Cutting Edge: Detection of Antigen-Specific CD4+ T Cells by HLA-DR1 Oligomers Is Dependent on the T Cell Activation State

Thomas O. Cameron, Jennifer R. Cochran, Bader Yassine-Diab, Rafick-Pierre Sékaly and Lawrence J. Stern

*J Immunol* 2001; 166:741-745; doi: 10.4049/jimmunol.166.2.741

http://www.jimmunol.org/content/166/2/741

References

This article cites 35 articles, 18 of which you can access for free at:
http://www.jimmunol.org/content/166/2/741.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Cutting Edge: Detection of Antigen-Specific CD4⁺ T Cells by HLA-DR1 Oligomers Is Dependent on the T Cell Activation State

Thomas O. Cameron,* Jennifer R. Cochran,* Bader Yassine-Diab,† Rafick-Pierre Sékaly,† and Lawrence J. Stern²*

Class I MHC tetramers have proven to be invaluable tools for following and deciphering the CD8⁺ T cell response, but the development of similar reagents for detection of CD4⁺ T cells based on class II MHC proteins has been more difficult. We evaluated fluorescent streptavidin-based oligomers of HLA-DR1 for use as reagents to analyze Ag-specific human CD4⁺ T cells. Staining was blocked at low temperatures and by drugs that disrupt microfilament formation and endocytosis. Cell-associated MHC oligomers were resistant to a surface stripping protocol and were observed by microscopy in intracellular compartments. This behavior indicates that detection of CD4⁺ T cells using class II MHC oligomers can depend on an active cellular process in which T cells cluster and/or endocytose their Ag receptors. T cells of identical specificity but in different activation states varied greatly in their ability to be detected by class II MHC oligomers. The Journal of Immunology, 2001, 166: 741–745.

Materials and Methods
Peptides
H₄₅₀₆–₃₁₈ (PKYVKQNTLKLAT), TT₄₃₀–₄₄₄ (QYIKANSKFIGITEL), and TI₅₀₆–₅₀₆ (RVEYHPLSPYVSPKESP) were synthesized using solid-phase Fmoc chemistry, and purified by C18 reversed phase HPLC. All peptides bind tightly to DR1 with dissociation constants below 100 nM (8).

Preparation of labeled Abs and streptavidin (SA)³
Murine mAbs OKT3 or OKT4 (American Type Culture Collection, Manassas, VA) purified from hybridoma supernatant or SA (ProZyme, San Leandro, CA) were incubated with 10-fold molar excess FITC (Sigma, St. Louis, MO), succinimidyl 6-(biotinamido) hexanoate (NHSLC-biotin; Pierce; Rockford, IL), or N-(6-(biotinamido)hexyl)-3’-(2 pyridyldithio) propionamide (Biotin-HPDP; Pierce) at pH 7.5 for 3 h at room temperature and isolated by gel filtration using Sephadex G-50 (Pharmacia, Piscataway, NJ). FITC-conjugated SA (SA-FITC) was prepared by preincubation of SA with 2-hydroxyazobenzene-4’-carboxylic acid (Pierce) before fluorescent labeling. R-PE-conjugated SA (SA-PE) was purchased from BioSource International (Camarillo, CA).

³ Abbreviations used in this paper: SA, streptavidin; SA-FITC, FITC-conjugated SA; SA-PE, R-PE-conjugated SA; 2-ME-SO₃⁻, 2-mercaptopethanesulfonic acid.
Preparation of fluorescent class II MHC oligomers

Soluble HLA-DR1 (B1*0101) peptide complexes carrying an α subunit C-terminal cysteine (9) were reacted with excess maleimide-oxyethylene-biotin (PEO-maleimide-activated biotin; Pierce) or pyridyldithio-propionamide-biotin (HPDP-biotin; Pierce) and isolated by gel filtration in PBS, pH 7.0. Oligomers were formed by stepwise addition of SA-FITC or SA-PE (BioSource International). SA-PE from other sources were tested and found to label cells less brightly. FITC-labeled tetramers were isolated by gel filtration using sequential SEC-3000 (Phenomenex, Belmont, CA) and Superdex 200 (Pharmacia) columns. SA and SA conjugates absorbed to the gel filtration matrix, but SA-FITC saturated with biotinylated DR1 eluted at its expected m.w.

T cell clones and line

T cell clones HA1.7 and Cl-1 were cultured as described (9) and rested six or more days before use. A short-term polyclonal CD4+ T cell line was raised by repeated in vitro stimulation of CD8-depleted PBMCs from a DR1-homozygous donor using autologous mitomycin C-treated PBMCs in the presence of 1 μM Ha peptide.

Flow cytometry

T cells (∼10^6/ml) were mixed with DR1-peptide oligomers for 3–5 h at 37°C, chilled for 5 min, supplemented with secondary Abs for 30 min, and washed twice with cold wash buffer (PBS, 1% FBS, 15 mM sodium azide). Inhibitors (stock solutions in DMSO, ethanol, or PBS; final concentration of solvent ≤0.5%) were preincubated with cells for 1 h, after which the cells were stained with oligomer as above. In cell surface stripping experiments, chilled and washed cells were resuspended in wash buffer containing 25 mM 2-mercaptoethanesulfonic acid (2-ME-SO₃⁻), incubated for 15 min at 37°C, and then washed twice with cold wash buffer. In inactivation experiments, cells were pretreated overnight in complete medium with peptide or with immobilized OKT3 (5 μg/ml in PBS, 2 h) and then were stained as above.

Fluorescence microscopy

Live HA1.7 T cells were isolated using a Ficoll gradient and incubated with 10 mg/ml FITC-dextran (average molecular mass 10 kDa; Sigma) and 70 μg/ml DR1-Ha SA-PE oligomer, or 35 μg/ml SA-PE alone, for 3 h at 37°C, seeded onto cold Cell-Tak (Becton Dickinson Labware, Mountain View, CA)-coated glass coverslips, washed, fixed, mounted in Fluoro-View, and viewed using a DeltaVision digital deconvolution microscope system (Applied Precision, Issaquah, WA).

Dynamic light scattering

Measurements were made at 22°C using a DynaPro-MX/X dynamic light scattering instrument (Protein Solutions, Charlottesville, VA). Protein samples were filtered through 0.2-micron spin filters (Corning-Costar, Cambridge, MA) before analysis. All samples were measured at two different protein concentrations and with identical results. Molecular mass equivalents (in Da) were estimated from hydrodynamic radii (R_h, nm) using an empirical model for globular proteins, log MW = 2.426 log (1.549 × E_h), as recommended by the manufacturer.

Results

DR1 oligomers detect Ag-specific CD4+ T cells

The oligomerization strategy used in this work relies on biotin covalently coupled to a cysteine residue at the C terminus of the HLA-DR1α subunit (10), with subsequent oligomerization using SA. Soluble DR1-peptide complexes, folded in vitro from subunits expressed in Escherichia coli (11), were biotinylated at the introduced cysteine with >90% efficiency, using a maleimide reagent carrying biotin at the end of a 29 Å hydrophilic linker (Fig. 1A). The DR1-peptide complexes were resistant to SDS-induced chain dissociation at room temperature, indicating quantitative peptide loading (Fig. 1A). For routine use, biotinylated DR1-peptide complexes were oligomerized with SA-PE. Such DR1-PE oligomers carrying the Ha peptide exhibited Ag-specific binding to two DR1-restricted, Ha-specific T cell clones, HA1.7 (12) and Cl-1 (13) (Fig. 1, B and C). Oligomers carrying unrelated peptides showed no significant binding (Fig. 1, B and C, A2 and TIR tracings). To confirm the specificity of oligomer binding we analyzed a series of dilutions of the HA1.7 clone mixed into nonspecific PBMCs (Fig. 1D). Monocytes present in the PBMC mixture exhibited nonspecific oligomer binding, but could be distinguished by their CD4+/medium phenotype. The fraction of T cells staining with the DR1-PE oligomers correlated closely with the fraction of Ag-specific cells in the mixture, highlighting the specificity of staining (Fig. 1D).

Oligomer staining requires an active cellular process

We examined the experimental conditions necessary for oligomer staining of the T cell clones. Both clones required relatively high oligomer concentration (Fig. 2A), with Cl-1 exhibiting saturating staining intensity at >300 μg/ml (~0.6 μM), whereas for HA1.7, staining did not appear to saturate even at 750 μg/ml (~2 μM). Staining intensity increased with increasing temperature for both clones (Fig. 2B). At 4°C, the temperature usually used for Ab staining, no staining was observed for either clone. The staining signal developed slowly, and continued to increase for at least 5 h after the addition of oligomer (Fig. 2C). These concentrations, temperature, and time requirements for efficient staining with these reagents are consistent with those described by other researchers using MHC class II oligomers (2, 4, 5). Our typical staining protocol uses 20–50 μg/ml oligomer reagent for 3–5 h at 37°C.

The elevated temperature and extended time required for efficient staining suggested that a metabolic process might be involved. We investigated the staining of T cells by DR-PE oligomers after treatments that interfere with various normal cellular functions (Fig. 2D). Treatments that inhibited conventional T cell signaling pathways, including genistein (14), staurosporine (15), PP2 (16), and methyl-β-cyclohextrin (17), weakly inhibited staining or had no effect. Agents that disrupt endosomal proteolysis, such as chloroquine, NH₄Cl, and monensin, had little or no effect. However, staining was substantially blocked by treatments that
interfere with endocytosis and cytoskeletal rearrangements, including the microfilament-disrupting drugs latrunculin A (18) and cytochalasin D (19), the phosphatase inhibitor phenylarsine oxide (20), sodium azide (80 mM), and paraformaldehyde fixation. TCRs are efficiently internalized following engagement by cell surface MHC-peptide complexes on other cells (21, 22). Because MHC oligomer staining was blocked by treatments that block endocytosis, we postulated that oligomers might be internalized along with TCR during the staining protocol.

**Bound class II MHC oligomers are present in internalized compartments**

To test whether MHC class II oligomers were internalized after binding, we performed fluorescence microscopy on HA1.7 T cells after incubation with DR-SA-PE oligomers. Oligomer staining was detected in intracellular compartments (Fig. 3A, red) colocalized with endocytic compartments as visualized by FITC-dextran (Fig. 3A, green). Cells incubated with SA-PE reagent alone (without MHC) showed normal FITC-dextran internalization but no detectable PE signal (data not shown). These results show that MHC class II oligomers are internalized efficiently by T cells.

We used a surface stripping protocol to evaluate the relative contributions of internalized and surface-bound oligomers to the staining signal observed by flow cytometry. A disulfide bond was introduced into MHC tetramers between the DR1 and biotin moiety (DR1-SA-PE) to allow cleavage by the membrane-impermeant reducing agent 2-ME-SO₃⁻ (r, reduced, open trace). Control staining (dotted trace) is shown for unstained cells (B and C) or for cells stained with oligomers carrying non-antigenic TT peptide (TT, D and E). 2-ME-SO₃⁻ strips Ab (αCD4-SS-bio) but not MHC oligomer (DR1-SA-SS-PE, DR1-SA-SS-FITC), or noncleavable Ab control (αCD4-bio). F and G, Dynamic light scattering. Hydrodynamic radii distributions were estimated by dynamic light scattering for DR1-SA-PE oligomer (average r = 34.4 nm) (F) and DR1-SA-FITC tetramer (G) (average r = 6.1 nm). Corresponding molecular masses are 15,000 and 230 kDa, estimated using a model for globular proteins.
DR1-SA-PE oligomers (Fig. 3D), even after expression of the staining results in terms of numbers of fluorophores bound per cell (data not shown). Nonetheless, the observed staining still required elevated temperature and was insensitive to surface stripping by 2-ME-SO₃ (Fig. 3E). Thus, the observed association of staining and endocytosis was not due to a reduced valency in the DR1-SA-PE oligomers.

A polyclonal T cell line contains subpopulations with different requirements for staining

To test the generality of the observation that staining with MHC class II oligomers requires an active cellular process, we tested a polyclonal T cell line restricted by HLA-DR1 and specific for the Ha peptide. Oligomer staining experiments were performed in parallel at 37°C and at 4°C (Fig. 4A). At 37°C, 67% of the total polyclonal cell population exhibited DR1-SA-PE staining (PE⁺), which was specific for the appropriate peptide. By contrast, only 12% of the cells were PE⁺ when stained at 4°C, and these exhibited ~2-fold reduced intensity. Both the minor PE⁺ population detected at 4°C and the major population detected at 37°C were oligoclonal, as shown by TCR Vβ3 and Cβ1 analysis (data not shown), and both had characteristics of CD4⁺ memory T cells (CD3⁺, CD4⁺, CD8⁻, CD25⁻, CD45RO⁻, and CD62L⁻). These results show that most of the polyclonal T cells share with HA.17 and CI-1 the requirement for an active cellular process to observe MHC oligomer staining, although some cells can stain in the absence of such processes.

**FIGURE 4.** Staining of a polyclonal T cell line. A, Cells were incubated with DR1-SA-PE carrying Ha peptide at either 37°C (open trace) or 4°C (shaded trace) for 3 h before analysis by flow cytometry. Staining with control oligomer is shown as dashed trace. When stained at 37°C, 67% of the cells were PE⁺, whereas when stained at 4°C only 12% were PE⁺. B, Treatments that anergize or inactivate T cells block staining. Cells were incubated overnight with the indicated concentration of control (TR) or specific (Ha) peptide, or with immobilized anti-CD3 Ab, before DR1-SA-PE staining. ■, oligomer staining intensity; ▶, staining after normalization for CD3 expression. C, MHC oligomer and CD3 staining levels after pretreatment with 10 μM control (TR) or specific (Ha) peptide.

Staining is reduced by treatments that induce a nonresponsive state

T cells can enter a nonresponsive or anergic state in response to a partial activation stimulus (23). We tested whether such treatments would effect T cell staining by MHC oligomers. Treatment with high concentrations of antigenic but not control peptides, or with immobilized anti-CD3, each dramatically reduced oligomer staining of the polyclonal T cell line (Fig. 4B). These treatments are known to induce T cell energy (24, 25). TCR surface expression was reduced somewhat by these treatments (Fig. 4C), but the effect was much smaller and accounted for <10% of the overall reduction in oligomer staining (Fig. 4B).

Discussion

The results presented here show that bound HLA-DR1 oligomers are internalized efficiently by two Ag-specific T cell clones. Oligomer staining was blocked by low temperature and by endocytosis inhibitors, suggesting that an active process requiring cytoskeletal rearrangement was required. Essentially all of the fluorescence of oligomer-stained T cells resulted from internalized oligomers, and cell surface staining was not detected. For a polyclonal CD4⁺ T cell line, most cells exhibited the same behavior as the T cell clones, with staining blocked at 4°C. (A subpopulation of CD4⁺ T cells capable of staining at 4°C was present, consistent with the observations of other investigators; W. Kwok and G. Nepom, personal communication). Staining was greatly reduced by treatments that anergize or inactivate T cells. These results indicate that class II MHC oligomer staining of CD4⁺ T cells requires active T cell processes, and depends on the T cell activation state. This behavior is not likely to be limited to the particular MHC-TCR interaction investigated here. Although a detailed kinetic analysis has not yet been reported for the MHC-TCR interactions investigated in this study (HLA-DR1, Ha peptide, and HA1.7 TCR), binding competition analysis suggests a monomeric K₅₇ ~10⁻⁹ M (10) within the range observed for other MHC-TCR interactions (10⁻⁴–10⁻⁷) (2, 6). In addition, a temperature dependence of staining similar to that observed here has been reported previously for murine class II MHC oligomers (2). Finally, similar behavior was observed in a short-term T cell line as well as in two Ag-specific T cell clones. Thus, a requirement for active cellular processes resulting in oligomer internalization may be a general (although not universal) characteristic of oligomer staining in the class II MHC system.

The requirement for active processes and association with internalization can be understood in terms of the effects of multivalent engagement of TCR. The MHC-TCR interaction is relatively weak, and would be expected to require multivalent engagement to survive the washing steps required for flow cytometry. Indeed, such considerations led to the initial development of MHC tetramers as staining reagents (26). Multivalent engagement is likely to require reorganization or rearrangement of TCR molecules in the plane of the membrane (10). Such rearrangements are likely to require cytoskeletal participation (27) and could be altered in nonresponsive or anergic T cells (28). For CD4⁺ T cells, multivalent engagement will trigger activation processes (9, 29, 30) leading to down-regulation (internalization) of activated TCR (22, 30) and internalization of bound MHC oligomers.

Staining of CD4⁺ T cells by class I MHC tetramers in general does not appear to require receptor internalization or other active T cell processes, as evidenced by their ability to be stained at 4°C (26, 31, 32) (although internalization has been reported to increase staining intensity and specificity; Ref. 33). The reason for this
One of the primary attractions of using MHC tetramers to detect Ag-specific T cells has been their ability to bind T cells independent of cellular response or activation state. This has been observed for class I MHC tetramers in several studies (26, 34, 35). If the activation dependence of class II MHC oligomer staining is a common feature of the system, it may preclude the use of class II MHC tetramers to investigate responding clones, and G. Paradis and staff at the Massachusetts Institute of Technology, W. H. Brissette, J. Rubenstein, and L. Wedderburn, A. Meyer, and K. Simons. 1997. Interaction of influenza virus haemagglutinin with sphingolipid-cholesterol membrane domains via its transmembrane domain. EMBO J. 16:5301.


