Use of Bispecific Heteroconjugated Antibodies (Anti-T Cell Antigen Receptor × Anti-MHC Class II) To Study Activation of T Cells with a Full Length or Truncated Antigen Receptor ζ-Chain

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ABSTRACT. Ligand-induced activation of T cells involves recognition of monovalent peptide Ag complexed with a cell surface MHC-encoded molecule. In contrast, antibody-induced activation of T cells typically requires external cross-linking of the TCR. To examine the mechanisms that underlie the ability of these different stimuli to signal, we have created bispecific chimeric antibody molecules (BA) that mimic Ag in several important aspects. Anti-TCR-α, -β, or anti-CD3-ε Fab fragments were covalently coupled to an anti-MHC class II Fab fragment. These BA elicited IL-2 production or proliferation from Ag-specific T cell hybridoma cells or splenic T cells, respectively, in the presence, but not the absence, of accessory cells expressing the appropriate MHC class II molecule. This response was prevented by soluble blocking antibodies against the TCR or MHC class II. When “presented” by MHC class II-bearing accessory cells, anti-TCR × anti-MHC class II BA, like cell surface Ag, elicited IL-2 production from T cell transfectants expressing full length TCR ζ-chain but not from otherwise identical cells expressing truncated ζ. When immobilized on a plastic surface these BA were potent stimulators that induced equal amounts of IL-2 from the same cells. Purified Ag/MHC complexes immobilized on plastic were able to induce IL-2 production from T cells expressing the full length, but not the truncated, form of ζ. We hypothesize that TCR-mediated T cell activation requires stable aggregation of the TCR. In this model, activation by mobile cell surface Ag/MHC or BA occurs in two steps, occupancy-induced TCR clustering followed by stable aggregation facilitated by the presence of a full length ζ-chain. Immobilized high affinity anti-TCR antibodies, but not low affinity Ag/MHC complexes, directly promote stable receptor aggregates, and thus would not require a full length ζ-chain. Journal of Immunology, 1993, 150: 2211.

Antireceptor antibodies have been widely used to simulate physiologic ligand binding. In particular, anti-TCR antibodies have been used to stimulate T cells in studies of the events that follow activation (1). The underlying assumption is that these antibodies elicit responses similar, if not identical, to responses initiated by the natural ligand, in this case the combination of Ag and an MHC-encoded glycoprotein. Both Ag/MHC complexes and anti-TCR antibodies induce phosphatidylinositol hydrolysis, increases in intracellular Ca2+, protein kinase C activation, tyrosine kinase activation,
and lymphokine secretion (2, 3). The parameters that influence the ability of anti-TCR antibodies to stimulate T cells have been extensively studied (4-11). Although some studies have demonstrated T cell activation in the absence of Ab \(^3\) cross-linking (12), soluble anti-TCR Ab have generally been found to be ineffective at inducing full T cell activation. External cross-linking (via FeR on accessory cells or secondary Ab, for example), or physical immobilization of the stimulatory Ab, is necessary and sufficient to activate T cell hybridomas (8). The requirements are less clear for resting normal T cells; some laboratories have reported that immobilized Ab alone are capable of stimulating resting T cells to proliferate, although others have found that an accessory cell-derived costimulatory signal is required (13-16). In any case, in the great majority of cases Ab multivalency seems to be necessary for initiating TCR-dependent activation. Similar results have been found in the unusual case of hapten-specific T cells; making the fluorescein molecule multivalent by coupling it to a large polymeric backbone such as polyacrylamide or dextran allows the soluble compound to activate fluorescein-specific T cell clones in the absence of MHC-encoded molecules (17, 18).

Based on the need for multivalent binding, TCR clustering or aggregation is generally thought to be a required intermediate stage for coupling the TCR to intracellular signaling pathways. However, it has also been proposed that conformational changes in the TCR are responsible for TCR-mediated cellular activation (19, 20). One key difference between Ab and physiologic ligand is that activation by the latter presumably involves TCR recognition of a monovalent Ag/MHC complex. Although Ag/MHC complexes are presented as an array on the surface of the APC, their monovalent nature precludes true external cross-linking of TCR; local aggregation of ligand and occupied TCR, however, does occur (21, 22). An unanswered question is, do anti receptor Ab stimulate T cells in a manner analogous to Ag/MHC, or do they initiate signaling in a fundamentally different way?

One tool to probe the mechanisms underlying TCR signaling has been provided by T cell hybridomas bearing partial or mutated TCR. Variants of 2B4.11, a pigeon cytochrome \(c\)-specific T cell hybridoma, that lack TCR-\(\zeta\) and \(-\eta\) chains have been characterized (23). One such cell, MA 5.8, was found to produce high levels of IL-2 in response to plate-bound anti-CD3 or anti-TCR-\(\alpha\beta\) Ab but not to Ag/MHC, although this result could not be unambiguously interpreted because these mutant cells expressed only 5% as much cell surface TCR as the wild type. However, when cell surface TCR expression was restored to normal by transfection of another \(\zeta\)-2B4.11 derivative with either a full length \(\zeta\) cDNA or a cDNA that encoded a truncated form of \(\zeta\) (lacking the C-terminal and intracellular 57 amino acid residues) (24), a similar dichotomy was observed: whereas both transfectants, FL (full length \(\zeta\)) and CT108 (truncated \(\zeta\)), responded well to immobilized anti-TCR-\(\alpha\beta\) or anti-CD3 Ab, only the full length \(\zeta\) transfectant produced IL-2 in response to pigeon cytochrome \(c\).

To address how Ab and Ag-stimulation differ, we have attempted to create Ab molecules that partially mimic Ag. To this end, Fab fragments of various anti-TCR chain Ab were ligated to the Fab fragment of an anti-MHC class II Ab. These bispecific heteroconjugated Ab constructs resemble Ag in that they interact in a monovalent fashion with the TCR and the MHC class II molecule and require the presence of APC bearing the proper MHC class II. We have used these BA and an Ag/MHC unresponsive T cell hybridoma expressing a truncated \(\zeta\)-chain to explore the mechanism by which TCR occupancy or perturbation initiates T cell activation.

**Materials and Methods**

**Animals**

B10.A mice were the kind gift of Dr. Ronald Schwartz (National Institute of Allergy and Infectious Diseases, NIH).

**Production and purification of Ab**

The Ab used to make BA are listed in Table I. A2B4, H57, and 2C11 were prepared from tissue culture supernatant and purified over a protein A-Sepharose affinity column. Purified Ab were dialyzed into borate buffer (0.17 M boric acid, 0.12 M NaCl, 0.2% sodium azide, pH 8.5) and concentrated by immersible-CX filters (Millipore Corp., Bedford, MA).

**Culture medium**

Cells were maintained and assayed in RPMI 1640 (Bio-fluids Inc., Rockville, MD) supplemented with 10% heat inactivated FCS, 4 mM glutamine, 100 U/ml penicillin, 150 \(\mu\)g/ml gentamicin, and 5 \(\times\) 10\(^{-5}\) M 2-ME (complete medium).

**Cells**

2B4.11, a cytochrome \(c\)-specific T cell hybridoma, was produced by polyethylene glycol-mediated fusion of lymph node T cells from Ag-primed B10.A mice to an AKR-derived thymoma, BW5147 (26-28). Although originally CD4\(^+\), the 2B4.11 cells and its variants are all now CD4\(^-\), as assessed by flow cytometry. 2 M.2, a \(\eta\)-deficient

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1 Abbreviations used in this paper: Ab, antibody; BA, b-specific Ab; SPDP, N-succinimidyl 3-(2-pyridyldithiol) propionate; Ag/MHC, Ag bound by MHC-encoded molecules; FceRI, Fc receptor for Ig E.
2B4.11 variant, was transfected with full length or cytoplasmic truncated TCR z-chain cDNA constructs to yield FL and CT108, respectively (24, 29). CT108 lacks the intracytoplasmic residues 108 to 164. L cells transfected with MHC class II genes of various allelic origins are listed in Table I, and were the kind gift of Dr. Ronald Germain (National Institute of Allergy and Infectious Diseases, NIH).

Bispecific Ab (BA) preparation

Fab fragments were prepared by digesting the Ab at 37°C with a 1:20 or 1:100 (w/w) ratio of papain/Ab in a buffer of 0.1 M sodium phosphate, 0.1 M NaCl, 1 mM EDTA, 20 mM cysteine, pH 8.0 (30). The reaction was terminated by adding iodoacetamide to a final concentration of 30 mM. The digested product was dialyzed against borate buffer, passed over protein A-Sepharose, and then applied to an Ultrogel AcA 34 column to remove Fc fragments and trace contaminants of intact IgG. Analysis by SDS-PAGE indicated that all of the material migrated at 50 kDa. Fab fragments were cross-linked using the heterobifunctional reagent, SPSP, as described (31, 32). Fab fragments (one anti-TCR, one 10–3.6.2) were each dialyzed against 0.1 M sodium acetate, 0.1 M NaCl, pH 4.5; dithiothreitol was added to a final concentration of 0.02 M. The reduced 10–3.6.2 Fab fragment was dialyzed against 0.1 M sodium phosphate, 0.1 M NaCl, pH 7.5 (coupling buffer), and incubated separately for 0.5 h at room temperature with twofold molar excess of SPDP in ethanol. The 10–3.6.2 Fab fragment was dialyzed against 0.1 M sodium acetate, 0.1 M NaCl, pH 4.5; dithiothreitol was added to a final concentration of 0.02 M. The reduced 10–3.6.2 Fab fragment was immediately passed through a Pharmacia PD10 column equilibrated with coupling buffer, and then added to the nonreduced anti-TCR Fab fragment. Reduction of the SPDP-anti-MHC class II Fab fragment before adding the SPDP-anti TCR Fab or SPDP-anti CD3 Fab fragment minimizes the production of anti-TCR × anti-TCR, anti-CD3-ε × anti-CD3-ε, or anti-MHC class II × anti-MHC class II Fab homodimers. After an 18-h incubation at 37°C, 1 mg of iodoacetamide was added and the protein eluted from an Ultrogel ACA 34 column (IBF Biotechnics, Savage, MD). Appropriate fractions were pooled and rechromatographed on a Pharmacia FPLC Superose 6 column, and the dimeric bispecific anti-TCR × anti-MHC class II Fab fragment was taken for the experiments. In some experiments, FPLC separation was performed twice to ensure purity of the Fab × Fab heteroconjugates. There were no differences in the functional characteristics of the heteroconjugate fractions that were purified once or twice by FPLC, indicating that one passage was sufficient to yield a pure preparation.

Preparation of MHC class II/pigeon fragment 81–104 complexes

Pigeon cytochrome c fragment 81–104 (pigeon fragment 81–104) was prepared as described (33). Eκ-3.6.2 was purified from detergent solubilized LPS-stimulated CBA/J B cell blasts using the 14–4–4 mAb for affinity chromatography as described (34). Purified Em-κ:Eβκ was stored in 30 mM octylglucoside in Tris (100 mM), NaCl (140 mM), sodium azide (0.02%), pH 8.3 at 4°C. Eκ-κ:Eβκ molecules were "loaded" with peptide as described (34). Briefly, purified Eκ-κ:Eβκ molecules (at a concentration of 1 mM) were adjusted to a pH of 4.5 in the presence of 100 μM pigeon fragment 81–104, and after a 60-min incubation at 37°C the reaction mixture was neutralized.

Stimulation of T cell hybridomas

For most experiments, T hybridoma cells were cultured in duplicate or triplicate in 96-well, flat-bottomed microtiter plates (no. 3596, Costar, Cambridge, MA) in a volume of 200 μl of complete medium at a density of 5 × 10⁴ cells/well. In some assays, the accessory cells RT, DCEK, or FT were added at a concentration of 5 × 10⁵ cells/well. In other experiments, the T cells were stimulated with Ab that was adherent to plastic microtiter wells (Costar no. 3596). This was accomplished by incubating the plates with the indicated purified intact Ab or BA at varying concentrations in 50 μl of PBS. After 2 h at room temperature, the wells were washed three times with complete medium and used. In some experiments, T cells were stimulated with purified Eκ-κ:Eβκ/pigeon fragment 81–104 complex. In these experiments, varying concentrations of preformed Eκ-κ:Eβκ/peptide complexes were directly placed in culture wells (Immunolon 4, Dynatech Laboratories, Inc., Chantilly, VA) to allow for binding to the plastic surface. The detergent concentration was diluted by adding 200 μl PBS, pH 7.2, followed by several washes with complete medium, and 5 × 10⁴ T hybridoma cells were added. Regardless of the means of stimulation, after an overnight culture aliquots of supernatant were removed and IL-2 content determined.

IL-2 assay

After approximately 20 h of coculture with the indicated stimuli, aliquots of supernatant were removed and frozen to ensure that no viable cells were included. IL-2 content was determined by incubating twofold serial dilutions of supernatant with an IL-2-dependent T cell, CTLL (4 × 10⁵ cells/well). After 16 to 18 h, 1 μCi of [³H]Tdr was added to each well for the final 6 to 8 h of culture. The incorporation of [³H]Tdr was determined by liquid scintillation counting, and the results are expressed as units of IL-2 activity. One U is defined as the dilution of supernatant causing CTLL cells to incorporate half-maximal amounts of [³H]thymidine (maximal levels were established in each
Table I
Antibodies and cells used

<table>
<thead>
<tr>
<th>Antibodies (Abbreviation)</th>
<th>Specificity</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>H57-597 (H57)</td>
<td>TCR-β chain</td>
<td>51</td>
</tr>
<tr>
<td>A2B4-2 (A2B4)</td>
<td>TCR-α chain</td>
<td>27</td>
</tr>
<tr>
<td>145-2C11 (2C11)</td>
<td>CD3-ε</td>
<td>52</td>
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<tr>
<td>10-3.6.2</td>
<td>MCC class II (I-Aκ)</td>
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</tr>
<tr>
<td>Transfected accessory cells</td>
<td>MHC Class II Expression</td>
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</tr>
<tr>
<td>RT7.3H3B (RT)</td>
<td>I-Aκ</td>
<td>54</td>
</tr>
<tr>
<td>FT7.1C6 (FT)</td>
<td>I-Aβ</td>
<td>55</td>
</tr>
<tr>
<td>DCEK.HC (DCEK)</td>
<td>I-ε</td>
<td>R. Germain and J. Miller, unpublished</td>
</tr>
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experiment using MLA 144 supernatant as a source of IL-2).

T cell proliferation assay

Freshly prepared spleen cells were cultured in triplicate in 96 well, flat-bottomed microtiter plates in a volume of 200 μl of complete medium at a density of 10^5 cells/well with indicated concentrations of BAs. After 48 hr the cells were pulsed with 1 μCi of [3H]TdR, incubated overnight, and harvested. The incorporation of [3H]TdR was determined by liquid scintillation counting, and the results expressed as the averaged cpm.

Results

BA stimulation of T hybridoma cells

BA were prepared from Fab fragments of the Ab listed in Table I, and experiments were performed to determine if these bispecific heterochimeric Ab molecules could stimulate T cells. The Ag-specific 2B4.11 T cell hybridoma was incubated with varying concentrations of three BA, each specific for the I-Aκ-encoded MHC class II molecule and a different TCR chain. In the presence of an I-Aκ-bearing accessory cell, all BA preparations, regardless of their TCR chain specificity, induced 2B4.11 cells to produce IL-2 in a dose-dependent fashion (Fig. 1). Several approaches were used to determine if BA corecognition of the TCR and MHC class II was important in this process (Fig. 2). When titered into microtiter wells containing AαβAβk2 (I-Aκ)-bearing L cells, the A2B4 × 10-3.6.2 BA stimulated IL-2 production. In contrast, in the absence of I-Aκ-bearing accessory cells, or in the presence of an L cell bearing the inappropriate MHC class II molecule (I-Aρ), the BA did not induce IL-2 from the T cell hybridoma. Inasmuch as A2B4 does not itself activate 2B4.11 T cells under these culture conditions (27), it was possible to test whether the intact Ab could prevent T cell activation by the BA by blocking the binding of the BA to the TCR. The addition of soluble A2B4 mAb, or soluble 10-3.6.2 mAb, which block BA corecognition of the TCR and MHC class II, respectively, prevented IL-2 production. Addition of equal amounts of an irrelevant anti-MHC class II Ab, Y-3P (anti-I-Aρ), had no effect. Similar results were also obtained with the other BA and 2B4.11 cells, and mere aggregation of 2B4.11 and class II-bearing L cells with anti-H-2Kk Ab did not cause IL-2 production (data not shown). These results attest to the "MHC-restricted" nature of the stimulus and the requirement for binding both the TCR and MHC class II.

BA activate normal T cells

To determine if normal T cells can respond to BA, B10.A spleen cells, whose B cells and macrophages bear the I-Aκ-encoded class II molecule, were incubated with varying concentrations of H57 × 10-3.6.2 (anti-TCR-β × anti-I-Aκ) and 2C11 × 10-3.6.2 (anti-CD3-ε × anti-I-Aκ). Both BA preparations induced excellent T cell proliferative responses in a dose-dependent manner (Fig. 3). Under these conditions, intact H57 and 2C11 cannot be used as potential blocking reagents, because they will activate the T cells as a result of external cross-linking by FcR present on splenic accessory cells (8). To circumvent this problem, H57 or 2C11 Fab fragments were used in an attempt to block T cell proliferation. The splenic T cells’ response to the BA was blocked by these soluble Fab fragments, as well as by anti-MHC class II Ab (Fig. 3). An irrelevant anti-MHC class II Ab had no effect on the proliferative response to BA. Moreover, spleen cells from B10.A (3R) mice, which express I-Aκ-encoded MHC class II (not recognized
FIGURE 2. Blocking Ab against the TCR or the appropriate MHC class II prevent BA activation. Five \( \times 10^4 \) 2B4.11 cells were cultured with the indicated concentrations of anti-TCR-\( \alpha \) x anti-MHC class II for 24 h under the following conditions: (○) alone (no accessory cells), (○) RT (I-A\(^b\)) accessory cells, (△) FT (I-A\(^b\)) accessory cells, (▲) RT + soluble A2B4 (1 mM final), (■) RT + soluble 10–3.6.2 (1 mM final), and (□) RT + Y-3P (anti-I-A\(^b\); 1 mM final). IL-2 was measured as in Figure 1.

by 10–3.6.2), did not proliferate in response to the same BA (data not shown). Together, these data prove that BA can indeed stimulate T cells, and that this stimulation is “MHC-restricted.”

Bivalent Fab heteroconjugates are responsible for stimulatory activity of BA

It is possible that contaminating heterotrimers of anti-TCR \( \times \) anti-MHC Fab \( \times \) 10–3.6.2 Fab or higher order multimers accounted for the stimulatory capacity of BA. To determine whether Fab multimers were involved in the T cell activation, an FPLC elution profile of the BA Ab preparations was generated and each fraction analyzed for its ability to stimulate 2B4.11 cells. One such elution profile, in which intact IgG (~150 kDa) and Fab fragments (~50 kDa) were used to calibrate the column, is shown (Fig. 4). The major protein peak, eluting with a retention volume of ~72 ml (solid line), corresponded to that predicted for Fab \( \times \) Fab dimeric heteroconjugates. A second smaller and trailing peak represented unreacted Fab fragments. No other species was detected; notably, no material was found that eluted with a higher apparent \( M_\text{r} \) than the Fab \( \times \) Fab BA. Concurrent bioassay demonstrated that the biologic activity in the sample was found in the Fab \( \times \) Fab dimeric heteroconjugate fraction. Similar elution and activity profiles were obtained with all three types of BA preparation. In addition, for many preparations the BA sequentially underwent two, rather than one, separation by FPLC. In those cases even the unreacted Fab peak was eliminated after the first FPLC run, and no unexpected high \( M_\text{r} \) material was detectable. For those preparations as well, all of the biologic activity was found in the single (Fab \( \times \) Fab) protein peak (data not shown). Therefore, BA formed by the conjugation of two monomeric Fab fragments were responsible for the T cell activating properties of the BA preparations.

BA stimulation is dependent on the mode of presentation and an intact TCR \( \zeta \)-chain

A \( \zeta \) negative variant of 2B4.11 has been transfected with either a full length \( \zeta \) (FL) or a mutated \( \zeta \) cDNA that encodes a \( \zeta \) protein truncated at residue 108 (CT108; missing the COOH-terminal 57 amino acid residues) (24). As shown in Figure 5, FL cells produced large amounts of IL-2 when stimulated with Ag in the presence of I-E\(^k\)-transfected L cells. As previously reported (24), CT108 cells did not respond in this experiment, even at the highest concentration of Ag tested. In other experiments, CT108 did begin to secrete IL-2 when the concentration of pigeon fragment 81–104 was increased to 100–300 \( \mu \text{M} \) (data not shown). This same dichotomy was evident when the BA were analyzed. Although 2C11 \( \times \) 10–3.6.2, A2B4 \( \times \) 10–3.6.2, and H57 \( \times \) 10–3.6.2 all induced IL-2 production by FL cells, they were ineffective at inducing a response from CT108 cells. There was a shift of ~100-fold in the BA dose-response curves between the two cells. The cell-dependent difference between the potency of the BA was not due to an intrinsic property of the BA themselves; when immobilized on a plastic surface, these BA induced both FL and CT108 cells to produce IL-2 (Fig. 6). Importantly, the dose-response curves to plastic-adherent BA were identical, ruling out a simple inherent quantitative difference in the potency of these BA to stimulate FL vs CT108 cells. Thus, the manner in which the BA is encountered by the TCR determines the cellular response; when “presented” by MHC class II-bearing accessory cells the BA resemble cell-bound Ag/MHC, and when immobilized they resemble Ab.
EFFECT OF TCR-ζ TRUNCATION ON T CELL ACTIVATION

FIGURE 3. Spleen T cells are activated by BA in an "MHC-restricted" manner. Whole spleen cell populations from B10.A mice were cultured with the indicated concentrations of either (a) anti-TCR-β × anti-MHC class II (H57 × 10–3.6.2) or (b) anti-CD3-e × anti-MHC class II (2C11 × 10–3.6.2) BA. The wells had the following additions: a, (●) medium alone, (○) H57 Fab (100 μM final), (□) 10–3.6.2 (100 μM final), (△) Y3P (100 μM final), b, (●) medium alone, (○) 2C11 Fab (100 μM final), (□) 10–3.6.2 (100 μM final), (△) Y3P (100 μM final).

Discussion

How receptor occupancy initiates signal transduction has been extensively studied. In many instances, cellular activation involves ligand binding followed by receptor aggregation, which in turn leads to signal generation. In the case of the receptor for IgE (FceRI), Ab that cross-link receptor-bound IgE, or multivalent ligands recognized by the FceRI-bound IgE, activate basophil/mast cells to degranulate (36). Although binding of bivalent Ab or (Fab')2 fragments against IgE causes degranulation, monovalent Fab fragments do not. There is evidence that the density of receptor aggregates and/or their degree of stability may be a determinant in cellular activation. In the case of Ab cross-linked FceRI, the smallest aggregate (the receptor-bound IgE dimer) is capable of triggering histamine release, but in some cells IgE trimers and higher order oligomers of IgE induce degranulation more effectively (37). Receptor mobility has also been implicated in this process; aggregations of two receptor-IgE complexes are predominantly mobile, although clusters of more than two rapidly become immobile after formation (38). In the case of the insulin receptor,
antireceptor Ab simulate insulin activity (39). Both anti-insulin receptor bivalent (Fab')2 and monovalent Fab fragments can inhibit the binding of insulin, but only the (Fab')2 has agonist properties. Furthermore, addition of anti-Fab Ab to cells treated with antinsulin receptor Fab fragments restores the agonist property. For growth factor receptors such as those for epidermal growth factor and platelet-derived growth factor, Schlessinger and coworkers (40–43) has proposed a model of "allosteric receptor oligomerization." In this model, ligand binding stabilizes oligomeric receptor complexes, which possess enhanced ligand-binding affinity and elevated protein tyrosine kinase activity. Such hetero- or homooligomers, involving receptors and perhaps accessory proteins, would bring about the activation of receptor tyrosine kinase activity by allosteric subunit interaction, resulting in intracellular signaling.

The mechanism by which the TCR transduces signals is not known. There are at least two qualitatively different mechanisms by which TCR occupancy might lead to signaling. In one model, occupancy of single TCR induces conformational changes in αβ that initiate signal propagation. In another scenario, TCR occupancy results in local clustering, which in turn initiates signaling events. Because of the complex nature of the TCR's ligand, and its requisite association with the plasma membrane of a second cell, much of our understanding in this area comes from work in which anti-receptor Ab have been used to trigger the T cell. Although the ability of a T cell to be stimulated by Ag/MHC or by Ab are usually inseparable, the ζ 2B4.11 variant and its derivatives expressing truncated ζ have provided a case in which the activities of these two stimuli diverge. Our experiments explore this difference with a stimulus that has some of the properties of both Ag/MHC and anti-TCR Ab. Using a T cell hybridoma or normal T cells, BA behaved like an "MHC-restricted" TCR ligand: IL-2 production and T cell proliferation required accessory cells bearing the correct MHC-encoded class II molecule, and blockade of either the TCR or MHC class II prevented T cell activation. In contrast to cells transfected with full length ζ, cells expressing truncated ζ responded very poorly to BA presented by MHC class II-bearing accessory cells. Thus, the ability of BA to stimulate normal and ζ-truncated T cells was strikingly similar to that of Ag/MHC.

FL and CT108 cells responded identically to plastic-immobilized BA. Therefore, although a full length ζ-chain was required for "occupancy"-generated activation, it was not needed for activation after TCR immobilization. A key observation is that although immobilized Ag/MHC is able to activate FL cells to produce IL-2, it is inefficient at activating CT108 cells. Assuming that the fundamental mechanism of activation is similar between Ab and Ag/MHC, how do these data conform to different models of the mechanism of T cell activation? The results do not fit well with a simple conformational mechanism (TCR occupancy resulting in a change in αβ conformation and then signaling), because anti-TCR × anti-MHC class II BA as well as intact anti-TCR Ab, regardless of which chain they bind (including the non-Ag-binding CD3-ε chain), can trigger cellular activation. The requirement for multivalent binding, of Ag (17, 18) as well as Ab, also argues against this type of conformational mechanism. The inability of Ag/MHC or BA presented by MHC class II-bearing accessory cells to

FIGURE 4. FPLC elution profile of BA and their T cell stimulatory activity. An A2B4 × 10–3.6.2 BA preparation was purified by sequential passage over an ACA 34 column followed by FPLC separation. The FPLC ODzso elution profile is shown as a solid line. The arrows indicate the expected elution fraction of BA trimers, dimers, and uncoupled Fab fragments. Aliquots were taken from each fraction and the ability to induce IL-2 production by 2B4.11 cells (2 × 10⁴/well) in the presence of RT accessory cells (2 × 10⁴/well) was measured (dotted line).
stimulate IL-2 production by CT108 indicates that in the absence of full length ζ, clustering alone is insufficient to trigger full activation. Yet, drawing the TCR into close proximity with immobilized BA, not immobilized Ag/MHC, is sufficient to stimulate cells that express truncated ζ. These differences suggest that TCR clustering and the formation of stable TCR aggregates are distinct phenomena. This can be understood if one considers the possibility that TCR signaling is initiated in two steps. First, occupancy results in TCR clustering at the interface of the T cell and the APC. Second, these clusters may be further modified in the presence of a full length TCR ζ-chain to form a stable TCR aggregate. It is an intriguing speculation that when in an occupancy-induced cluster, ζ might foster interactions between TCR themselves and/or, perhaps, other molecules such as cytoskeletal components, to promote stable aggregation. In the case of Ab stimulation, TCR drawn together at the cell/plastic interface would be physically prevented from moving in the membrane, thus stabilizing TCR associations without requiring the participation of ζ. This model would predict that in the absence of the ζ tail required for converting a TCR cluster into a stable aggregate, a relatively high affinity TCR-ligand interaction must be provided to maintain stable TCR associations. This pre-
FIGURE 7. Stimulation of FL and CT108 cells by purified Ag/MHC complexes or anti-TCR mAb immobilized on plastic. FL and CT108 cells were incubated in microtiter wells coated with either E\textsubscript{k,k}E\textsubscript{p,k}/pigeon fragment 81–104 complexes or 2C11 (anti-TCR).

Prediction is borne out in our studies using immobilized Ag/MHC complexes whose low affinity for the TCR might be expected to be insufficient to maintain such TCR aggregates. In contrast, cells with a full length ζ-chain, which would promote the conversion of clusters to stable aggregates, responded well to immobilized Ag/MHC. As 2B4.11 is CD4\textsuperscript+; it will be of interest to determine if the introduction of CD4 in its native or in an intracellularly truncated form would, by virtue of enhancement of TCR affinity for ligand, allow CT108 cells to respond to Ag/MHC immobilized on plastic.

Note that in this type of model, TCR conformation may still play an essential role in signaling. All of the data are compatible with the notion that initial aggregation leads to conformational changes in the TCR, perhaps ζ itself because of covalent modification such as phosphorylation (44) or ubiquitination (45), that in turn leads to stable aggregation. Thus, stable aggregate formation, the common event in the initiation of TCR signaling, may be achieved either by external immobilization or by conformation-induced and ζ-dependent mechanisms. This type of conformational effect is distinct from a conformational change in αβ that serves as a proximal initiator of signal generation.

In a recent study, Malissen and coworkers described a BW5147 transfectant that expressed TCR whose ζ-chains have a large internal deletion in the cytoplasmic portion (residues 66–157). These cells responded to stimulation with anti-TCR Ab and Ag/MHC, although not to normally stimulatory anti-Thy-1 and anti-Ly-6 Ab (46). Although the BW5147 transfectants appear to differ in their phenotype from CT108, the data obtained with the internal deletion and CT108 truncation are in fact compatible. The BW5147 transfectants were selected for very high expression of cell surface TCR, substantially higher than the levels expressed by the T cell hybridomas we have used (B. Malissen, personal communication). Inasmuch as Ag dose-response curves were not done, one cannot quantitatively compare the response of the BW5147 cells with a mutated ζ to those with full length ζ-chains. It is noteworthy that 2B4.11 derivatives lacking any ζ-chain at all produce IL-2 when the Ag concentration is sufficiently high (23), and CT108 cells respond to Ag at very high concentrations (100–300 μM, data not shown) as well as to cell surface-bound BA at concentrations 30- to 100-fold greater than those required to activate FL cells (Fig. 5) (because of limits in the amount of purified Ag/MHC that could be generated, we were unable to increase the amount of this ligand to equivalently high points in the dose-response curve). Furthermore, we have studied a CT108 subclone that expresses abnormally high levels of cell surface TCR and, as expected, it is more sensitive to Ag/MHC than the typical CT108 cells (responding to 30 pM pigeon fragment 81–104). Thus, all of the data are consistent with the notion that loss of the internal portion of ζ severely impairs the ability of a cell to respond to TCR occupancy, but does not completely prevent it if concentration of ligand (and thus the degree of occupancy) is sufficiently high. This may indicate that clustering of TCR at high density is functionally equivalent to formation of a stable aggregate.

Our findings allow us to build on a similar model that was considered to deal with the initial observation that ζ-deficient or ζ-truncated 2B4.11 cells responded well to anti-TCR mAb but not to Ag/MHC (24). The fact that anti-TCR × anti-MHC class II BA mimic Ag/MHC allows us to exclude theories that would explain the differences between Ab and Ag/MHC by invoking differences in the TCR subregions or epitopes involved in ligand binding. Interestingly, another study has used bispecific whole Ab directed against the TCR and an MHC-encoded molecule (in this case, class I) to study T cell activation (47). The
system differed from that used here in that the bispecific Ab bound TCR and MHC class I molecules on the same cell. However, the conclusion was that “immobilization” of the TCR is an important aspect in signaling, which is consistent with other attempts to trigger T cell activation by Ab-mediated cross-linking of the TCR to other T cell surface molecules (48).

The formation of stable receptor clusters might be advantageous for a number of reasons, including prolonging the time that individual TCR interact with one another (49). Indeed, coupled with the hypothesis that \( \zeta \) promotes stable aggregation, this possibility accounts for the inability of a low affinity ligand such as Ag/MHC to activate cells expressing a truncated \( \zeta \)-chain. In addition to interactions between TCR, stable aggregation will bring associated molecules involved in signaling (e.g., kinases, phospholipases, phosphatases, etc.) into closer proximity. Similar to our observations with cells expressing a truncated \( \zeta \), it has recently been reported that T cells expressing either the brain or the T cell form of the p59\( \text{fyn} \) tyrosine kinase respond to Ab, but only the latter respond to Ag (50). Taken together, these data may imply that the Ab vs Ag/MHC response phenotype is dependent on a TCR-tyrosine kinase(s) interaction that is dictated by a full length \( \zeta \)-chain. Studies to determine directly the effect of full length or truncated \( \zeta \) on TCR aggregate formation are currently being pursued.

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