Cutting Edge: Detection of Antigen-Specific CD4$^+$ T Cells by HLA-DR1 Oligomers Is Dependent on the T Cell Activation State[1]

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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.**

Tetramers of class I MHC proteins have been shown to bind to Ag-specific CD8$^+$ T cells with high specificity and sensitivity, thus providing a rapid in vitro assay for T cell detection and enumeration that in principle depends on neither the activation nor differentiation state of the T cell (1). The ability of class I MHC tetramers to detect low frequency Ag-specific T cells in mixed populations has led to increasing use of these reagents in studies of the immune response (1).

In recent reports, MHC tetramer technology has been extended to the class II system (2–5). These reports used relatively high concentrations of tetramer (20 μg/ml), extended incubation times (1–3 h), and elevated temperatures (22°C or 37°C). A requirement for such conditions would be surprising, given the avidity and fast on-rates expected for the oligomeric MHC-TCR interaction (6, 7). This suggests that class II MHC tetramer staining may reflect a more elaborate process than simple association of a soluble reagent with surface receptors.

In this study, we investigated the utility of oligomers of human class II MHC-peptide complexes in detection of Ag-specific CD4$^+$ T cells. We show by flow cytometry that fluorescent oligomers of DR1 in complex with a peptide from influenza virus specifically stain two DR1-restricted, influenza-specific T cell clones and an Ag-specific polyclonal T cell line, and that the oligomers are internalized efficiently. Treatments that interfere with cytoskeletal rearrangements and endocytosis block class II MHC oligomer staining, showing that an active cellular process is required. Implications of these results for the use of class II MHC oligomers in detection of Ag-specific CD4$^+$ T cells are discussed.

Materials and Methods

Peptides

$Ha_{306-318}$ (PKYVKQNTLKLAT), $TT_{830-844}$ (QYIKANSKFIGITEL), $A2_{103-114}$ (VGSDWRFLRGYHQYA), and $TfR_{680-696}$ (RVEYHFLSPYVSPKESP) 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 (NHS-LC-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).

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[3] Abbreviations used in this paper: SA, streptavidin; SA-FITC, FITC-conjugated SA; SA-PE, R-PE-conjugated SA; 2-ME-SO_3^-, 2-mercaptoethanesulfonic acid.

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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 ($\sim 10^7$/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 $\leq$0.5%) were preincubated with cells in PBS 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-SO_3^-), 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 HA.1.7 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 Fluoromount-G (Electron Microscopy Sciences, Fort Washington, PA), and visualized using a DeltaVision digital deconvolution microscope system (Applied Precision, Issaquah, WA).

Dynamic light scattering

Measurements were made at 22°C using a DynaPro-MS/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 $\times$ R_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. 1*A*). The DR1-peptide complexes were resistant to SDS-induced chain dissociation at room temperature, indicating quantitative peptide loading (Fig. 1*A*). For routine use, biotinylated DR1-peptide complexes were oligomerized with SA-PE. Such DR1-SA-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 TfR traces). To confirm the specificity of oligomer binding we analyzed a series of dilutions of the HA1.7 clone mixed into nonspecific PBMCs (Fig. 1*D*). 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-SA-PE oligomers correlated closely with the fraction of Ag-specific cells in the mixture, highlighting the specificity of staining (Fig. 1*D*).

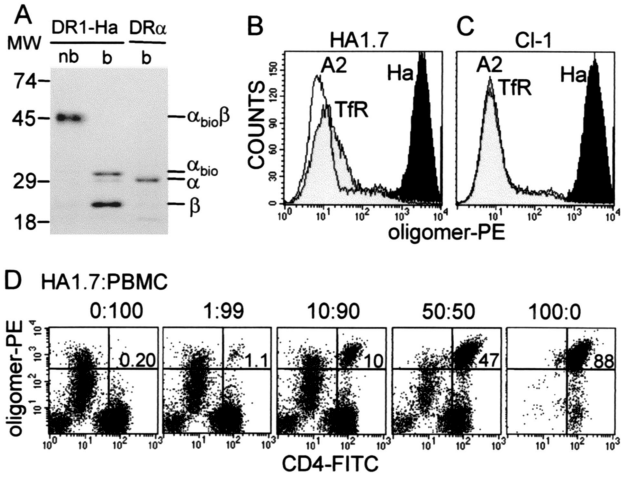

FIGURE 1. MHC class II oligomers specifically stain T cells. *A*, SDS-PAGE of biotinylated DR1-Ha complexes used in construction of oligomers. SDS resistance of $\alpha\beta$ band (not boiled, nb) indicates formation of DR1-Ha peptide complex. Decreased mobility of DRα in the complex (*middle*) relative to the free subunit (*right*) reflects quantitative biotinylation. Flow cytometry of HA1.7 (*B*) or Cl-1 (*C*) T cells stained with SA-PE oligomers carrying DR1-Ha (solid profile), DR1-TfR (shaded profile), or DR1-A2 (open profile) peptide complexes. *D*, Detection of HA1.7 T cells diluted into nonspecific PBMC mixtures at indicated ratios and stained with FITC-CD4 and DR1-SA-PE oligomer carrying Ha peptide. Percentage of DR1-SA-PE^+ $CD4^+$ cells in each mixture is indicated in the upper-right hand quadrant.

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. 2*A*), with Cl-1 exhibiting saturating staining intensity at >300 μg/ml ($\sim$0.6 μM), whereas for HA1.7, staining did not appear to saturate even at 750 μg/ml ($\sim$2 μM). Staining intensity increased with increasing temperature for both clones (Fig. 2*B*). 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. 2*C*). These concentration, 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-SA-PE oligomers after treatments that interfere with various normal cellular functions (Fig. 2*D*). Treatments that inhibited conventional T cell signaling pathways, including genistein (14), staurosporine (15), PP2 (16), and methyl-β-cyclodextrin (17), weakly inhibited staining or had no effect. Agents that disrupt endosomal proteolysis, such as chloroquine, NH_4Cl, and monensin, had little or no effect. However, staining was substantially blocked by treatments that

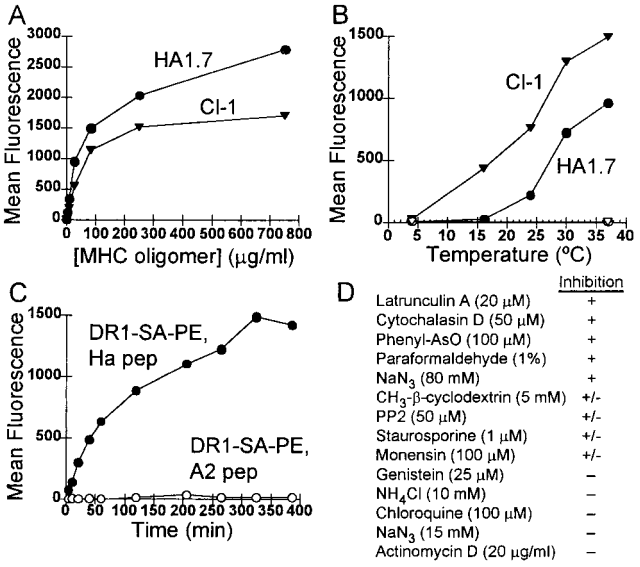

FIGURE 2. MHC oligomer staining depends on experimental conditions. Concentration (*A*), temperature (*B*), and time (*C*) dependence of staining for HA1.7 (●) and Cl-1 (▼) cells. Standard conditions (50 μg/ml, 37°C, 5 h) were varied as noted. *D*, Inhibitor analysis. Fraction of signal observed in the treated relative to an untreated sample is shown (+, <40%; +/−, 40–70%; −, >90%).

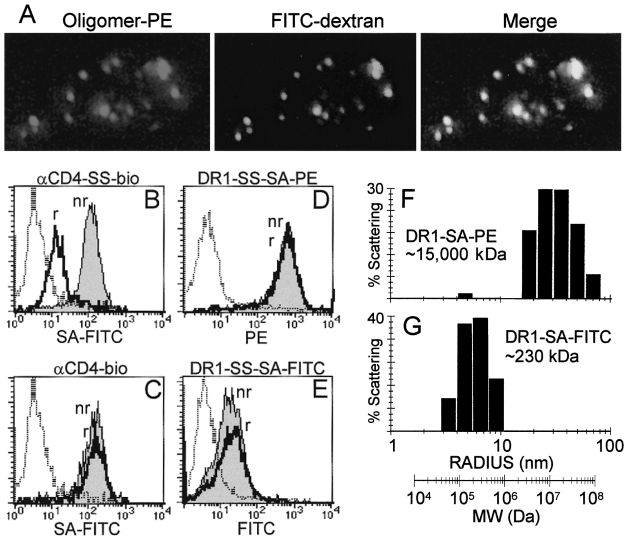

FIGURE 3. Cell-associated oligomers are not present at the surface. *A*, Fluorescence microscopy. Three-dimensional reconstruction of a single HA1.7 cell after incubation with DR1-Ha SA-PE oligomer (red) and the fluid-phase endocytosis marker FITC-dextran (green). *B–E*, Surface stripping. Oligomer- or Ab-stained HA1.7 T cells (nr, not reduced, shaded trace) were stripped with the membrane-impermeant reducing agent 2-ME-SO_3^- (r, reduced, open trace). Control staining (dotted trace) is shown for unstained cells (*B* and *C*) or for cells stained with oligomers carrying nonantigenic TT peptide (TT, *D* and *E*). 2-ME-SO_3^- strips Ab (αCD4-SS-bio) but not MHC oligomer (DR1-SS-SA-PE, DR1-SS-SA-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 = 34.4 nm) (*F*) and DR1-SS-SA-FITC tetramer (*G*) (average = 6.1 nm). Corresponding molecular masses are 15,000 and 230 kDa, estimated using a model for globular proteins.

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. 3*A*, red) colocalized with endocytic compartments as visualized by FITC-dextran (Fig. 3*A*, 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-SS-SA-PE) to allow cleavage by the membrane-impermeant reducing agent 2-ME-SO_3^-. DR1-SS-SA-PE oligomer staining of HA1.7 T cells was not affected by the 2-ME-SO_3^- treatment (Fig. 3*D*), indicating that cell-associated oligomers were not present at the cell surface. To demonstrate the effectiveness of the stripping protocol, we examined HA1.7 T cells that had been surface stained with an Ab carrying the cleavable linker (αCD4-SS-bio). In this experiment, staining was limited to the cell surface by incubation at 4°C. Fluorescent SA was efficiently stripped from the SS-bio Ab by the 2-ME-SO_3^- treatment (Fig. 3*B*) but not from a noncleavable biotinylated Ab (Fig. 3*C*). The insensitivity of the oligomer-binding signal to the 2-ME-SO_3^- surface stripping protocol and the lack of surface staining observed by fluorescence microscopy both indicate that essentially all of the oligomers associated with T cell staining are present in internal compartments.

One possibility for the correlation between MHC oligomer staining and internalization might be a low valency in preparations of DR1-SA-PE. The actual oligomeric state of MHC-SA-PE oligomers has not been reported. Moreover, physical characterization is difficult due to the large size of the PE moiety (~250 kDa) and heterogeneous cross-linking in commercial preparations of SA-PE. To obtain MHC oligomers with a well-defined valency, we prepared oligomers using SA labeled with FITC. Because several preparations of commercially available SA-FITC conjugates exhibited substoichiometric biotin-binding capacity, we developed a strategy to prevent damage to the biotin-binding sites during FITC labeling by protecting the biotin-binding sites using the weakly binding biotin analog 2-hydroxyazobenzene-4′-carboxylic acid (see *Materials and Methods*). DR1-SA-FITC oligomers prepared with this reagent exhibited an average molecular mass (230 kDa) consistent with one SA (60 kDa) and four DR1 (45 kDa), indicating that the desired tetrameric species had been formed (Fig. 3*G*). Gel filtration analysis gave a similar result (265 kDa, data not shown). By contrast, DR1-SA-PE oligomers exhibited a heterogeneous population distributed around a molecular mass of 15,000 kDa (Fig. 3*F*), indicating that they are composed of multiple SA and/or PE moieties; they are either large oligomers or noncovalent aggregates and, properly, they should not be referred to as "tetramers." T cell staining by the defined DR1-SA-FITC tetramers (Fig. 3*E*) was considerably less intense than that observed for the

DR1-SA-PE oligomers (Fig. 3*D*), 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-SO_3^- (Fig. 3*E*). 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. 4*A*). 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 Cl-1 the requirement for an active cellular process to observe MHC oligomer staining, although some cells can stain in the absence of such processes.

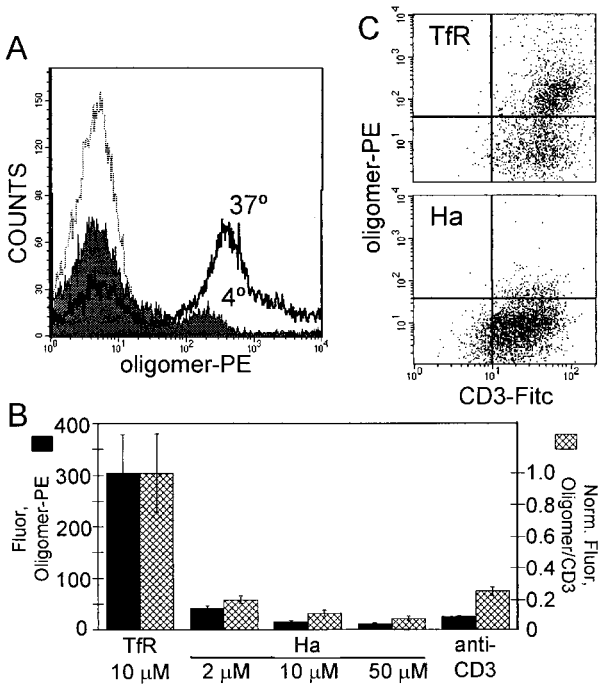

FIGURE 4. Staining of a polyclonal T cell line. *A*, Cells were incubated with DR1-SA-PE carrying Ha peptide at either 37°C (open trace) or 4°C (shaded trace) for 3 h before analysis by flow cytometry. Staining with control oligomer is shown as dashed trace. When stained at 37°C, 67% of the cells were 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 (TfR) 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 (TfR) 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. 4*B*). These treatments are known to induce T cell energy (24, 25). TCR surface expression was reduced somewhat by these treatments (Fig. 4*C*), but the effect was much smaller and accounted for <10% of the overall reduction in oligomer staining (Fig. 4*B*).

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 K_d ~10^{-6} 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 $CD8^+$ 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

difference between the behavior of class I and class II MHC oligomers is not clear. It may reflect differing roles for the coreceptors CD8 and CD4 in oligomer binding or cellular activation, or an intrinsic difference in the ability to cross-link or aggregate TCRs in $CD8^+$ vs $CD4^+$ T cells.

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 oligomers to characterize naive, inactive, or anergized $CD4^+$ T cells. These considerations may be relevant to recent reports using class II MHC oligomers to investigate responding frequencies for $CD4^+$ T cells (3–5).

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