Kinetics of T-cell Receptor Binding by Bivalent HLA-DR-Peptide Complexes That Activate Antigen-specific Human T-cells*

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Monovalent major histocompatibility complex-peptide complexes dissociate within seconds from the T-cell receptor (TCR), indicating that dimerization/multimerization may be important during early stages of T-cell activation. Soluble bivalent HLA-DR2-myelin basic protein (MBP) peptide complexes were expressed by replacing the F(ab) arms of an IgG2a antibody with HLA-DR2-MBP peptide complexes. The binding of bivalent HLA-DR2-peptide complexes to recombinant TCR was examined by surface plasmon resonance. The bivalent nature greatly enhanced TCR binding and slowed dissociation from the TCR, with a $t_{1/2}$ of 2.1 to 4.6 min. Soluble bivalent HLA-DR2-MBP peptide complexes activated antigen-specific T-cells in the absence of antigen presenting cells. In contrast, soluble antibodies to the TCR-CD3 complex were ineffective, indicating that they failed to induce an active TCR dimer. TCR/CD3 antibodies induced T-cell proliferation when bound by antigen presenting cells that expressed Fc receptors. In the presence of dendritic cells, bivalent HLA-DR2-MBP peptide complexes induced T-cell activation at $>100$-fold lower concentrations than TCR/CD3 antibodies and were also superior to peptide or antigen. These results demonstrate that bivalent HLA-DR2-peptide complexes represent effective ligands for activation of the TCR. The data support a role for TCR dimerization in early TCR signaling and kinetic proofreading.

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1 The abbreviations used are: TCR, T-cell receptor; MHC, major histocompatibility complex; PCR, polymerase chain reaction; HPLC, high performance liquid chromatography; CAPS, 3-(cyclohexylamino)propanesulfonic acid; mAb, monoclonal antibody; IL, interleukin; PAGE, polyacrylamide gel electrophoresis.

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T-cell receptor (TCR)$^+$ recognition of MHC-bound peptides is important for the induction of antigen-specific immune responses. Surface expression of the TCR requires coordinate expression and assembly with four associated proteins termed CD3 $\gamma$, $\delta$, $\epsilon$, and $\zeta$ (1, 2). CD3 $\epsilon$ associates with either $\gamma$ or $\delta$, resulting in the formation of $\gamma\epsilon$ and $\delta\epsilon$ heterodimers. It is well established that two CD3 $\epsilon$-containing complexes are incorporated into a TCR-CD3 complex (3, 4). Even though the CD3 $\gamma$, $\delta$, and $\epsilon$ chains are highly homologous, analysis of knockout mice has demonstrated that each subunit is important for development of $\alpha\beta$ T-cells (5–7).

The assembly of the TCR-CD3 complex follows discrete steps (1). An initial step is the association of the TCR $\alpha$ chain with CD3 $\delta\epsilon$, which can then pair with a CD3 $\gamma\epsilon$-associated TCR $\beta$ chain to form a disulfide-linked TCR $\alpha\beta$ heterodimer (8). Positively charged residues in the transmembrane regions of both TCR $\alpha$ and $\beta$ are essential for assembly with these CD3 subunits. The TCR $\alpha$ chain carries two positively charged amino acids (arginine and lysine), while the TCR $\beta$ chain transmembrane segment carries a single lysine residue. Site-directed mutagenesis of these residues abolishes assembly and transfer of an $\delta$-amino acid transmembrane segment of TCR $\alpha$ to an unrelated protein is sufficient for association with CD3 $\delta\epsilon$. Each CD3 subunit carries a negatively charged residue in the transmembrane region and site-directed mutagenesis experiments have demonstrated that these residues are required for TCR/CD3 assembly (9–12). As the final step in the assembly of the TCR-CD3 complex, the CD3 $\zeta\zeta$ homodimer associates, allowing transport to the cell surface (13).

Each of the CD3 $\gamma$, $\delta$, $\epsilon$, $\zeta$ chains carries a single immunoreceptor tyrosine-based activation motif. While TCR $\zeta$ chain is essential for TCR surface expression, its large cytoplasmic domain, which carries three immunoreceptor tyrosine-based activation motifs, is dispensable for signaling (14). $\zeta$ chain knockout mice are impaired in T-cell development, but T-cell development can be rescued by transgenic expression of a truncated $\zeta$ chain (15). Therefore, the TCR-CD3 complex consists of two signaling units, of which only CD3 $\gamma\epsilon$ and CD3 $\delta\epsilon$, but not CD3 $\zeta\zeta$, are essential (14). T-cell activation results in the recruitment of CD4 and its associated p56$^{1\text{IC}}$ kinase (16).

The binding of several TCRs to their MHC/peptide ligands has been examined by surface plasmon resonance. In all cases, the TCR dissociated rapidly from the MHC/peptide complex (17–20). For example, the $t_{1/2}$ of a TCR specific for I-E$^k$ and a moth cytochrome $c$ peptide was $\approx12$ s, while a $t_{1/2}$ of $10.8$ s was determined for a TCR specific for I-E$^k$ and a hemoglobin peptide (17–19). The MHC class I restricted 2C TCR had a slightly longer $t_{1/2}$ of $\approx27$ s (20). When partial agonist or antagonist peptides were examined for these TCRs, even shorter half-lives were observed (17–19). These data suggest that dimerization or a higher degree of multimerization is required for effective TCR signaling.

Interestingly, all TCR-associated components represent dimers ($\gamma\epsilon$, $\delta\epsilon$, $\zeta\zeta$). In addition, the extracellular domain of CD4 crystallized as a dimer in which the membrane-proximal D4 domain constituted the dimerization interface. This dimer was observed in three different crystal forms, indicating that it may not represent a crystallization artifact (21). For these reasons, dimerization of the TCR represents an attractive hypothesis that would account for important biochemical and biological properties of the TCR-CD3 complex. However, soluble bivalent
antibodies to the TCR or the CD3 complex do not induce activation in the absence of antigen presenting cells. Such antibodies only induce activation when they are further cross-linked by addition of a secondary antibody, by binding to a solid support or by binding to antigen presenting cells that express Fc receptors. In fact, soluble anti-CD3 antibodies were found to act as competitors for stimulation of T-cell clones by immobilized anti-CD3 (22, 23). In order to address this question, we expressed an immunoglobulin fusion protein in which the antibody F(ab) arms were replaced by HLA-DR-peptide complexes. The biological properties of these bivalent HLA-DR-peptide complexes were distinct from those of TCR/CD3 antibodies. Soluble bivalent HLA-DR-peptide complexes induced T-cell activation, even in the absence of antigen presenting cells. In the presence of dendritic cells, bivalent HLA-DR-peptide complexes activated antigen-specific T-cells at lower concentrations than antigen, peptide, or TCR/CD3 antibodies. Bivalent HLA-DR-peptide complexes had a much slower dissociation rate than those reported for monovalent MHC-peptide complexes. The biochemical and biological properties of bivalent HLA-DR2-peptide complexes indicate that TCR dimerization is sufficient for triggering T-cell activation.

EXPERIMENTAL PROCEDURES

Expression and Purification of Bivalent HLA-DR2-MBP Peptide Complexes—Drosophila Schneider cell transfectants were generated using the S2 cell line (24) and propagated in Schneider medium (Sigma) supplemented with 5–10% fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The Fc segment of murine IgG2a was attached to the previously described DRα-Fos construct (25) by overlapping PCR. This construct represented the signal peptide and extracellular domain of DRα, a 7-amino acid linker (Val-Asp-Gly-Gly-Gly-Gly), the Fos dimerization domain, a 3-amino acid linker (Ala-Ala-Ser), and the N-terminal part of the Fc segment. The DRα-Fos construct was amplified by PCR with the following oligonucleotides: forward primer 5′-AAATAATGATTTCCAGCAATGTG-GAGTC-3′ and reverse primer 5′-CCTCTGCGCTCACTACGTGAC-3′. The forward primer carried an EcoRI restriction site while the reverse primer created the overlap between Fos and the hinge region of IgG2a. The overlap was amplified by reverse transcriptase-PCR using RNA extracted from spleens of NOD mice using the following oligonucleotides: forward primer 5′-GGGCGGCCAGGATGAACT-3′ and reverse primer 5′-CTGGTTCGCGCGGATCACCAGC-3′. The overlapping PCR was done with the forward primer for TCRα and the reverse primer for the Fos segment. The TCRα-Fos chain was digested with BglII and cloned into the BomHI site of pAcAB3 baculovirus transfer vector, which carries two p10 promoters in opposite orientation. This allowed expression of soluble TCR with a single recombinant baculovirus. The TCRα segment that represented the signal peptide and the ectodomain as well as part of the linker was amplified with the following oligonucleotides: forward 5′-AAAAATCTAGATCTAGATGGAACATCTCGGGAGT-3′ and reverse 5′-AAAAATCTAGATCTAGATGGAACATCTCGGGAGT-3′. The segment representing part of the linker and the Fos dimerization domain was amplified with the following oligonucleotides: forward 5′-CTGGTTCGCGCGGATCACCAGC-3′ and reverse 5′-AAAAATCTAGATCTAGATGGAACATCTCGGGAGT-3′. The overlapping PCR was done with the forward primer for TCRα and the reverse primer for the Fos segment.

The TCRβ-Jun construct was made in the following steps. A free cysteine residue in Cβ was mutated to serine by overlapping PCR of the Cβ segment and a segment representing the Cβ segment and the Jun segment were amplified by overlapping PCR of the overlapping PCR was done with the forward primer for TCRα and the reverse primer for the Cβ segment. The Jun segment was amplified with the following oligonucleotides: forward 5′-AAAAATCTAGATCTAGATGGAACATCTCGGGAGT-3′ and reverse 5′-AAAAATCTAGATCTAGATGGAACATCTCGGGAGT-3′. The overlapping PCR was done with the forward primer for Vβ and the reverse primer for the 3′ segment of Cβ. The Jun segment was subcloned into the pAcAB3 vector, which carries two p10 promoters. A recombinant baculovirus was generated using a genetically modified baculovirus (BaculoGold, Pharmingen, San Diego, CA). TCR expression was examined by Western blot analysis with mAbs specific for TCRα and TCRβ (antibodies αF1 and βF1, Endogen, Woburn, MA).

TCR was expressed on a large scale in High Five cells infected with the recombinant baculovirus and purified from concentrated supernatants using the W4F5.B α-mAb (ATCC HB-9282) coupled to cyanogen bromide-activated Sepharose 4B beads. Supernatants were passed over a preclearing column and the W4F5.B column at a flow rate of <30 ml/h. The column was washed with 40 ml of 20 mM sodium phosphate, pH 7.0, followed by 40 ml of 20 mM sodium phosphate, 150 mM NaCl, and 40 ml of 20 mM sodium phosphate, pH 7.0, 500 mM NaCl. Prior to elution, the column was also washed with 20 ml of 50 mM CAPS, pH 10.0. These wash steps were necessary to reduce nonspecifically bound proteins. The protein was eluted with 20 ml of 50 mM CAPS, pH 11.5, and fractions were immediately neutralized by addition of 1 M sodium hydroxide. The protein was eluted with a linear gradient of 0–100% 1 M NaCl at a flow rate of 3 ml/min. The TCR peak was concentrated and the buffer was exchanged to 25 mM T-cell Receptor Binding by Bivalent MHC-Peptide Complexes
BIACORE Analysis of Binding of Bivalent HLA-DR2-MBP Peptide Complexes to Recombinant TCR—The binding of DR2/MBP-IgG to MHC-specific TCR was measured by surface plasmon resonance using BIACORE 1000 (Biacore AB, Uppsala, Sweden). HLA-DR2/MBP-specific Ob.1A12 TCR and HLA-DR1/HA-specific Y22 TCR (negative control) were immobilized using EDC/NHS coupling chemistry in 50 mM sodium acetate, pH 5.5, and 50 mM sodium acetate, pH 5.0, respectively. For those experiments comparing binding to both TCRs, Ob.1A12 immobilization was lowered to match that of Y22 (Biacore). Antibodies specific for the desmoglein(190–204) peptide bound to DR2 were immobilized in a 96-well flat bottom plate with 10^5 T-cells/well. T-cell proliferation was determined after 48 h of culture by [3H]thymidine incorporation as described above. The following TCR and CD3 antibodies were tested in these experiments: anti-CD3 antibodies OKT3 (34), UCHT1 and HT3a (Pharmingen, San Diego, CA), X35 and Cris-7 (Biodesign, Kennebunk, Maine) as well as TCR Vβ2.1 antibody (Cambridge Antibody Technology) and TNF-α antibody (Pharmingen) was used as a negative control since it carried the same Fe segment as the bivalent HLA-DR2-MBP peptide complex.

Recombinant MBP was expressed in E. coli with a N-terminal (His)_6-tag and purified by metal-chelate and ion-exchange chromatography. Recombinant cells were obtained by optimizing the adherent cell population of blood mononuclear cells from a HLA-DR2- normal donor for 4 days with granulocyte macrophage-stimulating factor and IL-4 (35). Dendritic cells were then washed and irradiated with 3000 rad. Experiments were set up in triplicates with 3.5 x 10^4 dendritic cells and 10^5 T-cells/well in a total of 200 μl of serum-free media (AIM-V, Life Technologies, Gaithersburg, MD). Bivalent HLA-DR2-MBP peptide complexes, recombinant MBP, MBP85–99 (peptide (ENPVVHFISSLKMLG) with a mouse IgG2a antibody specific for TNF-α (Pharmingen) was used as a negative control since it carried the same Fe segment as the bivalent HLA-DR2-MBP peptide complex. For those experiments comparing binding to both TCRs, Ob.1A12 immobilization was lowered to match that of Y22 (Biacore). Antibodies specific for the desmoglein(190–204) peptide bound to DR2 were immobilized in a 96-well flat bottom plate with 10^5 T-cells/well. T-cell proliferation was determined after 48 h of culture by [3H]thymidine incorporation.

RESULTS

Expression and Characterization of Bivalent HLA-DR2-Peptide Complexes—We previously reported that soluble HLA-DR2-peptide complexes can be expressed by replacing the hydrophilic transmembrane segments as well as cytoplasmic domains of DRα and DRβ with leucine zipper dimerization domains from the transcription factors Fos and Jun (36). The sequence of the MBP85–99 peptide that is recognized by T-cell clones specific for the DR2-MBP peptide complex was covalently linked to the N terminus of the mature DRβ chain (25), as reported for murine MHC class II molecules (37). In order to generate bivalent, antibody-like molecules, the Fe segment of murine IgG2a (hinge, C_{\text{H}2} and C_{\text{H}3} domains) was attached in-frame to the 3' end of the DRα chain construct (Fig. 1). The IgG2a sequence was chosen since it binds with high affinity to protein A, which has four IgG-binding sites (38). This strategy therefore allowed expression of soluble, bivalent molecules that could be further multimerized with protein A.

These constructs were cloned into the pRmHa-3 vector under the control of the copper-inducible metallothionein promoter and the plasmids were transfected into Drosophila Schneider cells. Transfected cells were cloned by limiting dilution and grown on a large scale in roller bottles. The protein was purified from concentrated supernatants by affinity chromatography using mAb L243, which is specific for the assembled HLA-DR heterodimer. Following elution and neutralization, the molecules were further purified by HPLC using a protein A column. SDS-PAGE under reducing conditions demonstrated two bands corresponding to DRα-IgG and DRβ/IgG. The identity of these bands was confirmed by Western blot analysis using polyclonal antibodies specific for the Fos and Jun dimerization domains (Fig. 2, lanes 2 and 3) and HLA-DR (Fig. 2, lane 4).

The biological activity of the DR2/MBP-IgG protein was examined in a T-cell proliferation assay using T-cell clones specific for the DR2-MBP peptide complex as well as control clones with other MHC/peptide specificities (Table 1). For these experiments, the DR2/MBP-IgG protein, mouse IgG2a antibody (negative control), or an anti-CD3 antibody (positive control) were immobilized in a 96-well plate. T-cell activation was assessed by [3H]thymidine incorporation following 48 h of culture. The DR2/MBP-IgG protein activated T-cells specific for the DR2-MBP peptide complex (clones Ob.1A12 and Ob.2F3), but did not activate four control clones specific for other MHC/peptide complexes.
T-cell activation in response to immobilized molecules was tested in order to determine if bivalent HLA-DR2-peptide complexes were properly folded. DR2/MBP-IgG, mouse IgG2a (negative control) and an anti-CD3 mAb (mAb HIT3a, positive control) were immobilized in a 96-well plate by overnight incubation of 200 ng/well in 100 mm bicarbonate, pH 9.6. Wells were washed with phosphate-buffered saline and T-cells (10^6 per well, triplicates) were added. T-cell proliferation was determined after 48 h by [3H]thymidine incorporation. Numbers represent the mean cpm of triplicate determinations. Clones Ob.2F3 and Ob.AI2 are specific for the MBP(86–99) peptide bound to HLA-DR2, while the other four control T-cell clones are specific for other MHC/peptide combinations: MBP(86–99) bound to HLA-DQ1 (clone Hy.1B11), tetanus toxoid (830–843) bound to HLA-DR2a (DRA, DRB5*0101) (clone KW.TT.1), desmoglein 3 (190–204) bound to HLA-DR4 (DRA, DRB1*0402) (clone GoP3.1), and HTLV-1 Tax (11–19) bound to HLA-A2 (clone 2G4) (30, 32, 33).

Binding Kinetics of Multivalent HLA-DR2-Peptide Complexes to Purified TCR—The binding of DR2/MBP-IgG to the T-cell receptor was directly examined by surface plasmon resonance (BIACORE). For that purpose, a TCR specific for the DR2/MBP complex (derived from clone Ob.1A12) was expressed as a soluble protein in the baculovirus system (Fig. 3). The purified recombinant TCR was immobilized on a BIACORE chip by standard EDC/NHS chemistry and soluble DR2/MBP-IgG was run over this surface at a flow rate of 5 μl/min. These experiments demonstrated specific, dose-dependent binding, with soluble DR2/MBP-IgG at concentrations of 0.3, 0.6, 1.2, and 2.4 μg (Fig. 4A). Several experiments were performed to determine the specificity of this interaction (Fig. 4B). First, DR2/MBP-IgG was run over an unmodified dextran surface. Second, a control surface was created with a recombinant TCR specific for the influenza hemagglutinin (306–318) peptide bound to HLA-DR1 (kindly provided by J. Hennecce and D. Wiley). Third, binding was assessed for mouse IgG2a, which carries the same Fc segment as the DR2/MBP-IgG protein. All of these controls confirmed that binding of the DR2/MBP-IgG protein to immobilized TCR was specific. We also attempted to examine the binding of monovalent DR2/MBP (expressed without the Fc segment of IgG2a) in this assay. Only a weak signal that was similar to a control protein was observed (data not shown). These results demonstrate that the bivalent nature greatly increased the binding of the DR2/MBP-IgG protein to an immobilized TCR.

The contribution of the valency of binding to the dissociation rate was further assessed by making complexes of DR2/MBP-IgG with recombinant protein A. Protein A has four IgG-binding sites and binds IgG with a 2:1 stoichiometry (IgG-protein A) (38). As shown in Fig. 4C, multimerization with protein A greatly enhanced binding of DR2/MBP-IgG to the immobilized TCR and further slowed dissociation from the TCR. The following complexes were examined: 1) DR2/MBP-IgG multimerized with protein A; 2) DR2/MBP-IgG without protein A; and 3) mouse IgG2a multimerized with protein A. Binding of DR2/MBP-IgG and the DR2/MBP-IgG-protein A complex was specific since little binding was observed with the complex of mouse IgG2a and protein A.

Based on these experiments, the half-life (t1/2) was calculated for the dissociation of DR2/MBP-IgG as well as the complex of DR2/MBP-IgG and protein A from the TCR. To a certain extent,
the calculated $t_{1/2}$ was dependent on the concentration of DR2/MBP-IgG that was injected over the TCR surface and ranged from 2.1 to 4.6 min for concentrations of 0.3 to 2.4 $\mu M$, respectively (Fig. 4A). The $t_{1/2}$ for the bivalent DR2/MBP-IgG complex was 2.9 min, compared with 43.1 min for the complex with protein A when both were injected at a concentration of 1.9 $\mu M$ and a flow rate of 5 $\mu l/min$ (Fig. 3C). At a slower flow rate (2 $\mu l/min$ instead of 5 $\mu l/min$) and a slightly higher level of TCR immobilization, the $t_{1/2}$ for the complex of DR2/MBP-IgG and protein A was even longer (>200 min) (Fig. 4D). At this flow rate, there may have been a certain degree of rebinding, even though the association rate was relatively low for the DR2/MBP-IgG-protein A complex.

Staining of Antigen-specific Human T Cells by Bivalent HLA-DR2-Peptide Complexes—Binding to the TCR on the surface of antigen-specific human T-cell clones was examined using two different approaches. As a first approach, T-cells were incubated with unlabeled molecules, followed by incubation with an anti-mouse IgG antibody conjugated to Alexa 488 (Fig. 5). As a second approach, bivalent HLA-DR2-peptide complexes were fluorescently labeled on carbohydrate groups following oxidation of carbohydrates with sodium periodate (data not shown). Both approaches demonstrated specific labeling of two T-cell clones (Ob.2F3, Ob.1A12) that recognize the HLA-DR2-MBP peptide complex (A and B in Figs. 5 and 6). In contrast, no

**FIG. 3. Expression of a soluble MBP-specific TCR.** A soluble TCR specific for the DR2-MBP peptide complex was expressed in the Baculovirus system. The TCR was purified from concentrated supernatants by affinity chromatography with a TCR-specific antibody (W4F5.B) and ion-exchange HPLC (see “Experimental Procedures”). A, SDS-PAGE under reducing conditions resolved the TCR $\alpha$ and $\beta$ chains (lane 1). Under nonreducing conditions, the disulfide-linked heterodimer represented a single band (lane 2). 4 $\mu g$ of purified protein were loaded per lane. B, Western blot analysis was performed with mAbs specific for TCR $\alpha$ (lanes 1 and 2) and TCR $\beta$ (lanes 3 and 4). Purified TCR was separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane and probed with mAbs $\alpha F1$ and $\beta F1$, which are specific for TCR $\alpha$ and $\beta$, respectively. Bound antibodies were detected by enhanced chemiluminescence using an horseradish peroxidase-conjugated anti-mouse IgG antibody. The band representing the TCR $\alpha \beta$ heterodimer was stained with both TCR $\alpha$ and $\beta$ chain-specific antibodies under nonreducing conditions (lanes 2 and 4). Under reducing conditions, the individual chains were detected (lane 1, TCR $\alpha$; lane 3, TCR $\beta$).

**FIG. 4. Binding of HLA-DR2/MBP-IgG to immobilized TCR.** The binding between TCR and DR2/MBP-IgG was examined by surface plasmon resonance using a BIACORE 1000. A soluble TCR specific for the HLA-DR2-MBP peptide complex (clone Ob.1A12) and a control TCR (Y22) specific HLA-DR1/HA (306–318) were used in these experiments. TCRs were immobilized on the BIACORE chip by standard EDC/NHS chemistry. Samples were run in 10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% Polysorbate 20 at a flow rate of 5 $\mu l/min$ unless noted. Binding was measured in resonance units (RU) relative to the level of TCR immobilization. A, dose-dependent binding of DR2/MBP-IgG to immobilized MBP-specific TCR. DR2/MBP-IgG was injected at concentrations ranging from 0.6 to 2.4 $\mu M$. The level of Ob.1A12 TCR immobilization was 12,522 RU. These data were fitted to a model using BIAevaluation version 3.0 (1:1 binding with drifting baseline; $X^2 = 9.38$) and adjusted for a bulk refractive index contribution. B, binding of DR2/MBP-IgG to MBP-specific TCR and control TCR. DR2/MBP-IgG (2 $\mu M$) was run over three surfaces: immobilized Ob.1A12 TCR (1), immobilized Y22 control TCR (2), and an unmodified dextran surface (3). The levels of Ob.1A12 TCR and Y22 TCR immobilization were 7,205 and 6,104 RU, respectively. C, dissociation rates of DR2/MBP-IgG and DR2/MBP-IgG multimerized with protein A. The complex of DR2/MBP-IgG and protein A (1), DR2/MBP-IgG without protein A (2), and a mouse IgG2a control antibody multimerized with protein A (3) were run over a Ob.1A12 TCR surface at a concentration of 1.9 $\mu M$. The level of Ob.1A12 TCR immobilization was 12,522 RU. D, binding of DR2/MBP-IgG multimerized with protein A to immobilized MBP-specific TCR. The association and dissociation phases of the DR2/MBP-IgG-protein A complexes were further examined by extending the time of analysis. DR2/MBP-IgG (1) and a control mouse IgG2a antibody (2) were complexed with protein A and run over the Ob.1A12 TCR surface at a concentration of 1.9 $\mu M$ and a flow rate of 2 $\mu l/min$. The level of Ob.1A12 TCR immobilization was 12,522 RU.
staining was observed with two control clones (Hy.1B11 and Go.P3.1, C and D). In both sets of experiments, mouse IgG2a was used as a control. The t1/2 of 2.1–4.6 min that was measured for bivalent HLA-DR2 peptide complexes by BIACORE explains why the T-cell staining was relatively weak compared with staining by the anti-CD3 antibody.

We also attempted staining with molecules that were multimerized with labeled protein A, since the BIACORE experiments had indicated that multimerization delayed dissociation from the TCR. However, the labeling reduced the IgG binding capacity of protein A and prevented effective multimerization. The complexes with labeled protein A were directly tested for TCR binding by BIACORE. The delayed dissociation that characterized the binding of unlabeled protein A complexes was not observed with labeled protein A. The labeling with fluorescein isothiocyanate probably modified a lysine residue that is important for IgG binding and thereby reduced the IgG binding capacity of protein A.

**Fig. 5. Staining of antigen-specific T-cell clones with bivalent HLA-DR2-peptide complexes and a labeled secondary antibody.** Two T-cell clones specific for the HLA-DR2-MBP peptide complex (A and B, clones Ob.2F3 and Ob.1A12) and two control clones (C and D, clones Hy.1B11 and Go.P3.1) were stained with mouse IgG2a (negative control, blue), DR2/MBP-IgG (red), and anti-CD3 (positive control, green). T-cells were incubated for 1 h at 4°C with 50 μg/ml mouse IgG2a or DR2/MBP-IgG. T-cells were then washed and bound molecules were detected with a polyclonal anti-mouse IgG antibody conjugated to Alexa 488. Samples were analyzed in a fluorescent-activated cell sorter (EPICS XL, Coulter Corp.). The x and y axes represent the fluorescence intensity (on a log scale) and the cell number at a given level of fluorescence, respectively.

Activation of MBP-specific T-cells by Soluble, Bivalent HLA-DR2-MBP Peptide Complexes but Not by Soluble TCR or CD3 Antibodies—MHC class II-peptide complexes may be useful for the induction of antigen-specific T-cell responses. The ability of soluble DR2/MBP IgG molecules to activate antigen-specific T-cells was therefore compared with antibodies against the TCR or the TCR-associated CD3 complex (Fig. 7). These experiments were performed with T-cell clones in the absence of antigen presenting cells, since binding to Fc receptors can create a multivalent surface. The two T-cell clones specific for the DR2/MBP-peptide complexes were activated by soluble DR2/MBP-IgG, but not by a soluble TCR antibody (directed against the Vβ2.1 segment expressed by these clones) or soluble antibodies directed against the CD3 complex. Six TCR/CD3 antibodies were tested, including a TCR Vβ2.1 antibody and five different antibodies against the CD3 complex. None of these antibodies activated the two HLA-DR2/MBP-specific T-cell clones when used in soluble form. However, all of these antibodies were biologically active since they activated the T-cell clones when immobilized on a solid support. These experiments were performed in media supplemented with 10% human serum. In serum-free media, soluble TCR/CD3 antibodies induced a moderate degree of T-cell stimulation, presumably because a fraction of the antibody could attach to the plate. The fact that the soluble TCR/CD3 antibodies were ineffective indicated that cross-linking of the TCR-CD3 complex by soluble, bivalent antibodies was not sufficient for activation of T-cells specific for the DR2/MBP peptide complex.

T-cell activation induced by bivalent HLA-DR2-peptide complexes was enhanced by cross-linking of CD28 or by addition of recombinant IL-2 (Fig. 8). In contrast, anti-CD3 antibodies did not activate these T-cell clones, even when costimulation was provided with the anti-CD28 antibody (data not shown). This indicated that the failure of anti-CD3 antibodies to induce T-cell activation was not due to a lack of costimulation.

One of the five T-cell clones that was examined in this set of
experiments showed a moderate degree of T-cell proliferation to three of the anti-CD3 antibodies (mAbs HIT3a, Cris-7, and X35). This T-cell clone was specific for a desmoglein 3 peptide (residues 190–204) bound to HLA-DR4 (DRA, DRB1*0402). The level of \[^{3}H\]thymidine incorporation that was induced by these soluble anti-CD3 antibodies was \(10^{-20}\%\) relative to the stimulation observed with immobilized antibodies (data not shown).

The stimulatory capacity of bivalent DR2/MBP IgG peptide complexes was also compared with the complex of DR2/MBP IgG with protein A (Fig. 9). Similar dose-response curves were observed, indicating that further multimerization did not enhance the ability of the bivalent molecules to induce T-cell activation. These results demonstrate binding of soluble, bivalent MHC-peptide complexes to the TCR is sufficient to induce T-cell activation.

**Effective T-cell Stimulation by Bivalent HLA-DR2-Peptide Complexes in the Presence of Dendritic Cells**—Numerous studies have demonstrated that soluble anti-CD3 antibodies are strong mitogens for T-cells in the presence of antigen presenting cells (22, 34). In such experiments, anti-CD3 antibodies are multimerized by binding to Fc receptors on the surface of antigen presenting cells. Dendritic cells are potent antigen presenting cells and express type I and type II FcyR (CD64 and CD32); ligation of FcyR has been shown to induce maturation of dendritic cells (39, 40). The ability to induce T-cell activation was therefore compared for DR2/MBP-IgG, recombinant MBP, MBP(85–99) peptide as well as a panel of anti-CD3/TCR antibodies.

Dendritic cells were differentiated from blood mononuclear cells of a normal HLA-DR2 donor by culture in granulocyte macrophage-colony stimulating factor and IL-4 and used as antigen presenting cells (34). Recombinant MBP and MBP(85–99) peptide induced strong T-cell stimulation at concentrations of 1 to 10 nM, while much higher concentrations were required for anti-CD3 antibodies. In contrast, picomolar concentrations of DR2/MBP-IgG induced T-cell activation (Fig. 10). Similar results were obtained with a second MBP-specific T-cell clone (clone Ob.1A12, data not shown). Three anti-CD3 and a TCR V\(\beta\)2.1 antibody were tested in these experiments. The anti-CD3 antibody shown in Fig. 10 (mAb UCHT1) induced the strongest stimulation of the TCR/CD3 antibodies that were tested (mAbs UCHT1, OKT3, X35, and MPB2D5). When dendritic cells were fixed with 1% formaldehyde, a similar degree of T-cell stimulation was induced by DR2/MBP-IgG molecules (data not shown), indicating that T-cell activation was not due to uptake and processing of the MBP peptide bound to DR2/MBP-IgG molecules. These results demonstrate that bivalent MHC-peptide complexes are superior to antigen, peptide, or TCR/CD3 antibodies in activating antigen-specific T-cells.

**DISCUSSION**

Soluble, bivalent HLA-DR2-peptide complexes were found to effectively activate antigen-specific human T-cells. In contrast, soluble bivalent TCR or CD3 antibodies were ineffective, indicating that cross-linking of the TCR by a soluble, bivalent antibody is not sufficient for activation. In the presence of dendritic cells that express Fc receptors, activation was induced both by bivalent HLA-DR-peptide complexes and TCR/CD3 antibodies. A striking difference in biological activity was observed since >100-fold lower concentrations of bivalent HLA-DR-peptide complexes than of TCR/CD3 antibodies were required for T-cell activation. Staining of human T-cell clones was much weaker with bivalent HLA-DR2-peptide complexes than with anti-CD3 antibodies, probably because the antibodies had a higher affinity for the TCR-CD3 complex. Nevertheless,
Activation of MBP-specific T-cells by bivalent HLA-DR2/MBP-IgG complexes but not by soluble TCR/CD3 antibodies. Activation of a MBP-specific T-cell clone (clone Ob.2F3) by soluble, bivalent HLA-DR2/MBP-IgG complexes and by soluble TCR/CD3 antibodies was examined in a T-cell proliferation assay in the absence of antigen presenting cells. Soluble molecules were used at concentrations ranging from 0.625 to 20 µg/ml. 10^5 T-cells were added per well in 200 µl of media containing 10% heat-inactivated human serum. T-cell proliferation was quantitated after 48 h of culture by [3H]thymidine incorporation. Bivalent HLA-DR2/MBP-IgG and recombinant protein A were used as controls. T-cell proliferation was quantitated after 48 h of culture by [3H]thymidine incorporation. Bivalent HLA-DR2/MBP-IgG and DR2/MBP-IgG multimerized with protein A were compared in a T-cell proliferation assay using clone Ob.2F3. DR2/MBP-IgG and recombinant protein A were incubated at a 2:1 molar ratio at 37 °C for 30 min. Molecules were tested at concentrations ranging from 0.625 to 20 µg/ml using 10^5 T-cells per well in 200 µl of media containing 10% heat-inactivated human serum. Mouse IgG2a and recombinant protein A were used as controls. T-cell proliferation was quantitated after 48 h of culture by [3H]thymidine incorporation.

Activation of a MBP-specific T-cell clone (clone Ob.2F3) by soluble, bivalent HLA-DR2/MBP-IgG complexes induced strong, dose-dependent T-cell activation. In contrast, soluble anti-CD3 antibodies (clones UCHT1 and HIT3a) and a TCR Vβ2.1 antibody (clone MPB2D5, Biodesign) did not activate these T-cells. These results indicated that soluble bivalent HLA-DR2/MBP-IgG complexes induced T-cell activation by dimerization of the TCR. In contrast, TCR cross-linking by soluble, bivalent antibodies to the TCR-CD3 complex was not effective. Small open square, mlG2a; filled circle, DR2/MBP-IgG; large open square, anti-TCR; open circle, anti-CD3 (clone UCHT1); open triangle, anti-CD3 (clone HIT3a).

Enhancement of T-cell proliferation induced by bivalent HLA-DR2/MBP-IgG complexes with an anti-CD28 antibody and rIL-2. MBP-specific T-cells (clone Ob.2F3) were stimulated with soluble, bivalent HLA-DR2/MBP-IgG complexes at a concentration ranging from 0.625 to 20 µg/ml. A soluble anti-CD28 antibody (clone 9.3, final concentration 1 µg/ml) and/or rIL-2 (final concentration 5 units/ml) were added to 10^5 T-cells per well in 200 µl of media containing 10% heat-inactivated human serum. T-cell proliferation was quantitated after 48 h of culture by [3H]thymidine incorporation. T-cell activation induced by bivalent HLA-DR2/MBP-IgG complexes was enhanced by cross-linking of CD28. In the absence of TCR stimulation, the anti-CD28 antibody did not induce T-cell proliferation (data not shown). mlG2a; ●, DR2/MBP-IgG; ▲, DR2/MBP-IgG + 5 units/ml IL-2; ○, DR2/MBP-IgG + anti-CD28; △, DR2/MBP-IgG + anti-CD28 + IL-2.

Bivalent and multivalent HLA-DR2/MBP-IgG complexes have a similar capacity to induce T-cell activation. The stimulatory capacity of DR2/MBP-IgG and DR2/MBP-IgG multimerized with protein A was compared in a T-cell proliferation assay using clone Ob.2F3. DR2/MBP-IgG and recombinant protein A were incubated at a 2:1 molar ratio at 37 °C for 30 min. Molecules were tested at concentrations ranging from 0.625 to 20 µg/ml using 10^5 T-cells per well in 200 µl of media containing 10% heat-inactivated human serum. Mouse IgG2a and recombinant protein A were used as controls. T-cell proliferation was quantitated after 48 h of culture by [3H]thymidine incorporation. mlG2a; ●, DR2/MBP-IgG; ▲, DR2/MBP-IgG + protein A; ○, protein A.

Bivalent HLA-DR2/MBP-IgG complexes are more potent stimulators than antigen, peptide or TCR/CD3 antibodies. Anti-CD3 antibodies are known to stimulate T-cells in the presence of antigen presenting cells that express Fc receptors. The stimulatory potential of bivalent HLA-DR2/MBP-IgG peptide complexes was therefore compared with recombinant MBP, MBP(85–99) peptide as well as antibodies to the TCR-CD3 complex. Dendritic cells from a HLA-DR2 normal subject were used as antigen presenting cells since such cells represent the most potent antigen presenting cells that have been described. Irradiated dendritic cells (3.5 × 10^4/well) were co-cultured with MBP specific T-cells (clone Ob.2F3, 10^5/well) for 48 h in AIM-V media and T-cell proliferation was determined by [3H]thymidine incorporation. Bivalent HLA-DR2/MBP-IgG peptide complexes were the most effective stimulus for the MBP-specific T-cell clone and induced T-cell activation at >10-fold lower concentrations than recombinant MBP or MBP(85–99) peptide. An even greater difference in biological activity was observed to the anti-CD3 antibody (clone UCHT1) which required >100 higher concentrations for a similar degree of T-cell proliferation. This anti-CD3 antibody induced stronger T-cell activation than two other anti-CD3 and a TCR Vβ2.1 antibody (data not shown). mlG2a; ×, anti-CD3; ●, DR2/MBP-IgG; ▲, rMBP; ○, MBP(85–99).
bivalent HLA-DR2-peptide complexes were more potent in activating antigen-specific T-cells than TCR/CD3 antibodies. These results demonstrate that binding of bivalent HLA-DR2-peptide complexes to the TCR is sufficient to induce T-cell activation.

Three major models of TCR activation, namely aggregation, multimerization, and dimerization, have been proposed (reviewed in Refs. 23, 41, and 42). The aggregation model is based on a large number of studies which demonstrated that soluble TCR or CD3 antibodies do not induce T-cell activation, unless further cross-linking is performed (reviewed in Ref. 23). Higher order cross-linking could be induced by an additional antibody against a different TCR/CD3 epitope, a secondary antibody, immobilization of TCR/CD3 antibodies on a solid support or by binding of antibodies to cells expressing Fc receptors. The comparison of soluble, bivalent HLA-DR2-peptide complexes and of a large panel of TCR/CD3 antibodies indicates that soluble antibodies fail to induce a biologically active TCR dimer that is induced by bivalent HLA-DR2-peptide complexes.

The multimerization model proposes that an active TCR ligand has a valency of greater than two (reviewed in Ref. 41). This model is based on T-cell activation experiments with biotinylated MHC-peptide complexes that were multimerized with streptavidin. In these experiments, tetrameric MHC-peptide complexes had a higher activity than dimeric or trimeric complexes. The streptavidin scaffold may provide limited mobility for bound MHC-peptide complexes and may therefore require a higher number of MHC-peptide complexes than the immunoglobin scaffold used in this study. An important feature of the immunoglobin scaffold is the hinge region which allows free mobility of the two F(ab) arms relative to the Fc segment.

The dimerization model proposes that TCR dimerization is required to initiate signaling (reviewed in Ref. 42). A key question is whether such a TCR dimer is formed during the encounter of appropriate MHC-peptide complexes or whether an inactive dimer is pre-assembled during biosynthesis. It is well established that two CD3 ε subunits are present in the TCR complex and that CD3 ε forms CD3 γε and CD3 δε heterodimers (3, 4). The CD3 γε and CD3 δε heterodimers may pair with two TCR heterodimers and form a (TCRβγε)3-CD3 complex in which the CD3 component consists of CD3 γε, CD3 δε, and CD3 ζε. This model is consistent with the requirement for CD3 γ, δ, and ε for TCR surface expression, a large body of biochemical data on the pairing of TCR chains with CD3 subunits and functional studies on the requirements for TCR signaling (1–12, 42–45). Dimeric murine MHC class II-peptide complexes also activate antigen-specific T-cells, indicating that both human and murine T-cells are activated by dimerization of the TCR (44, 45). In addition, a soluble bivalent TCR was shown to specifically bind to cells that displayed the appropriate MHC class I-peptide complex (46).

Crystallographic and functional studies of the erythropoietin receptor demonstrated a preassembled receptor dimer in which the individual membrane-spanning and intracellular domains were too far apart to permit signaling by the receptor-associated JAK2 kinases. Ligand binding induced a major conformational change of the extracellular domain that reduced the distance between the two transmembrane segments from -73 to 39 Å, allowing the associated JAK2 kinases to come into contact and autophosphorylate (47, 48). The crystal structure of the extracellular domain of tumor necrosis factor-R1 also demonstrated a preassembled dimer in the absence of tumor necrosis factor (49). In addition, fluorescence imaging studies have indicated that the IL-2 receptor and the epidermal growth receptor may occur as preassembled receptors on the cell surface. The formation of preassembled, inactive receptors is therefore observed in a number of transmembrane receptors (50, 51).

The model of a preassembled (TCRβγε)3-CD3 complex would account for the observation that soluble, bivalent TCR/CD3 antibodies are ineffective in inducing signaling. According to this model, antibodies could bind to different TCR complexes but fail to induce an active configuration of a pre-assembled (TCRβγε)3-CD3 complex. Conversely, bivalent HLA-DR-peptide complexes could bind to such a pre-assembled (TCRβγε)3-CD3 complex and induce an active configuration. A preassembled (TCRβγε)3-CD3 complex would also explain why T-cells are very sensitive to low densities of the appropriate MHC-peptide complex on the antigen presenting cell, even though the affinity of a monovalent TCR for a MHC-peptide complex is very low (17, 20).

Initial triggering of TCRs results in transport of TCR-MHC-peptide complexes to a small junction between the antigen presenting cell and the T-cell, which has been termed “immunological synapse” (52, 53). Within 5 to 30 min of T-cell activation, TCRs are concentrated in a small cluster that is surrounded by a ring of ICAM1. Interestingly, ICAM1 is excluded from the central cluster. An open question regarding the formation of such synapses is the precise nature of the structure that triggers transport. Since monovalent TCR-MHC-peptide complexes have a very short half-life (17–20), it is difficult to envision how a monovalent TCR could drag a MHC-peptide complex into the cluster. The dissociation rate observed in the BIACORE experiments for the bivalent HLA-DR-peptide complex indicates that a (TCR-MHC-peptide)2 complex would be more suitable for transport over such distances (τb of bivalent HLA-D2-peptide complexes of 2.1 to 4.6 min). The formation of immunological synapses as the final product of TCR activation results in a stable, highly multivalent TCR-MHC/peptide interface and sustained signaling (52, 53). Therefore, the dimerization and multimerization models may describe distinct phases in T-cell activation, namely the initial TCR triggering and transport of MHC/peptide TCR complexes into such clusters and the later phase of sustained TCR signaling.

The effective activation of T-cells by bivalent HLA-DR-peptide complexes may also be of practical value since such molecules are more effective than peptide, antigen, or TCR antibodies. Such complexes may therefore be useful for immunization against defined viral proteins or tumor antigens. Of particular importance may be their ability to bind to Fc receptor expressing antigen presenting cells, such as dendritic cells.

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