Conformation of Human Leukocyte Antigen Class II Molecules

EVIDENCE FOR SUPERDIMERS AND EMPTY MOLECULES ON HUMAN ANTIGEN PRESENTING CELLS*

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Corinne Roucard, Frédéric Garban, Nuala A. Mooney, Dominique J. Charron, and Mats L. Ericson‡

From the Laboratoire d’Immunogénétique Humaine, INSERM U396, Institut Biomédical des Cordeliers, 15 rue de l’École de Médecine, 75006 Paris, France

Subpopulations of human leukocyte antigen (HLA) class II molecules were studied in antigen presenting cells. We present evidence for double dimers or “superdimers” of HLA class II molecules that were stable in an SDS solution at room temperature but dissociated when heated to 50 °C into 60-kDa αβ heterodimers. Development of an immunofluorescence assay allowed us to quantify the expression of HLA antigens as reflected by the number of bound isotype-specific monoclonal antibodies per cell. The total expression of class II (DR, DQ, and DP) augmented 6-fold after a 36-h interferon-γ (IFN-γ) treatment of freshly isolated monocytes. Next, we used a recombinant and fluoroscein-conjugated form of the class II-associated invariant chain as a quantitative probe for empty peptide-binding sites. The fraction of empty class II molecules was 0.73–2.9% in resting monocytes but was reduced to 0.12–0.5% of the total after IFN-γ treatment. The fraction of empty sites in B lymphocytes was 0.09–0.36%. The mean number of empty sites per cell were: 6.3 × 10² (monocytes), 7.2 × 10³ (IFN-γ-activated monocytes), 5.2 × 10² (B lymphocytes), and 3.6 × 10³ (Raji B cells). A minor population (4.3–7.4% of total cells), which expressed a much higher number of empty sites, was consistently present in all cell types studied.

CD4+ helper T cells recognize antigen-derived peptides bound to major histocompatibility complex (MHC) class II molecules exposed on the cell surface of antigen presenting cells (APC) such as monocytes, macrophages and B cells. In man, the MHC polypeptides are referred to as HLA (human leukocyte antigen) molecules. Two non-covalently linked transmembrane chains form the HLA class II heterodimer: an α chain of 34 kDa and a β chain of 27 kDa. During their biosynthesis, the αβ dimers are transiently associated with a third molecule, the invariant chain (Ii). The Ii chain binds to the highly polymorphic MHC class II antigens regardless of isotype and functions as a chaperone to facilitate correct folding and assembly of the α and β chains, and also serves to prevent premature peptide binding in the endoplasmic reticulum (reviewed in Refs. 1 and 2). A nonameric complex composed of three αβIi trimers is transported from the endoplasmic reticulum presumably to the Golgi stack (3), where a signal sequence localized in the Ii cytoplasmic tail directs the complex from the exocytic pathway into an acidic intracellular compartment (4–6). This organelle is believed to be specialized in antigen processing and may be distinct from conventional early and late endosomes or dense lysosomes (7–10). However, a recent study proposed that the αβIi complexes move between distinct endocytic organelles suggesting that antigen processing and peptide loading might take place in several compartments (11). A stepwise proteolytic degradation of Ii allows the MHC class II molecules to associate with antigenic peptides (12). Accessory molecules, e.g. HLA-DM, probably serve as catalysts for the peptide loading process by enhancing the dissociation rate between class II molecules and remaining proteolytic fragments of Ii, the so-called CLIP-peptides (class II associated invariant chain peptides) (13–16). The newly assembled αβ-peptide complexes are then presented at the cell surface, where they can interact with T cell receptor on CD4+ T cells. Nonetheless, the fact that Ii can be observed on the cell surface of B cells suggests that a minor fraction of the αβIi complexes reach the cell membrane via the secretory pathway (17–19). The internalization of such molecules directed by an endocytosis determinant in the cytoplasmic domain of Ii could give them access to a peptide-loading compartment (20). In addition, recycling of cell surface αβ mature class II molecules appears to provide a mechanism for peptide exchange that may be important for a subset of antigens (21–23). The formation of a stable and long-lived complex between peptide and class II antigen correlates with resistance to denaturation by SDS at moderate temperatures (24).

Crystallographic studies of the class II DR1 antigen (25) revealed that the αβ heterodimers unexpectedly associated as parallel dimers of dimers or “superdimers.” It was hypothesized that the superdimers might form before, or during, recognition by helper T cells. Subsequent immunoprecipitation experiments carried out by Schafer and Pierce (26) confirmed the existence of a 120-kDa superdimer of the class II I-Ek molecule in mouse splenocytes; their results also suggested that the superdimers exist naturally on APC even in the absence of stimulator T cells. When exposed to sodium dodecyl sulfate (SDS), the superdimer remained stable at room temperature but dissociated into 60-kDa dimers at 50 °C. Further, a monoclonal antibody (mAb) preferentially recognizing the 120-kDa molecule was capable of blocking low affinity T cell responses, which supports the idea that the superdimer functions as an antigen presenting molecule (26). The concept of a
Superdimers and Empty HLA Class II Molecules

MHC class II superdimers are appealing since, at least theoretically, the superdimers could enhance the affinity for the T cell receptor complex and activate intracellular signaling pathways in both the antigen presenting cell and T cell (25). In addition to SDS-stable dimers and superdimers, several studies on B cells have suggested the presence of MHC class II molecules on the cell membrane that are not stably associated with peptide. It has remained unclear whether these data should be interpreted as the presence of empty class II molecules on the cell surface of or molecules that are loosely associated with peptide (27).

The present study is focused on two subpopulations of HLA class II molecules that were detected on the surface of human monocytes and B lymphocytes. We provide evidence for the existence of superdimers of the DR isotype on human APC, and we have determined the exact number of empty class II molecules in relation to the total number of HLA class II DR, DQ, and DP molecules.

EXPERIMENTAL PROCEDURES

Cells—Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 5 mM sodium pyruvate, 100 U/ml penicillin, and 100 mg/ml streptomycin (all from Biochrom KG, Berlin, Germany). Raji is a Burkitt’s lymphoma B cell line and was obtained from the American Type Culture Collection. Peripheral blood mononuclear cells were purified by apheresis from healthy donors (Blood Bank, Hotel Dieu, Paris). Mononuclear cells were isolated by density gradient centrifugation using a lymphocyte separation medium (dextran T70/Ficoll, Eurobio, Les Ulis, France) and kept in liquid nitrogen until further use. T cells were removed by rosetting with 2-aminoethylisouronium bromide-treated sheep erythrocytes followed by a Ficoll gradient centrifugation. B cells were isolated with magnetic beads coated with anti-CD19 mAb (Dynabeads, Dynal, Oslo, Norway) and recovered with DETACHabead (Dynal) according to the manufacturer’s instructions, yielding a 99% purity of CD19+ population as determined by cytofluorometry (data not shown). FACSscan analysis of monocytess was carried out after placing a live gate around the CD14+ population on the forward-scatter versus side-scatter dot-plot. For metabolic and cell surface labeling, monocytess were purified by depleting the mononuclear cells with an excess of Dynabeads coated with antiCD19 and anti-CD2 anti-mAbs. Activated monocytess were obtained by adherence on plastic of fresh mononuclear cells (after T cell depletion) in complete Dulbecco’s modified Eagle’s medium at 37°C during 1 h. Adherent cells were cultured in complete Dulbecco’s modified Eagle’s medium with 500 units/ml IFN-γ. After 36 h, cells were detached with cold PBS. Purification and analysis were performed as for fresh monocytess.

FITC Labeling—Three mAbs, D1.12 (anti-HLA-DR) (28), L2 (anti-HLA-DQ) (29), and B7.21 (anti-HLA- DP) (30), were purified from ascites liquid with a mAb Trap column (Pharmacia Biotech Inc., Uppsala, Sweden) and the concentration adjusted to 1.0 mg/ml. FITC was conjugated with anti-mAb (Becton Dickinson, Mountain View, CA) or anti-CD19 mAb (Immunotech, France) for 25 min at 4°C, which was followed by a second mAb: an anti-mouse IgG(ab) 2 fragment-FITC (Boehringer Mannheim, Germany). After two washes in cold PBS, 0.02% NaN₃, 10,000 cells were analyzed on a FACSscan (Becton Dickinson) using propidium iodide to exclude dead cells. A fluoresceinated trypsin inhibitor with a molecular weight similar to that of Ii sol-FITC was used as a negative control for Ii sol binding. To accurately quantify cell-associated fluorescence, the FACSscan instrument was calibrated before each experiment to distribute fluorescence over a 5-decade logarithmic scale. The mean fluorescence intensity was recorded for each experiment. The fluorescence intensity was calibrated with three fluorescence quantitation kits (Quantum™ 24p, 25p, and 26p, Flow Cytometry Standards Europe, Leiden, the Netherlands); each kit consists of five different microbead preparations coated with known amounts of MESF (molecules of equivalent soluble fluorochrome). A standard curve was constructed by plotting the mean intensity as a function of MESF. The curve permitted us to calculate MESF values for a given cell after labeling with mAb-FITC or Ii sol-FITC. The mean number of molecules bound per cell was calculated from the equation,

\[
\text{MESF} = \text{MESF}^* \times \frac{\text{eF/P}}{\text{eF/P}^*}
\]

where MESF = MESF per cell for specific binding protein, MESF* = MESF per cell for control (trypsin inhibitor-FITC or anti-mouse IgG(ab) 2 fragment-FITC). A modified purification scheme was used in this study. Briefly, 1 liter of SOB medium with 100 μg/ml ampicillin was inoculated with 10 ml of Ii sol-TOP 10 overnight culture and grown at 37°C (225 rpm) to an OD₆₀₀ of 0.3–0.4. Then isopropyl-α-D-thiogalactopyranoside was added to a final concentration of 1 mM, and incubation was allowed to proceed during 24 h at 32°C. The bacteria were harvested, cell pellets were resuspended in 150 mM Tris-HCl, pH 7.9, 6 mM urea, 150 mM NaCl, freeze-thawed three times, and sonicated three times for 20 s each. The lysate was cleared by centrifugation at 65,000 × g at 20°C for 20 min. The supernatant was separated on a 10-M column buffered at 0.5 M imidazole with Flow (Pharmacia Biotech Inc.) precharged with Ni²⁺ and equilibrated with binding buffer (20 mM Tris-HCl, pH 7.9, 5 mM NaCl, 500 mM NaCl, 0.5 mM imidazole). The column was sequentially washed with 100 ml of binding buffer and 60 ml of wash buffer (20 mM Tris-HCl, pH 7.9, 5 mM NaCl, 500 mM NaCl, 20 mM imidazole). Bound Ii sol was eluted with a step gradient of imidazole (10 mM, 150 mM, 177 mM, 201 mM, and 1 M). Fractions of 10 ml were collected and then analyzed by SDS-polyacrylamide gel electrophoresis. Ii sol eluted primarily in the 154 mM and 177 mM fractions, which were pooled. The Ii sol pool was first dialyzed against 3 mM urea and then renatured by adding slowly 50 mM Tris-HCl, pH 7.9 (1 ml/min) until the final concentration of 0.75 mM urea was reached. The renatured samples were finally dialyzed against PBS, pH 7.4 and stored at 4°C until further use. The renatured sample was used in the fluorescence analysis. For biophysical characterization, a flow cytometry instrument was calibrated before each experiment to distribute fluorescence over a 5-decade logarithmic scale. The mean fluorescence intensity was recorded for each experiment. The fluorescence intensity was calibrated with three fluorescence quantitation kits (Quantum™ 24p, 25p, and 26p, Flow Cytometry Standards Europe, Leiden, the Netherlands); each kit consists of five different microbead preparations coated with known amounts of MESF (molecules of equivalent soluble fluorochrome). A standard curve was constructed by plotting the mean intensity as a function of MESF. The curve permitted us to calculate MESF values for a given cell after labeling with mAb-FITC or Ii sol-FITC. The mean number of molecules bound per cell was calculated from the equation,

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where MESF = MESF per cell for specific binding protein, MESF* = MESF per cell for control (trypsin inhibitor-FITC or anti-mouse IgG(ab) 2 fragment-FITC). Production of Soluble—I An Escherichia coli TOP 10 strain containing an Ii sol-pTRCHisC expression plasmid has been described (31). A modified purification scheme was used in this study. Briefly, 1 liter of SOB medium with 100 μg/ml ampicillin was inoculated with 10 ml of Ii sol-TOP 10 overnight culture and grown at 37°C (225 rpm) to an OD₆₀₀ of 0.3–0.4. Then isopropyl-α-D-thiogalactopyranoside was added to a final concentration of 1 mM, and incubation was allowed to proceed during 24 h at 32°C. The bacteria were harvested, cell pellets were resuspended in 150 mM Tris-HCl, pH 7.9, 6 mM urea, 150 mM NaCl, freeze-thawed three times, and sonicated three times for 20 s each. The lysate was cleared by centrifugation at 65,000 × g at 20°C for 20 min, and the supernatant was separated on a 10-M column buffered at 0.5 M imidazole with Flow (Pharmacia Biotech Inc.) precharged with Ni²⁺ and equilibrated with binding buffer (20 mM Tris-HCl, pH 7.9, 5 mM NaCl, 500 mM NaCl, 0.5 mM imidazole). The column was sequentially washed with 100 ml of binding buffer and 60 ml of wash buffer (20 mM Tris-HCl, pH 7.9, 5 mM NaCl, 500 mM NaCl, 20 mM imidazole). Bound Ii sol was eluted with a step gradient of imidazole (10 mM, 150 mM, 177 mM, 201 mM, and 1 M). Fractions of 10 ml were collected and then analyzed by SDS-polyacrylamide gel electrophoresis. Ii sol eluted primarily in the 154 mM and 177 mM fractions, which were pooled. The Ii sol pool was first dialyzed against 3 mM urea and then renatured by adding slowly 50 mM Tris-HCl, pH 7.9 (1 ml/min) until the final concentration of 0.75 mM urea was reached. The renatured samples were finally dialyzed against PBS, pH 7.4 and stored at 4°C until further use. For biophysical characterization, a flow cytometry instrument was calibrated before each experiment to distribute fluorescence over a 5-decade logarithmic scale. The mean fluorescence intensity was recorded for each experiment. The fluorescence intensity was calibrated with three fluorescence quantitation kits (Quantum™ 24p, 25p, and 26p, Flow Cytometry Standards Europe, Leiden, the Netherlands); each kit consists of five different microbead preparations coated with known amounts of MESF (molecules of equivalent soluble fluorochrome). A standard curve was constructed by plotting the mean intensity as a function of MESF. The curve permitted us to calculate MESF values for a given cell after labeling with mAb-FITC or Ii sol-FITC. The mean number of molecules bound per cell was calculated from the equation,

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\text{MESF} = \text{MESF}^* \times \frac{\text{eF/P}}{\text{eF/P}^*}
\]
**RESULTS**

Presence of Two Distinct SDS-stable Forms of Class II on Raji Cells—The binding of peptide to the αβ heterodimer leads to the formation of stable dimers. Stabilization of the dimers can be monitored as an increased resistance to denaturation in the detergent SDS at room temperature (27, 33). In the SDS-stability assay, the 60-kDa αβ dimers migrate with an apparent molecular mass of 63 kDa in human (33) and 52–56 kDa for compact heterodimers in mouse (27). The variations in estimated size are likely to be due to slightly erratic migration of non-denatured samples in SDS-polyacrylamide gel electrophoresis. Class II dimers that are not associated with peptide, "empty" molecules, dissociate into free α and β chains under these conditions (27, 34). All SDS-unstable molecules are not necessarily empty, however, since they may be loosely associated with peptide in a way that does not efficiently stabilize the structure (27, 35). In addition, empty class II molecules form large aggregates during isolation (36–39). A Burkitt's lymphoma B cell line, Raji, was biosynthetically labeled, and HLA class II molecules were immunoprecipitated. After labeling, the cells were chased for 2 h to allow maturation of the class II molecules (40). The immunoprecipitates were analyzed by gel electrophoresis after exposure to SDS at various temperatures. The 2.06 mAb recognizes a conserved epitope present in the β chain of all three HLA isoforms but binds preferentially to the DRβ chain (32). This mAb as expected precipitates αβ dimers which are stable in SDS at room temperature but also immuno-precipitates αβIi complexes (Fig. 1A). The stable dimers dissociate on heating at 50 °C. Interestingly, when the immunoprecipitations were performed using another mAb (D1.12) that is believed to preferentially recognize mature and peptide-loaded HLA-DR αβ dimers (28, 41), a higher molecular form was detected (Fig. 1A). This form migrated with an apparent molecular mass of 120–150 kDa. We suggest that this form corresponds to the 120-kDa double dimers, or superdimers, described previously in mouse (26). The superdimers were stable at 37 °C (data not shown) but dissociated into αβ dimers when heated to 50 °C, which in turn dissociated into free α and β chains when boiled (Fig. 1A). This proves that the 120-kDa species represents class II superdimers and does not arise by association of the class II molecules with other intracellular proteins. The presence of superdimers on Raji cells was con-
confirmed using another antibody with a similar specificity, L243 (41) (not shown). Although SDS-stable αβ dimers could be readily detected in immunoprecipitates of DP molecules using the mAb B7.21 (30), we were unable to detect superdimers of this HLA isoform (data not shown). Attempts to precipitate DQ molecules were unsuccessful, possibly because of the low expression level of this isotype (see below).

A pulse-chase study was performed on biosynthetically labeled Raji cells. The monoclonal antibody D1.12 preferentially recognizes mature class II molecules, and so the gradual increase in band intensity with time reflects the maturation process. A slight reduction of the mobility of the HLA-DR α and β chains after 30 min of chase results from the maturation of the N-linked oligosaccharides and addition of sialic acids, which corresponds to the passage through the medial Golgi compartment (Fig. 1B, lane 4) (42). The maturation of the oligosaccharide side chains can also be monitored as an increased resistance to endoglycosidase H digestion. In a parallel experiment, the DR β chain became fully resistant to endoglycosidase H digestion after 1.5 h of chase (data not shown). The HLA-DR superdimers first appear after 1 h of chase (Fig. 1B, lane 7), which coincides with the first appearance of the p22 proteolytic fragment of the Ii chain (lane 7). These data are compatible with a formation of the DR superdimers in a post-Golgi compartment, similar or perhaps identical to the endosomal compartment where the SDS-stable 60-kDa dimers are formed (43).

Presence of Both SDS-stable Dimers and Superdimers on Human B Cells and Monocytes from Peripheral Blood—In order to study whether superdimers of class II DR molecules are also present on primary APC, human monocytes and B cells were isolated from peripheral blood and biosynthetically labeled. In preliminary studies, we had noticed that the maturation rate of class II molecules in these freshly isolated cells is considerably slower than in Raji cells and a 4-h chase was necessary to obtain fully glycosylated class II molecules devoid of Ii chain (data not shown). The SDS-stability patterns of class II molecules after immunoprecipitation with 2.06 and D1.12 were very similar to that of Raji cells (Fig. 2), demonstrating that (αβ)2 dimers are not cell type-specific. The results show a typical pattern of four independent experiments. We also observed that monocytes, but not B lymphocytes, expressed α and β chains migrating as doublet bands (Fig. 2). We do not present here an explanation for this phenomenon. IFNγ is an activator of HLA class II expression on monocytes (44, 45), and IFNγ-stimulated monocytes were therefore examined to investigate whether or not stimulation with IFNγ influences the proportion of SDS-stable versus SDS-unstable class II molecules. However, despite a general up-regulation of class II DR molecules after stimulation with IFNγ, the overall SDS-stability pattern remained unchanged (data not shown).

In order to detect surface class II molecule superdimers, we performed cell surface labeling with a [35S]sulfur labeling reagent followed by immunoprecipitation as before. As can be seen in Fig. 3, superdimers could easily be detected at the cell surface of both monocytes and B lymphocytes. The possibility that the superdimers resulted from some trivial artifact, e.g., co-migration of 60-kDa dimers with undissociated antibody was excluded after a Western blot analysis of whole cell lysates. Both the 60-kDa and 120-kDa species were detected in non-heated SDS-extracts using the DA6–147 anti-HLA-DR α antibody as a probe. A densitometric analysis of a typical Western blot revealed that the superdimers represent approximately 15% of the total amount of SDS-stable HLA-DR class II molecules in monocytes (data not shown).

Quantification of HLA Class II Isoforms on the Cell Surface—APC have characteristic patterns of class II antigen expression with regard to the relative amounts of the different isoforms (46), and the total amount of class II antigen depends on the activation state of the cell (46). By FACScan analysis using beads coated with standardized amounts of FITC, we have quantified the HLA class II molecules expressed on the surface of peripheral blood monocytes and B lymphocytes. The results are shown in Fig. 4 and in Table I and represent means from four separate donors. Raji B cells bound 1 × 106 anti-HLA-DR mAbs, 1 × 105 anti-HLA-DQ mAbs, and 5.4 × 105 anti-HLA-DP mAbs. Resting B lymphocytes on the other hand expressed approximately 10 times less of each of the three isoforms compared to Raji B cells (Fig. 4). The basic levels of HLA class II expression were similar on B lymphocytes and monocytes with the exception of the higher expression of HLA-DP in freshly isolated monocytes. IFNγ treatment of the monocytes considerably increased the cell surface level of class II antigens. Note that the increase in DR expression (9 ×) after IFNγ stimulation greatly exceeds that of DP (3 ×) or DQ (5 ×) (Fig. 4).

Presence of Empty HLA Class II Molecules on Fresh Monocytes—A recombinant and soluble form of human II (Ii sol) containing the CLIP region (14–16) and that binds to empty HLA class II molecules (31) has been produced. The Ii sol is inefficient in displacing bound peptide (31). Further, Ii sol does not bind to class II negative T lymphocytes (39). We decided to...
use the Ii sol labeled with FITC and cell fluorometry as a quantitative tool to probe APC for empty HLA class II molecules. Obtained fluorescence values were compared to those of a quantitative tool to probe APC for empty HLA class II molecules. We therefore denoted it is conceivable that the epitope recognized by 2.06 is inaccessible to the mAb in the superdimer form. The role of superdimers and empty HLA class II molecules on human APC. A mAb that recognizes a monomorphic region of the a chain outside the peptide binding groove (41). Accordingly, we did not observe any superdimers in immunoprecipitates of DP molecules. Another mAb (2.06) precipitated immature aβ1I complexes as well as mature, 60-kDa, aβ2 dimers and failed to precipitate 120-kDa molecule. The critical amino acid residues of the D1.12 epitope are localized on the a1 domain of the DRα chain outside the peptide binding groove (41). According to the previously described crystal structure of double dimers (25), the regions involved in dimer formation are distinct from the epitope recognized by D1.12 (41). The double dimers associated via protein surfaces localized on the β1-subunit but also via regions of a2, and a1 chains as well of dimers (aβ) and superdimers (aβ) are indicated.

**Fig. 3.** Superdimers are present at the cell surface of APC. B cells, monocytes, and Raji B cells were surface-labeled by incubation on ice in 50 μCi of a membrane-impenetrable [35S]sulfur labeling reagent, and HLA-DR molecules were then immunoprecipitated with D1.12. Samples were either incubated at room temperature (20°C) or at 50°C for 30 min or boiled (100°C) for 5 min before loading on a 10% discontinuous SDS-polyacrylamide gel and electrophoretic separation. The positions of molecular mass markers are shown to the right. The positions of free a, β, and Ii chains as well as dimers (aβ) and superdimers (aβ) are indicated.

**Fig. 4.** Quantification of HLA class II isotype expression on B cells and monocytes. The number of bound mAb molecules per cell is shown. Monocytes were activated with IFNγ as described under “Experimental Procedures.” Purified cells were incubated for 1 h with either of the following fluorescent mAbs: D1.12 (anti-HLA-DR), L2 (anti-HLA-DQ), or B7.21 (anti-HLA-DP). A goat anti-mouse-FITC conjugate was used as a negative control. Ten thousand cells were acquired on a FACScan cell cytometer, and dead cells were gated out. The instrument was calibrated to distribute fluorescence over a 5-decade logarithmic scale. Microbeads (Quantum kit) coated with calibrated amounts of FITC were analyzed after each experiment without changing the parameter settings, which permitted the calculation of the mean number of bound mAbs per cell. For monocytes and B lymphocytes, each value represents an average of four separate experiments ± S.D. For Raji cells, values are represented as means of duplicates ± S.D.

**Table I.** Multiple antigen binding sites of HLA class II molecules on various cell types

| Cell Type  | Mean Number of Molecules | Mean Number of Bound Molecules |
|------------|--------------------------|-------------------------------|
| B cells    | 1500 ± 200               | 1200 ± 120                   |
| Monocytes  | 1800 ± 220               | 1600 ± 160                   |
| Raji cells | 2000 ± 250               | 1800 ± 180                   |

**Density Binding (High).** The Low population of resting monocytes (93% of total cells) displayed 6.3 × 10^3 empty class II molecules on the cell surface (Table I). On IFNγ-activated monocytes, the number of empty sites increased only marginally despite a 6-fold increase in the total number of class II mAb binding sites (Fig. 4 and Table I). The number of empty sites on Low B lymphocytes (93% of total cells) was approximately 10 times less (5.2 × 10^2) compared to monocytes. Interestingly, the High populations of both B lymphocytes and monocytes display very high numbers of empty class II molecules (in the order of 10^3). We then estimated the size of the fraction of empty class II molecules as compared to the total number of class II molecules on a given cell type. The total numbers of HLA class II antibody binding sites for the different cell types are shown in Table I. The difficulty in directly converting the number of antibody binding sites into number of antigens resides in the fact that antibody fixation is likely to be an unpredictable combination of bivalent and monovalent binding (47). A further complication arises from the finding that class II DR molecules are represented by a mixture of dimers and superdimers on the cell surface. Thus, the minimum numbers are calculated assuming that one antibody binds one class II heterodimer. However, one antibody might, in theory, simultaneously bind two superdimers. Having taken into account these considerations, we could nonetheless estimate an upper and a lower limit of the number of class II (DR, DQ and DP) molecules on monocytes and on B cells from which the possible range of empty class II molecules for a given cell type and subpopulation could be calculated. By dividing the number of Ii sol binding sites per cell by the mean total number of class II molecules, the range of empty sites per cell could be calculated. For the High population of resting monocytes, between 36% and 100% of the HLA class II molecules are unassociated with peptide (Table I). In the Low population of the same cell type, between 0.73 and 2.9% of the cell surface molecules are empty. After activation of peripheral blood monocytes with IFNγ, the fraction of empty sites decreased. Compared to monocytes, the majority of B cells have low levels of empty sites. The mean percentage of empty sites on the total of Raji B cells never exceeds 0.21% (but can be as high as 17% for the minor High population).

**DISCUSSION**

We have studied the conformation and occupancy of HLA class II molecules on human APC. A mAb that recognizes mature DR heterodimers (28) immunoprecipitated an SDS-stable molecule with an apparent molecular mass of 120 kDa. This protein, which dissociated at 50°C in the presence of SDS into 60-kDa heterodimers, could therefore correspond to the 120-kDa superdimer of the mouse I-Ek molecule that was recently found in splenocytes (26). The mouse I-E^k molecule has extensive sequence homology to the DR molecule (48). In contrast, we did not observe any superdimers in immunoprecipitates of DP molecules. Another mAb (2.06) precipitated immature aβ1I complexes as well as mature, 60-kDa, aβ2 dimers and failed to precipitate 120-kDa molecule. The critical amino acid residues of the D1.12 epitope are localized on the a1 domain of the DRα chain outside the peptide binding groove (41). According to the previously described crystal structure of double dimers (25), the regions involved in dimer formation are distinct from the epitope recognized by D1.12 (41). The double dimers associated via protein surfaces localized on the β1-subunit but also via regions of a2, and a1 chains as well of dimers (aβ) and superdimers (aβ) are indicated.
evidence that they generate low-affinity T cell responses (26). The CD4 glycoprotein is present on a subset of T cells and binds simultaneously to the T cell receptor and to the MHC class II molecule, thereby stabilizing their interaction. Second, it recruits the intracellular p56^lck^ tyrosine kinase to the T cell receptor complex (reviewed in Ref. 49). Only dimerized or oligomerized CD4 molecules stably bind to MHC class II molecules (50). It has been suggested that class II superdimers might facilitate the formation of co-aggregates with CD4 molecules, a step that may in turn be necessary to initiate a transmembrane signaling cascade (50, 51). The demonstration of preformed superdimers on murine splenocytes (26), on human cord blood B cells (39), and on human APC (this paper) in the absence of T cells seems to argue against the idea that dimerization is induced by helper T cells binding as suggested by Brown et al. (25). However, an equilibrium might exist between dimeric and superdimeric forms, and T cell recognition could favor the formation of the latter. However, in order to cross-link two T cell receptors, the two individual HLA dimers would probably have to present two identical peptides. How, and if, this can be achieved in an APC simultaneously presenting a mixture of peptides remains to be elucidated.

### Table I

Average number of HLA class II binding mAbs and empty sites per cell

| Cell type       | Average number of HLA class II binding mAbs | Ii sol sites (High) | Relative % of empty HLA class II molecules (High)^a | Ii sol sites (Low) | Relative % of empty HLA class II molecules (Low)^a |
|-----------------|---------------------------------------------|--------------------|----------------------------------------------------|--------------------|----------------------------------------------------|
| Monocytes       | 2.3 ± 0.7 × 10^6                           | 3.4 ± 1.4 × 10^5   | 36-100%                                            | 6.3 ± 2.8 × 10^1   | 0.73-2.9%                                            |
| Activated monocytes | 1.4 ± 0.3 × 10^6                       | 3.6 ± 0.7 × 10^5   | 6.7-27%                                            | 7.2 ± 3.3 × 10^1   | 0.12-0.5%                                            |
| B cells         | 1.6 ± 0.2 × 10^6                           | 1.4 ± 0.1 × 10^5   | 23-91%                                             | 5.2 ± 0.5 × 10^2   | 0.09-0.36%                                            |
| Raji cells      | 1.7 ± 0.3 × 10^6                           | 2.7 ± 0.1 × 10^5   | 4.2-17%                                            | 3.6 ± 0.2 × 10^3   | 0.05-0.21%                                            |

^a Depending on antibody mono- or bivalency and on proportion of dimers-superdimers (see “Results”).

^b Indicates S.D.

^c Percent gated cells of the total population.

![Graph](http://www.jbc.org/Downloaded from http://www.jbc.org/)

**Fig. 5.** Two APC subpopulations show differential binding to Ii sol-FITC. A, monocytes and B cells were incubated for 1 h with Ii sol-FITC (solid line) or trypsin inhibitor-FITC (dotted line) as negative control. Ten thousand cells were analyzed using a FACScan flow cytometer, and dead cells were excluded. The majority of the cells bound a low amount of Ii sol-FITC (L), but a minor cell population consistently bound a high amount (H). B, FL1 (green fluorescence) versus FL2 (red autofluorescence) diagram showing Ii sol-FITC Low density binding and High density binding populations on freshly isolated B lymphocytes and monocytes. The windows used for the quantitative estimations described in Table I were as shown.
A considerable portion of the αβ heterodimers on the cell surface of B cells are nonetheless SDS-unstable (27, 33). These molecules are not necessarily devoid of peptide, but they do not contain strongly bound peptide (27, 35). The percentage of SDS-unstable dimers on B cells has been estimated as approximately 70–75% for mouse AαAβ molecules and 10–20% for EαEβ molecules (27). The fraction of truly empty molecules could be considerably smaller (52). We have used a different approach to determine the number of empty HLA class II molecules on antigen presenting cells. This method is based on the use of a soluble and FITC-labeled human I chain. We have shown previously that binding of this molecule and peptide to HLA class II antigens are mutually exclusive but also, importantly, that the soluble I chain does not affect the peptide dissociation rate (31). These findings confirmed earlier reports demonstrating that immunoaffinity-purified αβIi complexes were devoid of peptides (53). Measurement of empty class II molecules using soluble Ii as a probe might constitute a more accurate means than peptide binding/antigen presentation assays. In fact, the fraction of empty class II molecules on freshly isolated peripheral B cells was as low as 0.09–0.36% of the total class II molecules and 0.05–0.21% on Raji B cells (about 500 empty sites on B cells and 3,600 on Raji cells). Compared with B cells, the frequency of empty dimers on freshly isolated monocytes was higher and represented 0.73–2.9% of the total number of HLA class II DR, DQ and DP molecules at the cell membrane. In absolute numbers, this corresponds to approximately 6,000 empty class II molecules/cell. After activation of the monocytes with IFNγ, the total expression of class II isotypes augmented by a factor of 9 (DR), 5 (DQ), and 3 (DP), respectively. In contrast, the proportion of empty class II molecules on activated monocytes diminished to about 0.5% (7,000 sites) for activated monocytes. It is known that exposure to IFNγ increases monocyte presentation of exogenous antigen (45). The increased stimulatory ability is not only due to an increase of class II expression but is also believed to depend on changes in the capacity of antigen processing (45). The decrease in the percentage of empty class II molecules compared with total number of class II molecules after stimulation with IFNγ observed here is therefore compatible with previous results. However, by closely analyzing the pattern of Ii sol binding on B lymphocytes and monocytes, we identified a minor cell population binding an exceptionally high level of Ii sol-FITC. We speculate that these cells may represent a cell-cycle intermediate. An attempt to identify and characterize the High population is currently under way. It is conceivable, where loosely associated peptides on endocytosed class II molecules are gradually replaced by peptides of higher affinity.

An interesting possibility is the use of empty cell surface class II molecules as targets for synthetic peptides for vaccination. Exogenous peptide can be directly loaded onto surface class II molecules independently of cell metabolism or of endocytosis (52, 54, 55), although binding at neutral pH seems to be inefficient for some peptide and class II isotype combinations (56). Moreover, cell surface class II molecules can capture denatured protein antigen and protect T cell epitopes from proteolytic degradation (57). The number of empty molecules on B cells (500) or monocytes (6,000) exceeds the lower limit of peptide-displaying class II molecules on B cells needed to elicit an appropriate helper T cell response, which has been estimated to be only 100–400 (58, 59). A single peptide-MHC complex can in turn sequentially engage and trigger up to 200 T cell receptor molecules (60). It would be interesting to investigate whether the empty class II molecules have access through endocytosis to a compartment offering optimal conditions for the formation of stable and long-lived peptide-class II complexes. If that were the case, empty class II molecules could in principle function both as receptors for soluble antigen and as antigen presenting molecules.
Superdimers and Empty HLA Class II Molecules

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