

Antibody Recognition of an Immunogenic Influenza Hemagglutinin–Human Leukocyte Antigen Class II Complex

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Summary

The A/Japan/57 influenza hemagglutinin (HA) peptide HA 128-145, when bound by human histocompatibility leukocyte antigen-DRw11 cells, is recognized by the human CD4⁺ T cell clone V1. A rabbit antiserum has been raised against HA 128-145 which recognizes not only the free peptide, but also the HA 128-145/DRw11 complex on a solid matrix, in solution, or on the surface of viable cells. The detection of these complexes on viable cells was shown to be class II specific, DRw11 restricted, and commensurate with the level of DRw11 expression. The identity of DRw11 as the cell surface molecule binding HA 128-145 was confirmed by immunoprecipitation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and tryptic peptide mapping. Using this antiserum, HA 128-145/DRw11 complexes could be detected on the cell surface as soon as 30 min after the peptide was added, and increased up to 24 h. Dissociation kinetics showed these complexes were long-lived, with a half-life of approximately 14 h. This anti-HA peptide antiserum represents the first direct means of studying antigenic peptide–human leukocyte antigen class II complexes on the surface of living cells without the addition of a non-amino acid moiety to the peptide. The properties of this antiserum thus provide the potential to study naturally processed antigenic peptides as well as the mechanism of processing itself in a physiologically relevant system.

The immune response of CD4⁺ T cells is thought to be triggered by immunogenic MHC class II molecule-peptide complexes present on the surface of APC's (1–5). Previous reports have demonstrated in vitro association of peptide antigen with purified class II molecules (5–10), and proliferation of cloned T cells exposed to these complexes (6, 11).

While antigen-specific T cells have been useful for qualitatively demonstrating the presence of such complexes, the low number of surface peptide-class II complexes needed to activate a T cell, and the all-or-none nature of the recognition event have made such assays less useful for studying the kinetics and nature of peptide-class II binding (12–14). Studies of APC antigen processing and class II loading have been hampered by the inability to directly detect peptide bound in the antigen-binding groove of these molecules on the surface of intact cells. Whole cell binding assays using radiolabelled peptide antigen have been plagued by nonspecific uptake of radiolabel into the cells. Those studies which promote meta-

bolic antigen-processing have suffered from unacceptably high levels of nonspecific binding which have made it difficult to separately study the complexes of peptide antigen bound to surface class II molecules. Similar studies done under conditions which block APC intracellular antigen uptake and processing have demonstrated a high level of nonspecific cell surface binding and only a low level of binding to class II molecules (7, 15–20).

A series of rather elegant studies using biotinylated peptides and a fluorescent-avidin detection system has emerged from the laboratory of J.B. Rothbard, Immunologic Pharmaceutical Corporation (Palo Alto, CA) (12, 21, 22), allowing valuable insights into the association of immunogenic peptides with cell surface class II molecules. However, the interpretation of these studies is complicated by the potential of the biotin moiety to alter the conformation of the peptide bearing the biotin, or to affect the binding of the peptide to the class II molecule when attached to an amino acid in the MHC-interacting region of the antigen.

An antibody reagent which could detect unmodified peptide-MHC class II complexes on the cell surface would clearly be advantageous, and also has the potential to detect naturally processed peptides derived from intact antigen. A series of studies in the late 1970's and early 1980's initially sought to raise such antisera (17, 23-26). Although these studies did raise several antisera capable of binding to free peptide antigen, they were unsuccessful in obtaining binding of peptide to class II after the antisera had bound the peptide, or in demonstrating antisera binding to antigen after antigen uptake onto class II had occurred. Among the possibilities suggested to explain this failure were the denaturation or removal during antigen processing of epitopes on the peptide that are recognized by the antisera, masking of the epitope by the class II molecule, or alteration of the epitope upon binding of peptide to class II (17, 23).

The human, cytolytic, CD4-positive T cell clone, V1, has been shown to recognize a fragment of hemagglutinin protein from the influenza strain A/Japan/57 (Brown, L.R., N. Nygard, M.B. Graham, C. Bono, V.L. Braciale, J. Gorka, B.D. Schwartz, and T.J. Braciale, manuscript submitted for publication). This hemagglutinin peptide includes the amino acid residues 128-145, and is recognized by V1 only in the context HLA of DRw11.

We report here the ability of a rabbit antiserum raised against this influenza hemagglutinin (HA)¹ peptide to directly detect the complex recognized by the human T cell clone V1, composed of an antigenic HA peptide without any artificial linkage element and bound to HLA-DRw11 on the surface of an APC.

Materials and Methods

Synthetic Peptides. All peptides used in this study were synthesized on an Applied Biosystems (Foster City, CA) automated solid phase peptide synthesizer. The photoprobe peptide was also made on the Applied Biosystems instrument by incorporating the photoreactive group 4-benzoylbenzoic acid on the ϵ -amine of the N-terminal lysine according to the method of Gorka et al. (27). The sequences of all peptides used are given in Fig. 1.

Cell Lines and Clones. The T cell clone V1 was generated and maintained as previously described (28). It is specific for the hemagglutinin peptide encompassing amino acid residues 128-145 (HA 128-145) from influenza strain A/Japan/57 when presented in the context of HLA-DRw11 (Brown, L.R., N. Nygard, M.B. Graham, C. Bono, V.L. Braciale, J. Gorka, B.D. Schwartz, and T.J. Braciale, manuscript submitted for publication). EBV-transformed B lymphoblastoid cell lines (B-LCLs) GM3104 (DR1/DR1), GM3161 (DR2/DR2), GM3098 (DR3/DR3), and GM3164 (DR4/DR4) were obtained from the Mutant Cell Repository (Coriell Institute for Medical Research, Camden, NJ). The B-LCL Swei (DRw11/DRw11) was originally obtained from Dr. John Hansen (Fred Hutchinson Cancer Research Center, Seattle, WA), and has been maintained in our laboratory for the past 12 yr. TB-LCL (DR2/DRw11) was transformed from normal B cells autologous to clone V1. SJO, a B-LCL prepared from a patient with the class II-negative phenotype, bare lymphocyte syndrome was the generous gift of

¹ Abbreviations used in this paper: B-LCL, B lymphoblastoid cell line; HA, influenza hemagglutinin; PP, photoprobe peptide.

Dr. Jack Gorski (Milwaukee Blood Center, Milwaukee, WI). The murine L cell transfectants bearing DRw11, DRw52, DQw7, or DPw4 were the generous gift of Dr. Robert Karr (VA Medical Center, Iowa City, IA).

T Cell ⁵¹Cr Release Cytotoxicity Assay. The ⁵¹Cr-release cytotoxicity assay was performed as previously described (28).

α HA Peptide Antiserum. α HAP rabbit antiserum was prepared as in references 29 and 30 with the following modifications. 5 mg of HA 128-145 photoprobe peptide (HA 128-145 PP, Fig. 1) was mixed with 4.5 mg of BSA or KLH and exposed to 362 nm UV light at a distance of 2 cm for 3 h at which time >65% of HA 128-145 PP was conjugated to the protein. 350 μ g of peptide-protein conjugate in 200 μ l PBS was emulsified in 500 μ l CFA, and used to immunize rabbits by s.c. injection at three sites. The rabbits were boosted at 2 and 4 wk by injection of 350 μ g of peptide-protein conjugate in 500 μ l IFA, and were bled 1 wk thereafter. Negative control antiserum was prepared in an identical fashion with irrelevant DN10 peptide (Fig. 1).

Mouse Monoclonal Antibodies. The hybridoma producing the anti-DR framework antibody L243 was obtained from the American Type Culture Collection (Rockville, MD). The L243 was affinity-purified using protein A-Sepharose, and used at a concentration of 0.4 mg/ml for whole cell lysate immunoprecipitation. FITC-conjugated L243 was purchased from Becton Dickinson & Co. (Mountain View, CA) for use in FACS analysis. OKDR (anti-DR framework mAb) was purchased from Ortho Diagnostic Systems (Raritan, NJ). SFR3-DR5 (31), an anti-DRw11 mAb was the gift of Dr. Susan Radka (Oncogene, Seattle, WA). Control antibodies used included MKD6 (BD #1360), a murine anti-IA^d mAb, and G2CL, a murine anti H-2K^k mAb (BD #9051), both obtained from Becton Dickinson and Co. A511, a murine monoclonal IgG2a antibody made in our laboratory, was also used in some experiments as a negative control.

DRw11 Class II Purification. DRw11 haplotype HLA class II protein was affinity-purified from Swei cells by the method of Turkewitz et al. (32) using L243 mAb.

α HA Peptide Antiserum Enzyme-Linked Immunosorbent Assay. Nunc #439454 microtiter plate wells (Nunc, Inc., Naperville, IL) were coated with peptide or protein overnight at 4°C and washed with PBS/1% BSA. Where indicated, the wells containing plate-bound protein were then incubated with 2 μ g peptide in 100 μ l PBS for 3 h at room temperature and rewashed in PBS/1% BSA. 100 μ l of rabbit serum diluted 1/100 in PBS/1% BSA were added to each well, the incubation continued for 45 min at room temperature, and the wells then washed. The second antibody added was 100 μ l of a 1/1000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma Chemical Co., St. Louis, MO) followed by a 45 min incubation at room temperature. After washing, the ELISA was developed by addition to each plate well of 100 μ l of a 1 mg/ml substrate, *p*-nitrophenyl disodium phosphate, (Sigma #104-105; Sigma Chemical Co.). Following a 1 h room temperature incubation, the OD was read at 410 nm on a Dynatech MR700 ELISA plate reader.

Fluorescence-Activated Cell Sorter Assay with α HAP. 10⁶ indicated cells were incubated for 24 h at 37°C in 7% CO₂ atmosphere in 1 ml culture medium containing 5% dialyzed FCS and 21 μ M of the indicated peptide. The cells were washed twice in PBS/1% FCS and resuspended at 5 \times 10⁷/ml in wash buffer. Equal aliquots of peptide-pulsed cells were incubated for 20 min at 4°C with 100 μ l of 1/100 dilution of preimmune rabbit serum or immune rabbit α HA peptide antiserum, or the indicated mAb in PBS/1% FCS. After incubation the cell aliquots were washed in PBS/1% FCS, and then incubated in 100 μ l of FITC-conjugated

goat anti-rabbit IgG (Fisher Biotech #OB1400; Fisher Scientific, St. Louis, MO) for rabbit sera, 100 μ l of FITC-conjugated goat anti-mouse IgG (Fisher Biotech #OB1420-FITC) for unconjugated murine mAb, or no secondary reagent for FITC-conjugated mAb. After rewashing twice at 4°C in PBS/1% FCS, cells were fixed in 200 μ l 1% formaldehyde and stored at 4°C until FACS analysis. Cell pellets were brought up in 200 μ l saline and run on a FACS[®] Analyzer (Becton Dickinson and Co.), counting 10,000 cells/plot.

Immunoprecipitations of Radiolabeled HA (128-145)PP-Pulsed Swei Cell Lysates. 70 \times 10⁶ Swei cells were incubated in 35 ml culture medium containing 5% FCS and 250 μ g ¹²⁵I-labeled HA 128-145 photoprobe peptide. The peptide was labeled by the iodobead method (Pierce Scientific, Rockford, IL) to a specific activity of 18.7 μ Ci/ μ g. After incubation for 24 h at 37°C, the cells were washed twice in cold PBS and exposed to 350 nm wavelength UV light at 4°C for 1 h. The cells were washed two more times in cold PBS and lysed in 1 ml cold PBS containing 1% NP-40, 50 μ g/ml N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 50 μ g/ml N α -p-tosyl-L-lysine chloromethyl ketone (TLCK), and 200 μ g/ml PMSF. The lysate was centrifuged at 13,250 RPM for 15 min, and pre-cleared with 100 μ l packed protein A-Sepharose (Pharmacia, Uppsala, Sweden) for 30 min at 4°C with agitation. After centrifugation, the supernatant was divided into equal aliquots, and 50 μ l of NRS, α HAP, MKD6, OKDR, or L243 were added. Overnight incubation at 4°C was followed by addition of 100 μ l packed protein A-Sepharose for 30 min at 4°C with agitation every 5 min. After centrifugation the pellets were washed three times with cold 0.25% NP-40, then boiled for 2 min in 165 μ l 2% mercaptoethanol SDS elution buffer and run on an 11% SDS-PAGE gel. After fixation and drying, the gel was subjected to autoradiography.

Trypsin Digestion and Peptide Mapping. The proteins to be compared were eluted from an excised piece of gel, subjected to trypsin digestion, and peptide mapped as previously described (33).

Results

Native and Modified Forms of HA 128-145 Are Both Recognized by V1. The human CD4⁺ cytolytic T cell clone V1

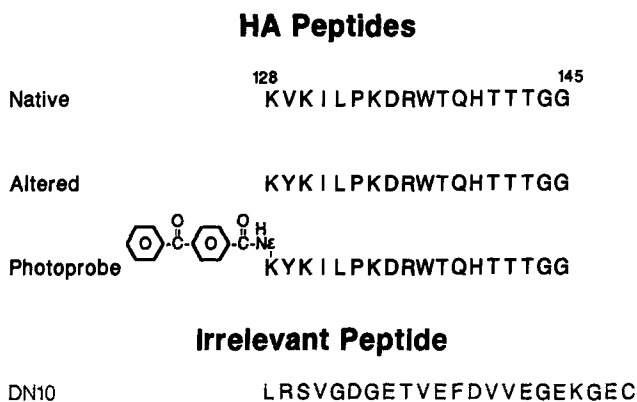


Figure 1. Sequences of peptides used in the study. (Top) native peptide sequence from hemagglutinin residues 128-145 of influenza strain A/Japan/57. (Second from top) altered nonphotoprobe hemagglutinin peptide (HA 128-145) showing tyrosine substitution at position 129. (Second from bottom) altered photoprobe peptide (HA 128-145 PP) showing photoreactive 4-benzoylbenzoic acid side chain on N-terminus lysine. (Bottom) irrelevant peptide DN10.

recognizes a specific 18-amino acid fragment (residues 128-145) of HA from influenza strain A/Japan/57, when presented on HLA-DRw11 APC's (Brown, L.R., N. Nygard, M.B. Graham, C. Bono, V.L. Braciale, J. Gorka, B.D. Schwartz, and T.J. Braciale, manuscript submitted for publication). We modified this peptide by substituting tyrosine for valine at position 129 to allow ¹²⁵I-radioiodination in some experiments, and for other experiments by adding a photoreactive side chain residue, 4-benzoylbenzoic acid to the ϵ -amine of the N-terminal lysine (Fig. 1). The recognition by clone V1 of the altered synthetic HA peptide (HA 128-145) was found to be identical to that of the native unmodified HA peptide when presented on DRw11 APC's (Fig. 2). In addition, the HA 128-145 photoprobe peptide (PP) (Fig. 1) was recognized equally well by clone V1 when presented on HLA-DRw11 cells (data not shown).

Rabbit Antiserum α HAP Recognizes Both Free HA Peptide and HA Peptide Bound to DRw11. When assayed by ELISA, rabbit antisera α HAP raised against HA 128-145 PP coupled to BSA or KLH did not react with the irrelevant peptide DN10 nor with affinity-purified DRw11 class II molecules alone (Fig. 3, A and B). However, the antisera did react with plate-bound HA 128-145 at a titer >1:200,000, (Fig. 3 A) as well as with complexes formed when plate-bound, affinity-purified HLA-DRw11 molecules were first exposed to soluble HA 128-145 (Fig. 3 B). In contrast, these antisera did not recognize plate-bound DRw11 molecules were first exposed to soluble HA 128-145 (Fig. 3 B). In contrast, these antisera did not recognize plate-bound DRw11 molecules exposed to

V1 Lysis with Altered HA Peptide

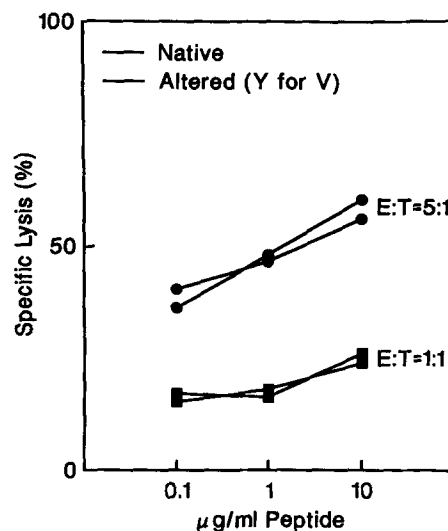


Figure 2. A comparison of V1 T cell recognition of DRw11 positive target cells pulsed either with native or with altered nonphotoprobe HA 128-145. Recognition is measured on the ordinate as percent specific lysis over background of ⁵¹Cr-labeled, peptide-pulsed APC's exposed to V1 at indicated effector to target (E/T) ratios. Concentrations of HA peptide used in APC antigen-loading incubation are given on the abscissa.

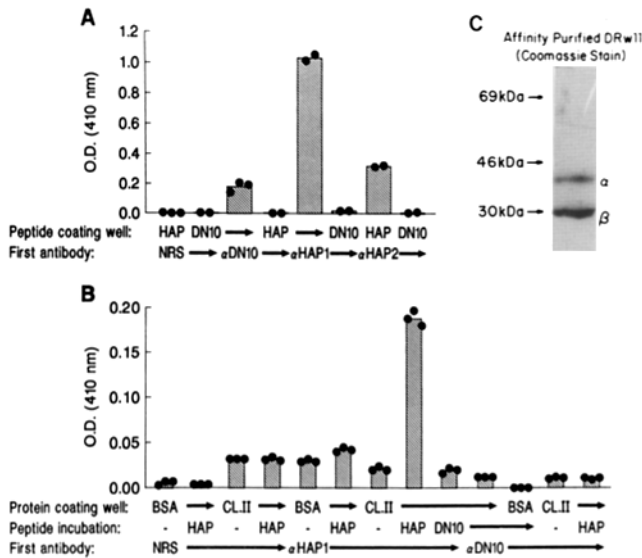


Figure 3. Rabbit antisera recognition of HA 128-145 by ELISA. (A) Rabbit sera (First antibody) were incubated with the indicated plate-bound peptide. (B) Indicated peptides were incubated over the various plate-bound proteins, then incubated with rabbit serum (First antibody). (C) SDS-PAGE Coomassie stain of the affinity-purified DRw11 class II preparation used in Fig. 3 B, demonstrating the relative purity of the class II protein preparation. Mol wt positions are indicated on the left. HAP: altered A/Japan/57 influenza hemagglutinin peptide, residues 128-145. DN10: irrelevant, non-HLA, non-influenza peptide. NRS: normal preimmune rabbit serum. αDN10: rabbit antiserum prepared against DN10 peptide conjugated to KLH. αHAP1: immune rabbit anti-HAP antiserum prepared against HA 128-145 peptide conjugated to BSA. αHAP2: rabbit antiserum prepared against HA 128-145 peptide conjugated to KLH. CL.II: DRw11 haplotype HLA class II molecules, affinity-purified from Swei cells using mAb L243. Each dot in Fig. 3 A and B represents an individual ELISA well OD reading shown on the ordinate.

an irrelevant peptide, nor plate-bound BSA incubated with HA 128-145. These results indicate that HA 128-145 bound to the plate-bound DRw11 molecules, and that the antisera were recognizing HA 128-145 complexed to DRw11. Because the antisera raised against the BSA and KLH-conjugated HA 128-145 yielded similar results, only the experiments using antiserum raised against HA 128-145 conjugated to BSA (denoted αHAP1 in Fig. 3 and αHAP in subsequent figures) are presented.

αHAP Detects HA 128-145 Uptake Onto Intact DRw11-Expressing B Cells by FACS. To determine if αHAP could detect HA 128-145 on the surface of a DRw11-expressing APC, cell flow cytometry (FACS) analysis was done (Fig. 4 A). The homozygous DRw11 BLCL Swei was incubated with no peptide (Fig. 4 A, panel b) or with HA 128-145 (Fig. 4 A, panel c) for 24 h, was reacted with αHAP, stained with FITC-conjugated goat anti-rabbit immunoglobulin, and analyzed by FACS. A class II negative, bare lymphocyte syndrome BLCL, SJO, was similarly treated (Fig. 4 A, panel a). A significant fluorescence shift was seen with αHAP for the HA peptide-pulsed, class II-positive Swei cells (Fig. 4 A, panel c) but not for the HA peptide-pulsed SJO cells, nor for the Swei cells not exposed to HA peptide. In addition αHAP did not react with Swei cells which had been exposed to the

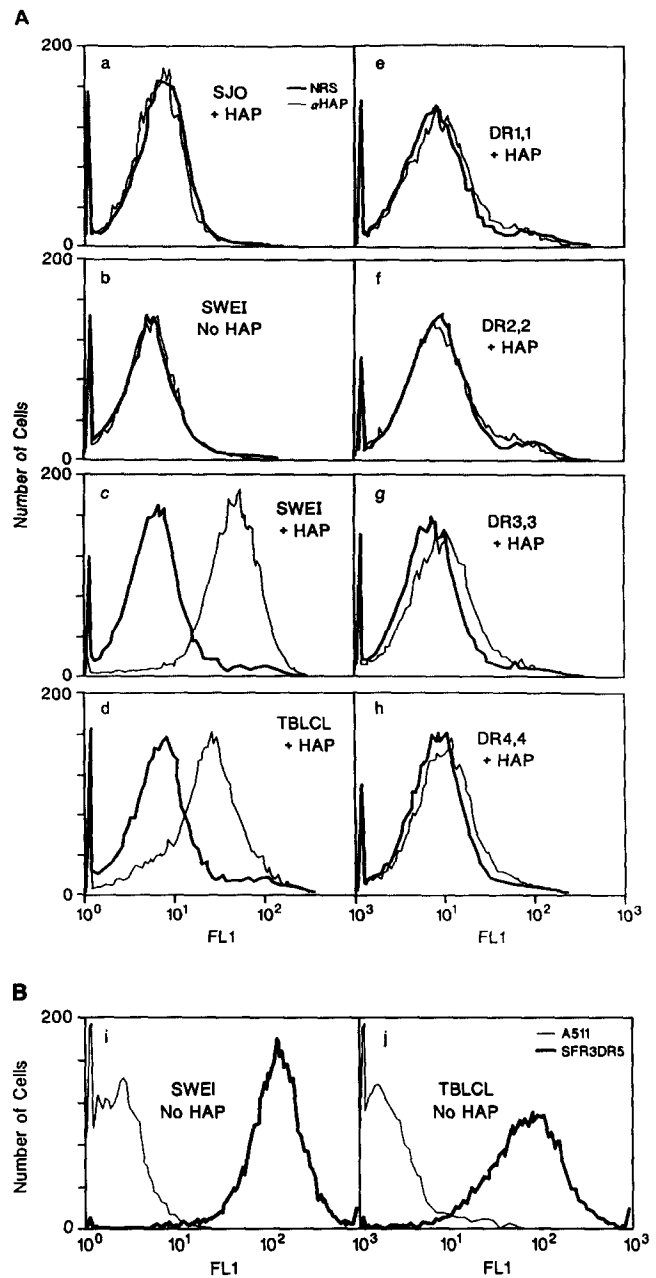


Figure 4. (A) HA 128-145 is specifically expressed on DRw11 positive BLCLs after 37°C incubation. Thick lines indicate initial staining in normal preimmune rabbit serum (NRS). Thin lines indicate initial staining in rabbit anti-HAP antiserum (αHAP). (a) SJO (class II negative); (b) Swei; (Drw11, w11) without HA peptide; (c), (d), (f), (g), and (h) Swei (Drw11,w11); TBCLL (DRw11,2); 3104 (DR1,1); 3161 (DR2,2); 3098 (DR3,3); and 3164 (DR4,4) lymphoblastoid cell lines, respectively, incubated with HA peptide. Staining with αDN10 resulted in FACS patterns indistinguishable from those obtained with NRS. (B) (i) Swei, and (j) TBCLL, without HA peptide, stained with control mAb A511 (thin line) or for DRw11 expression with the anti-DRw11 mAb SFR3-DR5 (thick line).

irrelevant DN10 peptide (data not shown). These results suggest that HA peptide uptake is specific for class II-expressing cells, and that αHAP can detect the peptide on such whole, living cells directly by FACS assay.

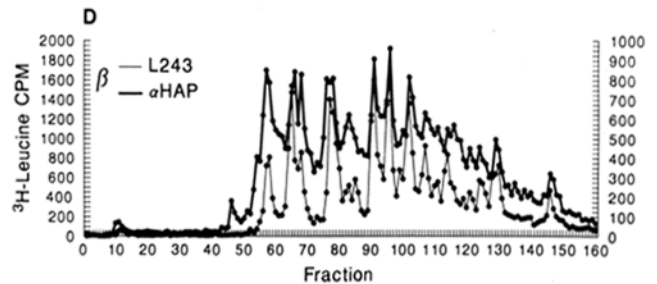
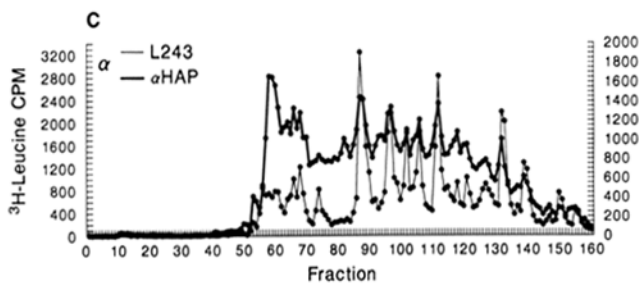
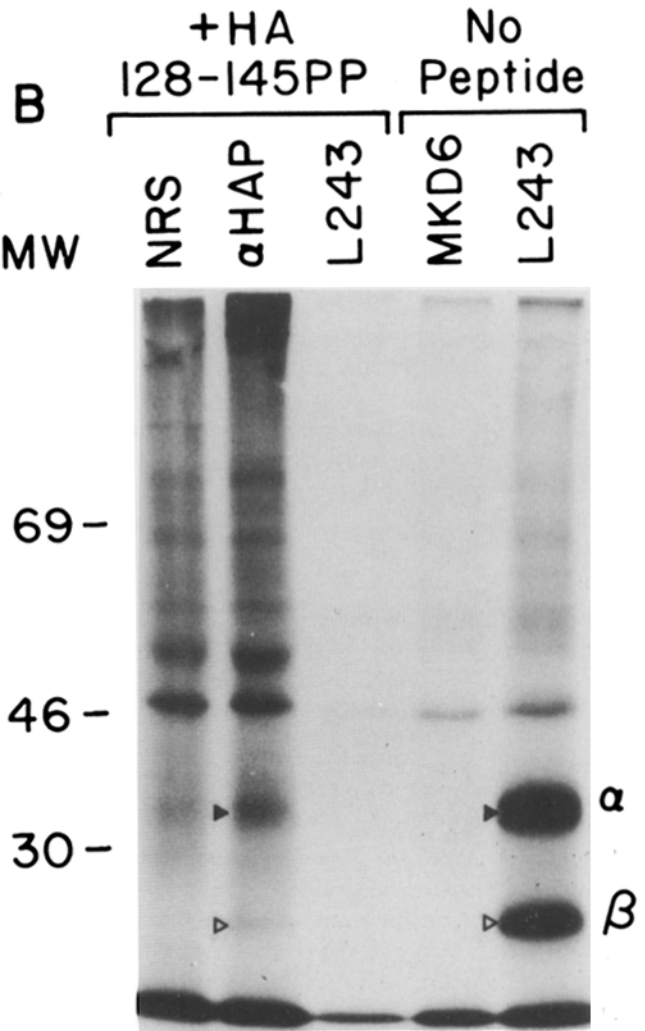
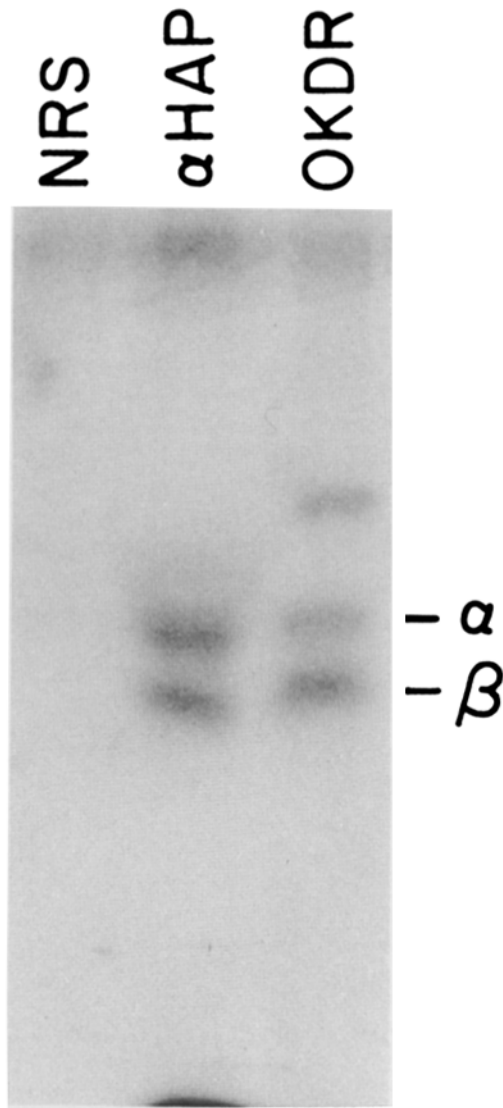
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Figure 5. (A) SDS-PAGE gel autoradiograph of immunoprecipitates of Swei whole cell lysates pulsed with ^{125}I -labeled HA 128-145 PP (photoprobe peptide), and UV-exposed before immunoprecipitation with normal rabbit serum (NRS), immune rabbit anti-hemagglutinin peptide antiserum (αHAP), or anti-DR mAb OKDR and protein A-Sepharose. Bands corresponding to the α and β chain proteins of DR class II are indicated. (B) SDS-PAGE autoradiograph of immunoprecipitates of whole cell lysates from ^3H -leucine labelled Swei cells precipitated with NRS or αHAP followed by protein A-Sepharose after HA 128-145 PP incubation and UV exposure (+HA 128-145 PP), or with MKD6 or L243 without HAP incubation (no peptide). The positions of the indicated mol wt markers are shown on the left. The closed arrow heads mark the α chain protein bands of class II, and the open arrow heads the β chain protein bands. (C and D) HPLC of tryptic digests from bands in Fig. 5 B corresponding to α and β chain class II proteins, respectively, seen on SDS-PAGE of L243 and αHAP immunoprecipitates.

The Surface Expression of HA 128-145 on Antigen-Presenting Cells is DRw11-Restricted. Homozygous BLCLs bearing DR1, DR2, DR3, or DR4, and one heterozygous (DR2,w11) BLCL (TBLCL) were incubated sequentially with non-photoprobe HA 128-145, with α HAP, and with FITC-goat anti-rabbit antiserum. The marked fluorescence shift observed with Swei cells (Fig. 4 A, panel c) was not seen with SJO, nor with any of the other homozygous HLA class II-expressing lines studied (Fig. 4 A, panels e-h), indicating allelic specificity in the formation of the DRw11-HA 128-145 complex. The DR2,w11-positive TBLCL showed approximately half the fluorescence obtained with Swei cells when both were stained with α HAP (Fig. 4 A, panels d and c) and with the anti-DRw11 mAb SFR3-DR5 for DR expression (Fig. 4 B, and Brown, L.R., N. Nygard, M.B. Graham, C. Bono, V.L. Braciale, J. Gorke, B.D. Schwartz, and T.J. Braciale, manuscript submitted for publication). This result indicates that α HAP detected quantitative differences of DRw11/HA 128-145 complexes commensurate with the level of DRw11 surface expression. The DRw11 dependence for detection of HA 128-145 was further demonstrated by the binding of α HAP to DRw11-bearing L cell transfectants which had been incubated with HA 128-145, but by the absence of α HAP binding to the parental L cells which had been similarly exposed (data not shown).

α HAP Immunoprecipitates DRw11-HA Complexes from DRw11 Cells Incubated with HA 128-145 PP. The previous results strongly suggested that α HAP was detecting HA 128-145/DRw11 complexes on the surface of Swei cells. To further demonstrate this association an immunoprecipitation experiment was performed. Swei cells were incubated with 125 I-labeled HA 128-145 PP for 24 h at 37°C. The cells were washed, exposed to UV light to form covalent class II-photoprobe linkages, and were then detergent-lysed. HA 128-145 PP-conjugated molecular complexes were immunoprecipitated from whole cell lysates with α HAP or anti-DR mAb OKDR and Sepharose-linked protein A, and were then analyzed on SDS-PAGE under reducing conditions (Fig. 5 A). Although we had initially expected HA 128-145 PP to be associated with a number of cell components, autoradiographs demonstrated only two bands at \sim 36 kD and 28 kD, corresponding to the expected approximate sizes of the \sim 2.3 kD HA 128-145 photoprobe peptide bound to DRw11 α (\sim 34kD) and β (\sim 26 kD) chains. The OKDR mAb immunoprecipitated two bands of identical size from the same Swei lysates. The absence of any other bands immunoprecipitated by α HAP from the DRw11-bearing cell lysate argues against the existence of any long-lived cell surface complexes formed between HA 128-145 and non-class II molecules.

To further confirm the identity of these bands as DRw11 α and β chains, 3 H-leucine labeled Swei cells were incubated in the presence or absence of HA 128-145 PP and were then UV-light exposed. After cell lysis the 3 H-leucine labeled proteins photoconjugated to HA 128-145 PP were immunoprecipitated by α HAP from lysates of the cells incubated with HA 128-145 PP, and 3 H-leucine labeled α and β chains of class II were immunoprecipitated by anti-DR mAb L243 from lysates of the cells not incubated with HA 128-145 PP. After

SDS-PAGE (Fig. 5 B), the corresponding bands (arrow heads) were excised, eluted, and subjected to trypsin digestion. Comparative tryptic peptide analysis indicated that the HA 128-145 PP-conjugated proteins were indeed the DRw11 α and β chains (Fig. 5 C and D).

DRw11-Binding of HA 128-145 Inhibits L243 Binding. FACS analysis of Swei cells incubated in the absence or presence of HA 128-145 before staining with FITC-conjugated L243 indicated that preincubation with HA 128-145 inhibited recognition of DRw11 by L243 and then protein A-Sepharose (Fig. 6 A). This finding was confirmed by immunoprecipitation. When the lysate of Swei cells photoconjugated with 125 I-labeled HA 128-145 PP was reacted with L243, (Fig. 6 B) no bands were seen, despite the presence of bands with

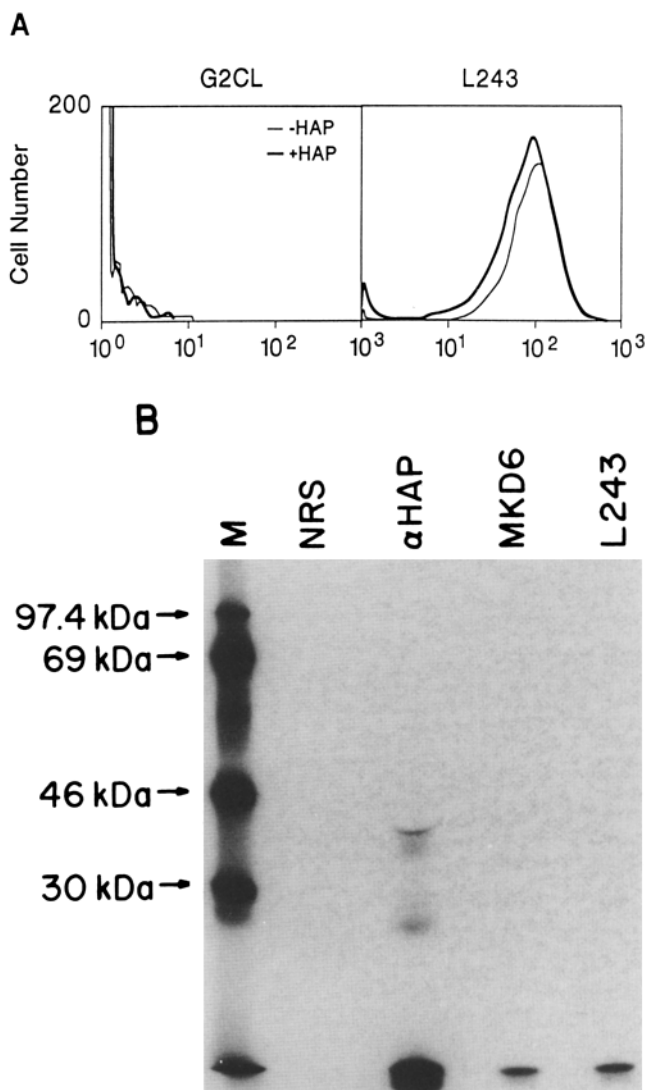


Figure 6. (A) FACS analysis of Swei cells with (+HAP) and without (-HAP) preincubation in HA 128-145 stained with control mAb G2CL or with α DR mAb L243. (B) Autoradiogram of SDS-PAGE analysis of whole cell lysates from Swei cells photoconjugated with 125 I-HA 128-145 photoprobe and immunoprecipitated with NRS, α HAP, anti-murine I-A^d mAb MKD6, and anti-HLA-DR mAb L243 followed by protein A-Sepharose. Mol wt markers are in the first lane.

α HAP (Fig. 6 B), and with OKDR (Fig. 5 A). These results indicated that the epitope recognized by L243 is either located near the DRw11 antigen-binding site or is conformationally altered when HA 128-145 is bound.

The observation that α HAP was capable of directly recognizing HA 128-145/DRw11 complexes on the surface of living APC's indicated that α HAP presented the most direct means to date of studying antigen-class II interaction in a human T cell/antigen/APC system and allowed functional studies of MHC-peptide interaction with viable APCs.

Kinetics of Association of HA 128-145 with HLA-DRw11. Swei cells were incubated with HA 128-145 for increasing time periods, then stained with FITC-goat anti-rabbit IgG, and analyzed by FACS. HA 128-145/DRw11 complexes were detectable within 30 min and increased for approximately 20–24 h with a 50% saturation time of 5 1/2 h (Fig. 7). No further increase was seen at 48 h.

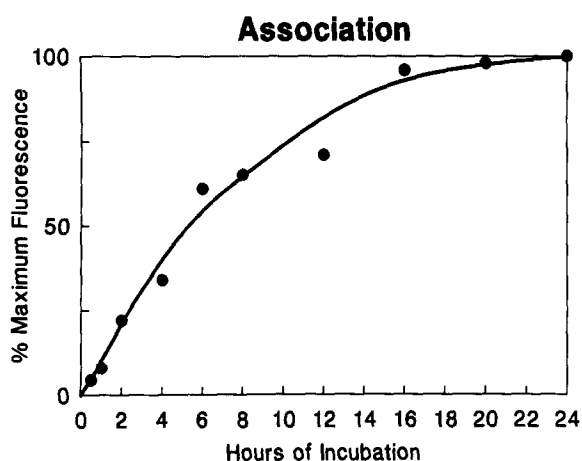


Figure 7. Association plot of the percent of maximum increase in mean fluorescence above background occurring on Swei cells pulsed with HA 128-145 for progressively longer time periods prior to α HAP staining and FACS analysis.

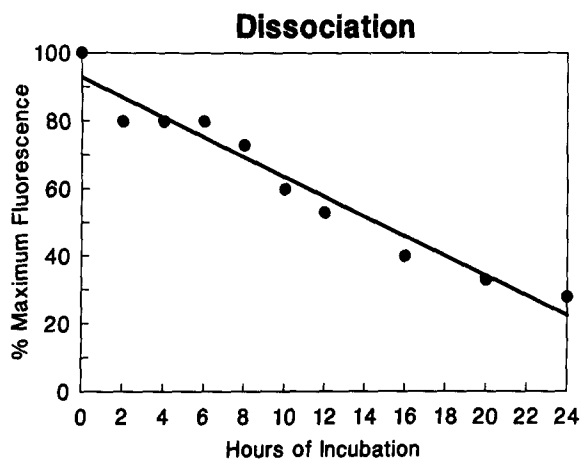


Figure 8. Dissociation plot of the decay in percent mean fluorescence increase above background when Swei cells are pulsed with HA 124-145 for 24 h, washed, and then incubated for progressively longer time periods without HA peptide prior to α HAP staining and FACS analysis.

Kinetics of Disappearance of HA 128-145/DRw11 Complexes. Swei cells were incubated with HA 128-145 for 24 h, placed in HA peptide-free media for increasing time periods, and then assayed by FACS for cell surface HA peptide. A prolonged half-life of approximately 14 h was seen for the surface HA 128-145/DRw11 complexes (Fig. 8), indicating that the complexes are relatively long-lived.

Discussion

The immunogenic complex composed of the hemagglutinin peptide HA 128-145 and HLA-DRw11 is recognized by the cytotoxic CD4⁺ T cell clone V1. The experiments presented here provide direct biochemical evidence that the same cell surface immunogenic complex can be detected by an antiserum produced against the peptide. This antiserum thus provides the first direct means of studying MHC class II-peptide complexes in which the peptide has not been modified by the addition of any non-amino acid moiety. The absence of any such non-amino acid moiety on the peptide eliminates the possibilities that the affinity of the peptide for class II or the recognition of the complex by a T cell will in any way be altered.

Antibody recognition of cell surface complexes of HA 128-145/DRw11 complexes was validated by demonstrating antibody binding to DRw11 positive B-LCLs exposed to HA 128-145, but not to DRw11 negative cells similarly exposed. Antibody also bound to DRw11 positive L cell transfectants incubated with HA 128-145, but not to parental untransfected L cells nor to L cells bearing a different class II molecule that were similarly incubated with peptide. In addition, the number of complexes detected was always commensurate with the level of DR expression.

The complex could also be detected in lysates of cells both when the peptide was unmodified, and after the DRw11 α and β chain peptide interactions had been stabilized by photoconjugation with a photoreactive moiety attached to the peptide. The identity of the DRw11 molecule bound to the peptide under these conditions was confirmed through immunoprecipitation of the complexes by both α HAP and the anti-DR mAb OKDR, by mobility on SDS-polyacrylamide gels, and by tryptic peptide mapping.

Our ability to detect unmodified peptide-class II complexes on the cell surface with anti-peptide antibody successfully for the first time most likely depends on at least three parameters which are optimized in our system. First, Swei cells have between 5×10^5 and 10^6 DRw11 surface molecules/cell (J. Pollack, Washington University, St. Louis, MO, unpublished observations), presenting a large number of potential binding sites for the HA peptide. Second, dose-response curves suggest a high affinity between HA 128-145 and DRw11. Third, an epitope on HA 128-145 recognized by the α HAP antiserum remains available to the antiserum when the peptide is bound in the DRw11 antigen-binding cleft. It is possible that in other systems these conditions were not coincident.

The ability of α HAP to recognize the HLA-DRw11/HA 128-145 complex without the addition of non-amino acid

moieties to the peptide allows delineation of the kinetics of formation and disappearance of complexes without concerns that the kinetics may be affected by the presence of a foreign element. In addition, because detection does not depend on the presence of a foreign element, the antiserum detection system for the first time presents the potential for following the kinetics of complex formation during intracellular uptake and antigen processing.

Initial kinetic characterization has been accomplished using the HA 128-145 peptide and detection of cell surface complexes by FACS. The association curve of HA 128-145/DRw11 complexes (Fig. 7) shows an appearance of the complex by 30 min that is consistent with previous reports (34, 35). The continued complex accumulation to 24 h is longer than that previously reported by Ceppellini et al. (34) for living APC's and is consistent with the report of Busch et al. (12, 22), who showed a lack of surface peptide-class II saturation after 16 h of APC antigen incubation using a biotinylated HA 307-319 peptide on DR1-expressing cells. Studies of peptide saturation using solubilized, purified class II protein have also demonstrated a slow accumulation of the complex peaking at extended intervals of from 6 to 50 h (7, 8, 34). The very rapid plateau in binding of radiolabeled peptide on whole B-LCL's reported by Ceppellini et al. (34) may be a reflection of cell peptide internalization with the inability of the radiolabel assay to separate surface from internal peptide uptake.

The dissociation plot of Fig. 8 indicates that the cell surface complex of HA 128-145 and DRw11 class II is very stable. The stability of these complexes has been shown previously with purified class II molecules (5, 8, 12, 22, 36), but the half-life of these complexes on intact cells has not been as well characterized (12, 13, 22, 34, 36).

The precise epitope(s) recognized by the α HAP antiserum when the HA 128-145 peptide is bound in the DRw11 cleft has not yet been identified, and this identification may have implications for recognition of the naturally processed peptide. While the size of the naturally processed peptide bound

to a class I molecule has recently been determined (37), no similar determination has yet been made for peptides bound to class II molecules. However, preliminary studies demonstrating that the α HAP antiserum blocks V1 recognition of DRw11 positive target cells exposed to HA 128-145 (Mary Beth Graham, Washington University School of Medicine, St. Louis, MO, unpublished results) underscore the physiologic relevance of the antibody recognition, and suggest that α HAP may well recognize the naturally processed peptide.

A number of technical difficulties have prevented demonstrating whether or not α HAP can recognize naturally-processed A/Japan/57 hemagglutinin on DRw11-expressing cells using cytofluorometry. The exposed position of HA 128-145 on the A/Japan/57 virion allows α HAP to detect the intact virion. The inability to clear unprocessed virions either enzymatically or by natural turnover from the membranes of target cells pulsed with UV-treated virus has resulted in high levels of α HAP staining by the FACS assay even on non-DRw11 APC's. Intact hemagglutinin and a truncated 45 amino acid HA peptide containing the 128-145 epitope also bind nonspecifically to non-DRw11 cells and are also detected by α HAP, again resulting in high background. Distinguishing the DRw11-associated HA peptide surface staining from this high background level has not been possible. Studies are currently underway using HA peptides coupled to transferrin to circumvent these difficulties.

The ability for the first time to directly detect peptide-MHC class II surface complexes on APC's with α HA peptide antiserum allows a number of studies that previously have been difficult or impossible to perform. Indeed, it may be possible to characterize the naturally processed peptide and the intracellular compartments in which naturally processed peptide interacts with MHC class II antigens. These studies are currently underway, and should provide new opportunities for extending our knowledge of human MHC class II function.

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