (Becton Dickinson).

**T-Cell Assays.** Purified T cells were isolated from peripheral blood of normal human volunteers (18). T cells, mitomycin-treated fibroblasts, and the various staphylococcal toxins were incubated at the indicated concentrations in RPMI 1640 containing 10% fetal bovine serum, glutamine, and antibiotics in round-bottom 96-well plates. After 48 hr, 1 μCi (37 kBq) of [*methyl*-$^3$H]thymidine (6.7 Ci/mmol; ICN Biomedicals) was added to each well. The cells were harvested onto glass fiber filters 16 hr later and the incorporation of label into DNA was determined by liquid scintillation counting. Cytotoxic T-cell assays with clone E1.9 were carried out as described (7).

**Quantitative PCR of TCR $V_\beta$ Usage.** Total RNA was extracted from guanidinium isothiocyanate homogenates of cultured T cells (24). cDNA was synthesized by avian myeloblastosis virus reverse transcriptase using oligo(dT) primers in the presence of actinomycin D (50 μg/ml). $V_\beta$ gene expression was determined by analysis of the cDNA with the 22 family-specific 5′ primers (25) and the reverse primer 3′Cβ4: 5′-ACCCACCAGCTCAGCTCCA-3′. In each experiment a negative control, with no cDNA added, was run in parallel for all primer pairs. The PCR amplifications were carried out in triplicate using a 96-well poly(vinyl chloride) microtiter plate in the PTC-100-96 thermal controller (MJ Research, Watertown, MA). A sample of each PCR amplification mixture was transferred to nylon membrane by alkaline transfer, using a slot blot apparatus. The specific β-chain products were then detected with a $^{32}$P-labeled probe, 5′-ATTCTCCCACACCCAAAAGG-3′, derived from the β-chain constant ($C_\beta$) region. Hybridized probe was quantified with the AMBIS radioanalytic imaging system (AMBIS Systems, San Diego). The specificity of these amplifications was confirmed by electrophoresis of samples of products in agarose gels, transfer to nylon membranes, and probing as described above. All V-region products detectable on slot blots produced a single band of the correct size on the agarose gel blots. The data are expressed as the mean of the triplicate samples of each $V_\beta$ amplification divided by the sum of the 22 $V_\beta$ amplifications.

## RESULTS

**Ii Does Not Block Binding of Staphylococcal Toxins to HLA-DR.** The binding of the staphylococcal toxins SEA, SEB, SED, and TSST was tested on transfected human fibroblasts expressing the DR1 αβ heterodimer alone (4N5), DR1 αβ together with the major p33 form of Ii (4N5-Ii), or surface αβIi complexes (4N5-IiΔ20) (Fig. 1). Ii is synthesized in excess over the α and β chains in both 4N5-Ii and 4N5-IiΔ20 and is present intracellularly at comparable levels in each cell type. In 4N5-IiΔ20, the amino-terminally truncated Ii does not dissociate from the α and β chains of DR and is present at the cell surface as part of a stable αβIi complex (7). The level of DR at the cell surface is increased ≈2-fold on cells transfected with Ii or IiΔ20 (Fig. 1 *A*, *F*, and *K*). The exact mechanism for this difference is unknown but may represent differences in either transit of class II out of the endoplasmic reticulum or stability of the molecule.

The surface expression of transfected Ii is also shown in Fig. 1 *A*, *F*, and *K*. There is a small amount of wild-type Ii at the cell surface. This is detected with POP.I, an IgM monoclonal antibody, with a subsequent two-step signal amplification. Hence, the true level of surface Ii is actually much less than that of the class II αβ dimers and in accordance with other studies demonstrating a low level of cell surface Ii (26). A small fraction of Ii molecules in 4N5-Ii has thus escaped endosomal degradation. In contrast, loss of the endosomal targeting signal of Ii leads to a level of IiΔ20 on the cell surface that is ≈100 times that seen with full-length Ii. This includes both Ii that is complexed to class II and an excess of free Ii (7).

SEA (Fig. 1 *B*, *G*, and *L*), SEB (*C*, *H*, and *M*), SED (*D*, *I*, and *N*), and TSST (*E*, *J*, and *O*) bind to all three cell types. For SEA, SEB, and SED, the level of binding increases

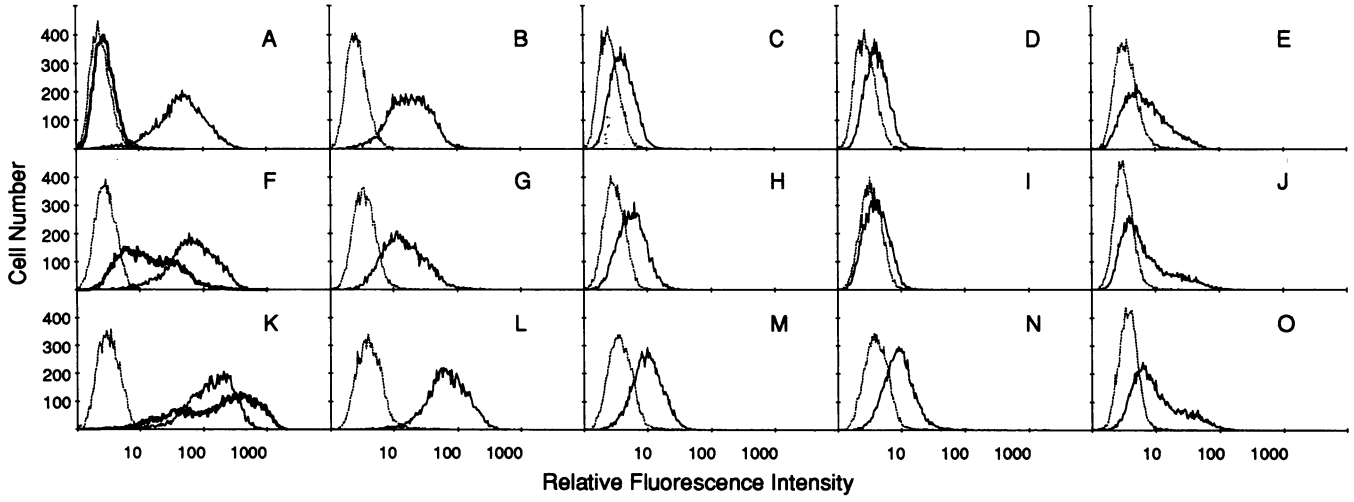


FIG. 1. Microcytofluorimetric analysis of staphylococcal toxin binding. The cell types 4N5 (*A–E*), 4N5-Ii (*F–J*), and 4N5-IiΔ20 (*K–O*) were stained for surface DR expression, surface Ii expression, and binding of SEA, SEB, SED, and TSST and analyzed by flow cytometry. (*A*, *F*, and *K*) Dotted line (left-most) peak, control monoclonal antibody; thin line, L243 (anti-DR); thick line, POP.I4.3 (anti Ii). (*B*, *G*, and *L*) SEA binding vs. control. (*C*, *H*, and *M*) SEB binding vs. control. (*D*, *I*, and *N*) SED binding vs. control. (*E*, *J*, and *O*) TSST binding vs. control. In each case, the control consisted of anti-toxin and fluorescent-reagent staining of the DR-positive cell in the absence of toxin. These control histograms were indistinguishable from an analysis of toxin binding to the untransfected (DR-negative) M1 cells.

roughly in proportion to the level of DR expressed on the cell surface. The presence of Ii complexed to the $\alpha\beta$ dimer of DR does not have a significant effect on the binding of those toxins. In addition, the toxins $SEC_3$, SEE, and exfoliative toxin were tested with similar results (data not shown). Similar results were seen with 4N5-IiΔ15 (data not shown). The binding of TSST to 4N5-IiΔ20 is less than expected for the increase in DR, although it is still easily detectable. The ratio of the mean channel fluorescence for toxin binding to that for L243 binding is given in Table 1. This ratio is fairly constant for SEA, SEB, and SED on the DR-expressing cells, regardless of the presence of Ii. However, the value obtained for TSST binding to 4N5-IiΔ20 is approximately one-fourth that obtained for either 4N5 or 4N5-Ii.

**Cells Expressing $\alpha\beta$IiΔ20 Complexes Stimulate T Cells in Association with Staphylococcal Toxins.** Highly purified peripheral blood T cells were cultured with mitomycin C-treated transfected human fibroblasts and stimulated with increasing concentrations of SEA, SEB, or TSST. All three transfectants had similar ability to stimulate T cells in the presence of maximal amounts of SEA (Fig. 2*A*). This suggests either that the TCR can recognize the $\alpha\beta$IiΔ20 complex efficiently or that SEA-mediated stimulation does not involve MHC/TCR contacts. However, the maximal stimulation by SEB (Fig. 2*B*) and TSST (Fig. 2*C*) bound to 4N5-IiΔ20 was lower than that seen with either 4N5 or 4N5-Ii. For TSST, the decrease is ≈50%. This can be explained by the lower affinity of $\alpha\beta$IiΔ20 for TSST, although a conformational difference between $\alpha\beta$IiΔ20 and the $\alpha\beta$ dimer that is unable to efficiently activate the TCR in association with TSST cannot be ruled out. There is also a shift in the dose–response curve for 4N5-Ii with TSST, although the maximal level of stimulation is the same. This may represent competition between the small amount of cell surface Ii and the toxin. It is possible that the 4N5-IiΔ20 cells were deficient in some costimulatory signal that was necessary only for activation by TSST. Purified T cells were incubated in the presence of various amounts of 4N5-IiΔ20 and 10 μg of Con A per ml. There was no difference in the level of stimulation among the three cell types (data not shown), and no stimulation was seen in the absence of the class II-positive fibroblasts.

**The Presence of Surface Ii Does Not Alter $V_\beta$ Selection by TSST.** It was possible that a qualitative as well as quantitative difference existed in the repertoire of T cells that were stimulated by 4N5, 4N5-Ii, and 4N5-IiΔ20. It is known that TSST stimulates predominantly $V_\beta 2$-bearing human T cells (25). Quantitative PCR was used to test whether the higher level of stimulation with TSST using 4N5 and 4N5-Ii was due to utilization of other $V_\beta$ genes. Purified peripheral blood T cells were cocultured for 6 days with each of the antigen-presenting cell types plus TSST, or with anti-CD3 to assess total $V_\beta$ usage. The percentage of each $V_\beta$ used is shown in Table 2. The anti-CD3 antibody stimulates the T cells in a polyclonal fashion, indicating the potential repertoire of this sample. Approximately 60–70% of the T cells responding to TSST in conjunction with HLA-DR are $V_\beta 2$, regardless of the presence of surface Ii. These results suggest that the interaction of Ii with the class II $\alpha$ and $\beta$ chains does not mask or significantly alter those regions of class II that are required for recognition by the TCR in association with TSST.

Table 1. Ratio of staphylococcal toxin binding to HLA-DR expression on transfected human fibroblasts

| | Toxin binding/anti-DR fluorescence | | | |
|---|---|---|---|---|
| Transfectant | SEA | SEB | SED | TSST |
| 4N.5 | 0.49 | 0.09 | 0.10 | 0.26 |
| 4N.5-Ii | 0.33 | 0.10 | 0.11 | 0.21 |
| 4N.5-IiΔ20 | 0.51 | 0.08 | 0.07 | 0.05 |

Transfectants expressing DR1 in the absence or presence of Ii were analyzed by microcytofluorimetry as in Fig. 1. Value given is the ratio of the mean channel fluorescence of the toxin binding histogram to the mean channel fluorescence of the anti-DR histogram. Data are representative of four experiments.

**Class II Conformation Can Influence SEA-Mediated T-Cell Stimulation.** It is not known whether the surface $\alpha\beta$Ii complex can interact directly with the TCR. If it does not, the T-cell proliferation mediated by SEA bound to $\alpha\beta$Ii complexes would mean that MHC/TCR interactions are not important in the response to this superantigen. This interpretation is unlikely based on the results obtained with the DR1-restricted T-cell clone E1.9. E1.9 is specific for the epitope comprising amino acids 307–318 of influenza hemagglutinin H3. It also responds to SEA presented by DR1 (18). However, E1.9 is unable to respond to SEA bound to 4N5 cells even though it responded to SEA bound to either 4N5-Ii or 4N5-IiΔ20 cells (Fig. 3). The 4N5 cells are not defective in T-cell stimulation, as they efficiently present synthetic peptide to E1.9 (Fig. 3 and ref. 7). The 4N5 cells do present SEA to another H3-specific T-cell clone (data not shown) and to polyclonal T-cell population (Fig. 2). This strongly suggests that direct MHC/TCR contacts are involved in superantigen-mediated T-cell stimulation and that the proper class II conformation required for SEA recognition by clone E1.9 is present in $\alpha\beta$Ii complexes and in $\alpha\beta$ dimers that have been associated with Ii during biosynthesis. This conformation is not present in $\alpha\beta$ dimers that are synthesized in the absence of Ii.

## DISCUSSION

The data demonstrate that the seven staphylococcal toxins tested interact with class II molecules at a site(s) distinct from the binding site for Ii. A transfected cell line expressing a stable $\alpha\beta$Ii complex on the cell surface was capable of binding six of the toxins with levels comparable to cells expressing free surface $\alpha\beta$ heterodimers. The low degree of homology observed between certain staphylococcal toxins and Ii (15) therefore does not reflect coevolution for a common binding site in class II.

The reduced binding of TSST in the presence of surface Ii provides additional evidence for the distinction among different toxins in the way they interact with class II. SEA and SEE bind to a conserved region in the $\alpha$-helical portion of the DR $\beta$ chain (18, 27), while TSST interacts with sequences in the $\alpha 1$ domain of DR and in the $\alpha$-helices of both $\alpha$ and $\beta$ chains in mouse Ia (19, 20). However, there may be a degree of overlap among these sites as demonstrated by partial cross-inhibition of binding (28). In this study, the binding of SEA and SEE was unaffected by the presence of surface Ii. TSST binding was diminished but not abrogated on 4N5-IiΔ20. This suggests that Ii is not physically associated with a large region of the $\alpha$-helices of either the $\alpha 1$ or the $\beta 1$ domain. Ii would either have to bind at the "end" of the $\alpha$-helices, away from the toxin binding sites, or extend directly into the peptide binding site.

In addition to their binding to $\alpha\beta$Ii complexes, the staphylococcal toxins retained their ability to stimulate T cells in the presence of IiΔ20. This suggests that Ii binds to $\alpha\beta$ molecules in such a way that TCR recognition of MHC molecules can still occur. An alternative interpretation, however, is that MHC/TCR contacts are not necessary during T-cell stimulation by superantigen and that toxin interaction with $V_\beta$ was insensitive to the Ii chain bound to $\alpha\beta$. A role for MHC structure in superantigen-mediated T-cell responses was suggested by a study of multiple class II isotypes and alleles (27). On the other hand, certain $CD8^+$ T-cell clones can be stimulated by class II-negative cells by high concentrations of staphylococcal toxins, suggesting that MHC/TCR interactions may not be essential (29). Strong evidence in favor of

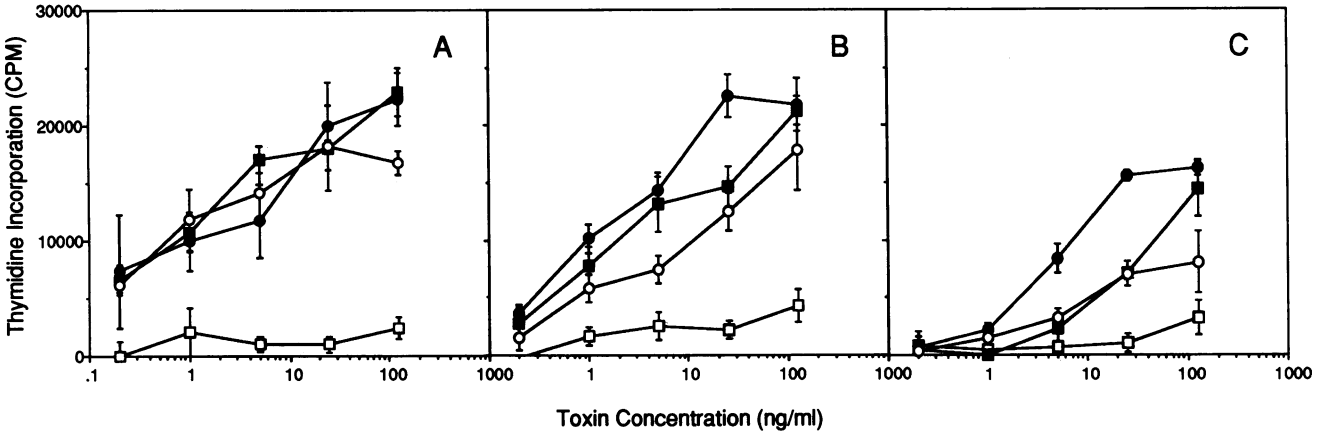


FIG. 2. Stimulation of T cells by staphylococcal toxins and transfected human fibroblasts. Mitomycin C-treated M1 (□), 4N5 (●), 4N5-Ii (■), and 4N5-IiΔ20 (○) cells were cultured with highly purified human T cells and various concentrations of SEA (*A*), SEB (*B*), or TSST (*C*). At 56 hr, [$^3$H]thymidine was added and the cells were harvested 16 hr later. Mean ± SE of triplicate determinations for each point is plotted. Data are representative of three separate experiments with different T-cell donors.

MHC/TCR interactions during SEA-mediated stimulation was obtained by using the DR1-restricted, antigen-specific T-cell clone E1.9. This clone failed to respond to SEA when bound to $\alpha\beta$ chains expressed in a cell line lacking Ii, even though it recognized a specific peptide presented by the same cells. E1.9 is efficiently stimulated by SEA in association with class II on a human B-cell line (18) or with a transfected line expressing $\alpha\beta$ chains in the presence of Ii (Fig. 3). The different conformations of $\alpha\beta$ chains expressed in the presence or the absence of Ii had previously been suggested by antibody reactivity, ability to load peptides, and heterodimer stability (30, 31). The failure of clone E1.9 to recognize SEA bound to DR on 4N5 suggests that class II molecules that are synthesized and mature in the absence of Ii differ substantially from class II molecules that exchange Ii for peptides in endosomes. Furthermore, class II conformation obviously affects E1.9 responses to superantigen. The ability of E1.9 to recognize SEA bound to 4N5-IiΔ20 cells implies that Ii itself does not interfere with the MHC/TCR interactions and that Ii provides the proper $\alpha\beta$ conformation for recognition.

Finally, the ability of superantigen to bind $\alpha\beta$Ii complexes has implications for the cellular biology of endogenous superantigens. Products of endogenous retroviruses in mice have been identified as superantigens, causing neonatal deletion of T cells expressing particular $V_\beta$ sequences (32–34). Because these endogenous superantigens also stimulate T cells via $V_\beta$, it is likely that they bind to class II in a manner similar to exogenous superantigens. Thus, it is quite possible that endogenous superantigens bind to class II molecules directly in the exocytic pathway, prior to the dissociation of

Table 2. TCR $V_\beta$ usage by peripheral blood T cells responding to TSST and transfected human fibroblasts

| | % $V_\beta$ usage | | | |
|---|---|---|---|---|
| $V_\beta$ family | Anti-CD3* | 4N5† | 4N5-Ii† | 4N5-IiΔ20† |
| 1 | 7.31 | 1.43 | 2.10 | 0.84 |
| 2 | 3.17 | 57.80 | 66.94 | 72.05 |
| 3 | 1.61 | 0.75 | 0.78 | 0.52 |
| 4 | 2.45 | 2.52 | 3.06 | 1.53 |
| 5.1 | 1.99 | 1.11 | 1.15 | 0.87 |
| 5.2-3 | 3.03 | 1.36 | 0.14 | 0.44 |
| 6.1-3 | 11.82 | 4.55 | 2.59 | 2.34 |
| 7 | 17.32 | 5.27 | 3.76 | 2.94 |
| 8 | 6.31 | 2.09 | 2.85 | 1.04 |
| 9 | 3.41 | 2.08 | 1.10 | 0.70 |
| 10 | 3.15 | 1.37 | 2.76 | 2.04 |
| 11 | 0.89 | 0.44 | 0.54 | 0.23 |
| 12 | 0.11 | 0.64 | 1.18 | 1.03 |
| 13.1 | 9.80 | 4.12 | 2.93 | 3.11 |
| 13.2-3 | 8.16 | 3.14 | 2.04 | 1.94 |
| 14 | 4.63 | 1.44 | 1.30 | 1.07 |
| 15 | 2.74 | 1.01 | 0.38 | 1.03 |
| 16 | 1.82 | 0.98 | 0.67 | 0.63 |
| 17 | 1.60 | 2.14 | 2.23 | 2.00 |
| 18 | 2.26 | 1.41 | 0.20 | 0.79 |
| 19 | 3.92 | 1.94 | 0.85 | 1.81 |
| 20 | 2.51 | 2.41 | 0.46 | 1.07 |

Values (means of triplicates) indicate the percent of total $V_\beta$ usage in a responding T-cell population following a 6-day culture of $6 \times 10^6$ purified T cells and either anti-CD3 or $2 \times 10^6$ transfected fibroblasts plus toxin.
*Immobilized monoclonal antibody 64.1 (10 μg/ml).
†Cultured with TSST (125 ng/ml).

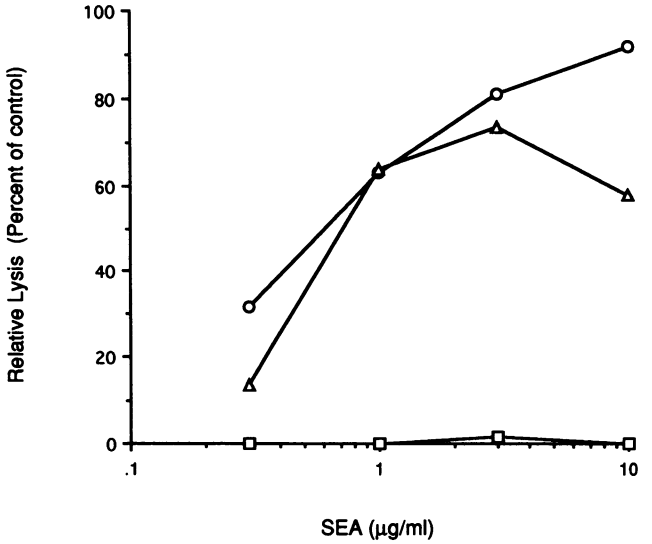


FIG. 3. T-cell clone E1.9 fails to respond to SEA bound to 4N5. Rested E1.9 cells (DR1-restricted, human T-cell clone specific for residues 307–318 of influenza hemagglutinin) were cocultured with 4N5 (□), 4N5-Ii (○), or 4N5-IiΔ20 (△) cells that had been $^{51}$Cr-labeled and pulsed with either SEA or hemagglutinin-(307–318) peptide. Cytotoxic T-cell activity was measured by $^{51}$Cr release 8 hr later. The specific lysis obtained with SEA-pulsed target cells is expressed as a percentage of the specific lysis obtained with saturating hemagglutinin-(307–318) peptide (relative lysis). At saturating peptide concentrations, all three target cells are recognized by the clone E1.9 (7). Data represent the mean of triplicate determinations.

Ii from $\alpha\beta$ in endosomes. Because of the importance of potential pathogen-encoded superantigens in understanding disease, many human pathogens are currently being screened for superantigen activity. The search for such superantigens must obviously include endogenous proteins.

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