

Turnover of Ia–peptide complexes is facilitated in viable antigen-presenting cells: Biosynthetic turnover of Ia vs. peptide exchange

(antigen processing/major histocompatibility complex/macrophage/B lymphocyte/endocytosis)

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ABSTRACT Macrophages and B cells process antigens to produce antigenic peptides that associate with class II major histocompatibility complex molecules (e.g., Ia molecules); these Ia–peptide complexes are recognized by CD4⁺ T lymphocytes. Processing of the antigen hen egg white lysozyme was inhibited by cycloheximide in peritoneal exudate cells (PECs, largely macrophages), but not in TA3 B-lymphoma cells. The uptake and metabolism of hen egg white lysozyme was largely intact in cycloheximide-treated PECs, implicating a blockade in other steps in the formation of Ia–peptide complexes. Turnover of Ia–peptide complexes was markedly enhanced in viable antigen-presenting cells (TA3 and PEC) as compared to such complexes studied on fixed cells or in isolated preparations of Ia and peptide. In B cells the half-life of Ia–peptide complexes was much shorter than the half-life of the Ia molecules, implying turnover of Ia–peptide complexes by dissociation and peptide exchange. In PECs, the dissociation of Ia–peptide complexes was more limited; the enhanced Ia–peptide turnover in viable PECs reflected in part biosynthetic turnover of Ia molecules. Specific mechanisms may exist in TA3 cells to facilitate exchange of peptides bound to Ia, allowing recycling of Ia to present another antigenic peptide; such Ia recycling would explain the ability of these cells to process and present antigen in the absence of Ia synthesis.

CD4⁺ T lymphocytes recognize processed antigen associated with class II major histocompatibility complex (Ia) molecules. Antigen processing involves internalization, denaturation, and proteolysis of antigen, with subsequent binding of antigen-derived peptides to Ia molecules (1). The site of formation of Ia–peptide complexes remains unclear, but some evidence suggests that these complexes may form within intracellular vesicular compartments (i.e., endosomes). Ia is internalized and sequestered within endosomes (2), although the necessity of Ia endocytosis for antigen processing is unproven. B-lymphoma cells process antigen in the absence of protein synthesis (2). Thus, newly synthesized Ia is not necessary in these cells. In contrast, protein synthesis inhibitors block antigen processing by adherent peritoneal macrophages (2, 3).

In vitro studies of peptide binding to isolated Ia molecules demonstrate a K_d of $\approx 10^{-6}$ M and very slow binding and dissociation kinetics (4–6) [e.g., $t_{1/2}$ for dissociation = 30 hr; 24–48 hr is required for equilibrium binding of ovalbumin-(323–339) to I-A^d at room temperature] (5). Dissociation is enhanced by acidity close to pH 5 (similar to endosomal pH) (7) in some studies (5) but not others (see below). In addition, other unknown conditions or factors inside endosomes may serve to increase the rates of dissociation and association of Ia–peptide complexes, possibly facilitating turnover of the

peptides bound to Ia molecules (i.e., peptide exchange). Accordingly, the present data explore the rates of turnover of Ia–peptide complexes on viable cells and fixed cells in comparison to the rates of association and dissociation observed with isolated Ia and peptide. Ia–peptide turnover was strikingly facilitated in viable cells. In peritoneal exudate cells (PECs), this may be largely explained by turnover of Ia by biosynthesis and degradation. In B-lymphoma cells, however, turnover of Ia–peptide may occur by peptide exchange (with one Ia molecule, therefore, being “recycled” to present multiple peptides). Thus, B-lymphoma cells may continue to process antigen after treatment with cycloheximide by recycling pre-existing Ia to present newly generated antigenic peptides.

METHODS

Cells. The murine B-lymphoma hybridoma TA3 (H-2^{kxd}) (8), the CH-27 B lymphoma (H-2^k) (9), and the 3A9 T-cell hybridoma were maintained in RPMI 1640 medium supplemented with 10% (vol/vol) fetal or newborn calf serum and antibiotics. 3A9 cells are specific for the hen egg white lysozyme (HEL) peptide residues 46–61 [HEL-(46–61)] bound to I-A^k and secrete interleukin 2 when stimulated by this complex (10). Murine PECs were harvested from CBA/J (H-2^k) retired breeders (The Jackson Laboratory) after successive i.p. injections with *Listeria monocytogenes* and peptone (11). About 70% of the elicited PECs were Ia⁺ macrophages. PECs were allowed to adhere to culture dishes for 2 hr before washing or were maintained in suspension in Teflon vials with constant gentle agitation. Unless indicated, all incubations with cells were performed at 37°C.

Reagents. The anti-I-A^k monoclonal antibody 10.3.6.2 was affinity-purified by using protein A; HEL was from Sigma. Both proteins were iodinated by the chloramine-T method. HEL-(46–61) and related peptides were synthesized as described (6).

Antibody Binding and Antigen Uptake Assays. The level of intracellular I-A^k in PECs was estimated by the binding of ¹²⁵I-labeled 10.3.6.2, as described for TA3 cells (2), in the presence and absence of 0.02% saponin. To measure antigen uptake and metabolism, PECs were incubated with ¹²⁵I-labeled HEL (50 µg/ml) at 37°C, washed, and incubated in medium at 37°C for various periods. The culture supernatants were harvested and the cells were solubilized in 1% Triton X-100; the amount of trichloroacetic acid-soluble and -insoluble radioactivity was then determined in each cell fraction (12).

Antigen-Presentation Assays. PECs or TA3 cells were incubated with intact HEL or the peptide HEL-(46–61) for specified periods at 37°C. For peptide dissociation studies,

the cells were then washed and further incubated in medium. Next, the cells were lightly fixed in 1% paraformaldehyde for 10–15 min, washed, and incubated (2×10^5 cells per well) with 10^5 3A9 cells per well for 18 hr. The culture supernatants were harvested and incubated with CTLL cells (10) to measure antigen presentation. [^3H]Thymidine incorporation by the interleukin 2-dependent CTLL cells was used to assay antigen-stimulated interleukin 2 release by the 3A9 cells.

Immunoprecipitation Studies. PECs or TA3 cells were incubated in medium containing [^3H]leucine for 1 hr, washed, and incubated in medium for various periods. The cells were then solubilized in isotonic phosphate-buffered saline (pH 7.3; PBS) containing 1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, leupeptin (10 $\mu\text{g}/\text{ml}$), and 10 mM iodoacetamide and immunoprecipitated with 10.3.6.2 and protein A-Sepharose CL-4B (2). The samples were analyzed by SDS/PAGE under reducing conditions and autoradiography (13), and the amount of I-A was quantitated by densitometry.

Ia–Peptide Binding Assays. Isolated I-A^k was prepared and its interaction with HEL-(46–61), HEL-(52–61), and derivatives of these peptides was studied using methods described in detail elsewhere (6). Two immunogenic derivatives were utilized for these experiments, Tyr-Glu-HEL-(52–61)-amide and Tyr-HEL-(46–61). Briefly, I-A^k was immunoaffinity isolated from detergent-solubilized membrane preparations from TA3 or CH-27 cells. Radioiodinated peptides (6) were incubated with isolated I-A^k in PBS plus 20 mM octanoyl-N-methylglucamide (Mega 8) and 20 mM nonanoyl-N-methylglucamide (Mega 9) for periods as long as 48 hr. Peptide bound to I-A^k was separated from free peptide by Sephadex G-50 column chromatography. Ia–peptide complexes were incubated for various periods at room temperature in PBS plus detergent (as above) with or without 5 mM lysophosphatidylserine or in PBS plus 4 mM citrate (pH 4.5) and detergent, followed by Sephadex G-50 column chromatography.

RESULTS

Antigen Processing Is Sensitive to Cycloheximide in Macrophages but Not B-Lymphoma Cells. In previous experiments cycloheximide was found to inhibit antigen processing by adherent PECs but not TA3 B-lymphoma cells (2). Whereas both TA3 cells and PECs analyzed before adherence contained 20–40% of their complement of Ia intracellularly, PECs contained little measurable intracellular Ia after adherence. We have now examined Ia distribution and antigen processing in PECs maintained in suspension *in vitro* to test whether there was a correlation between loss of intracellular Ia and the sensitivity of antigen processing to cycloheximide. When PECs were incubated for 1–2 hr in suspension (sufficient time for adherence-associated Ia redistribution to occur) intracellular Ia showed only a small decline from 30% to 23% of the total cellular Ia. The presence of cycloheximide (10 $\mu\text{g}/\text{ml}$) during this incubation resulted in little change in Ia distribution (28% intracellular), indicating that maintenance of the intracellular pool of Ia in PECs, as in TA3 cells (2), was not dependent on protein synthesis and, therefore, this was probably not newly synthesized Ia in transit to the plasma membrane.

Despite the presence of an intracellular pool of Ia in suspension PECs, as in TA3 cells, antigen processing by PECs in suspension was inhibited by cycloheximide (Fig. 1). This inhibition was always observed with PECs, whether adherent or in suspension, but was not observed with TA3 cells, therefore, representing an inherent difference between these cell types not simply related to Ia distribution. Full inhibition of antigen processing in suspension PECs required a 2- to 3-hr preincubation with cycloheximide (10 $\mu\text{g}/\text{ml}$)

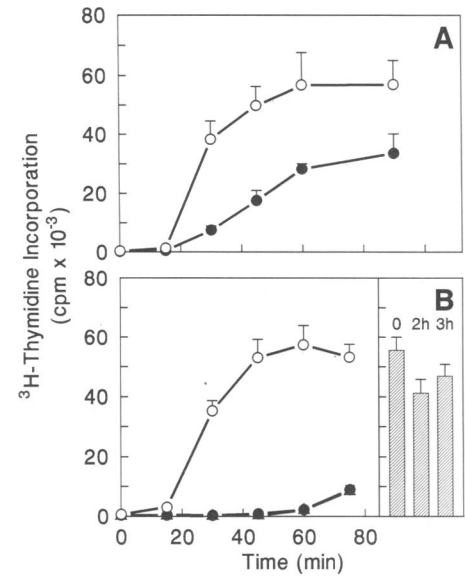


FIG. 1. Cycloheximide inhibits antigen processing by PECs in suspension. PECs were incubated in suspension at 37°C alone or with cycloheximide (10 $\mu\text{g}/\text{ml}$) for 1–3 hr. HEL (0.5 mg/ml) was then added for various periods, and the cells were finally fixed, washed, and incubated with 3A9 cells. The CTLL assay was used to quantitate the release of interleukin 2 by 3A9 cells, a measure of antigen presentation. (A) Partial inhibition was observed with a 1-hr cycloheximide treatment before addition of antigen. \circ , Control untreated cells; \bullet , cells treated with cycloheximide. (B) Complete inhibition was observed after a 2- to 3-hr treatment with cycloheximide. \circ , No cycloheximide; \bullet , 2-hr cycloheximide treatment; \blacktriangle , 3-hr cycloheximide treatment. (Inset) Response to 10 μM HEL-(46–61) added to PECs fixed after 0–3 hr of treatment with cycloheximide was not affected.

before the addition of antigen (Fig. 1), similar to results obtained with adherent PECs.

To clarify the mechanism of this effect, the influence of cycloheximide on the metabolism of HEL by PECs was investigated. After incubation with cycloheximide (10 $\mu\text{g}/\text{ml}$) for 3 hr, PECs were exposed to ^{125}I -labeled HEL for 30 min at 37°C, washed, and then incubated in medium for 1–5 hr. The amounts of acid-soluble and acid-insoluble material were determined in the medium and in cell fractions. The total HEL uptake after 30 min was decreased by only 16% in the presence of cycloheximide. The production of intracellular and extracellular trichloroacetic acid-soluble HEL metabolites declined by about 50% in the presence of cycloheximide (data not shown).

Dissociation of Ia–Peptide Complexes Is Facilitated in Viable Antigen-Presenting Cells. The half-life of Ia–peptide complexes was investigated in PECs and TA3 cells. The antigen-presenting cells were incubated for 20 min at 37°C with the peptide HEL-(46–61), which binds directly to I-A^k and does not require processing for presentation. The cells were then washed and incubated in medium for various periods and then fixed and washed. The relative level of I-A^k–HEL-(46–61) complexes remaining on the cells was then assayed using the 3A9 T-cell hybridoma specific for this Ia–peptide complex. The expression of Ia–peptide complexes decreased rapidly in viable TA3 cells ($t_{1/2} = 15$ –53 min in four experiments; Fig. 2 shows one representative experiment). In contrast, if these cells were fixed immediately after exposure to the peptide, the Ia–peptide complexes were stably expressed for at least 27 hr. In PECs, expression of Ia–peptide complexes also declined (albeit somewhat more slowly) in viable cells, with a half-life of 5.5 hr (3.4–7.5 hr), but remained stable for at least 69 hr on fixed PECs (Fig. 3). Similar results were obtained if the PECs were first fixed,

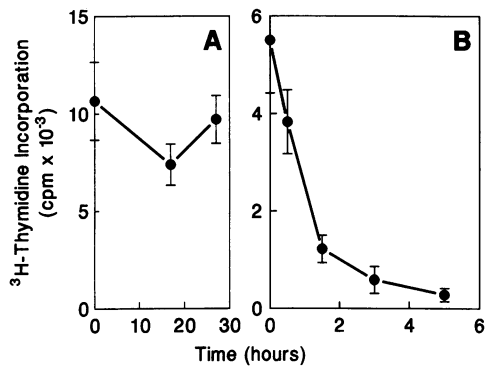


FIG. 2. Turnover of Ia-peptide complexes is markedly enhanced in viable TA3 cells. TA3 cells were incubated with 10 μ M HEL-(46-61) for 20 min at 37°C. The cells were then either fixed, washed, and incubated in medium for various periods at 37°C or simply washed and incubated, while viable, in medium for various periods before fixing and washing. The presence of I-A^k-HEL-(46-61) was assayed by the response of 3A9 cells (Fig. 1). (A) I-A^k-HEL-(46-61) complexes remained stably expressed on fixed TA3 cells. (B) I-A^k-HEL-(46-61) complexes disappeared rapidly from viable TA3 cells.

washed, then exposed to peptide, washed, and finally incubated at 37°C for various periods; under these conditions Ia-peptide complexes were stably expressed for at least 81.5 hr (Fig. 3), indicating that stability of the complexes on fixed cells was not due to cross-linking of peptide to Ia during fixation.

Contribution of Biosynthesis and Degradation of Ia to Ia-Peptide Turnover. The loss of Ia-peptide complexes could occur by degradation of the Ia and peptide molecules or by dissociation of peptide from Ia to allow association of another peptide molecule. To assess the contribution of Ia degradation to Ia-peptide turnover, we examined the half-life of Ia

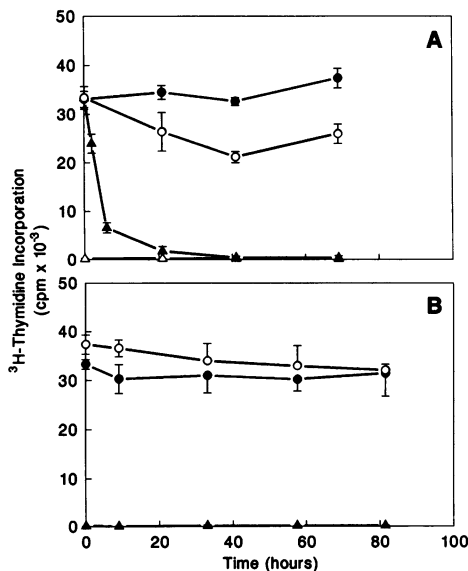


FIG. 3. Ia-peptide complexes are expressed stably on fixed PECs but disappear rapidly from viable PECs. (A) The disappearance of I-A^k-HEL-(46-61) complexes from PECs was monitored (as in Fig. 2) after a 15-min incubation with 1.5 μ M HEL-(46-61). \blacktriangle , Disappearance of I-A-peptide complexes from viable PECs; \triangle , response to PECs without peptide incubation; \circ , dissociation of I-A-peptide complexes from fixed PECs pulse-labeled with HEL-(46-61); \bullet , positive control in which 1.5 μ M HEL-(46-61) was added at each time point after fixation and washing. (B) PECs were first fixed, washed, and then incubated with 2 μ M HEL-(46-61) overnight. \circ , Cells washed and incubated for the indicated periods in medium at 37°C; \bullet , peptide present continuously; \blacktriangle , response to PECs without peptide.

molecules in PECs and TA3 cells (Fig. 4). The cells were incubated in medium containing [³H]leucine for 1 hr, washed, further incubated in medium for various periods, and solubilized. I-A^k was then immunoprecipitated and analyzed by SDS/PAGE under reducing conditions. In TA3 cells, Ia molecules exhibited a half-life of 11.3 hr; in PECs the Ia half-life was 9.3 hr. Additionally, in TA3 cells, unlike PECs, a significant proportion of the Ia exhibited an even longer half-life and remained after the longest chase (22.5 hr). In PECs, the half-life of Ia during the 19-hr incubation was unaffected by the continuous presence of recombinant interferon- γ (100 ng/ml) beginning 2 hr before the addition of [³H]leucine.

Dissociation of Isolated Ia-Peptide Complexes Is Extremely Slow. The binding and dissociation of radio-labeled peptides to detergent-solubilized, affinity-purified I-A^k was monitored by first incubating I-A^k with peptide and then separating I-A^k-peptide complexes from free peptide by Sephadex G-50 column filtration (Fig. 5) (6). Binding was inhibited by the addition of excess unlabeled peptide (6). Similar data were obtained with I-A^k isolated from both TA3 and CH-27 cells. Fractions containing the I-A^k-peptide complex were pooled and incubated at room temperature for various periods. Subsequent analysis by Sephadex G-50 column filtration showed that only 1–10% of bound peptide dissociated over 72 hr at pH 7.3 (Fig. 5). At pH 4.5 dissociation was only slightly enhanced; 10–20% of the bound peptide dissociated over a 40-hr period.

DISCUSSION

It is important, in trying to understand the biology of antigen presentation, to determine a number of properties of Ia molecules in viable cells (i.e., their availability and binding properties, cellular distribution, degree of occupancy by self or foreign peptides, turnover rates, etc.). It appears that a significant amount of Ia molecules expressed on the cell surface are already occupied by peptides, either self or foreign, and that only a fraction of them may be available for binding to new peptides. In our recent experiments 1–10% of the isolated Ia molecules could be occupied by HEL peptides

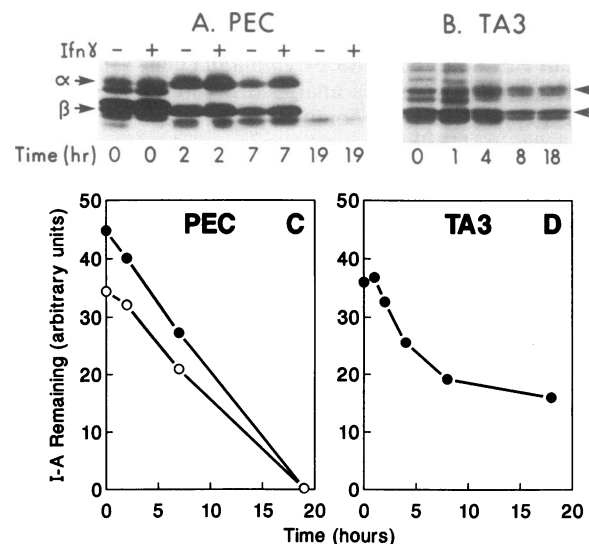


FIG. 4. Half-life of I-A^k in PECs and TA3 cells. Cells were labeled with [³H]leucine for 1 hr, washed, incubated in medium at 37°C for various periods, and solubilized. I-A^k was immunoprecipitated and analyzed by SDS/PAGE under reducing conditions and autoradiography. (A) PECs were incubated for 2 hr alone (-) or with (+) interferon- γ (Ifn γ , 100 ng/ml) before labeling. (B) TA3. [³H]leucine-labeled I-A^k was quantitated by densitometry. (C) PECs. \circ , (-) interferon γ ; \bullet , (+) interferon γ . (D) TA3 cells.

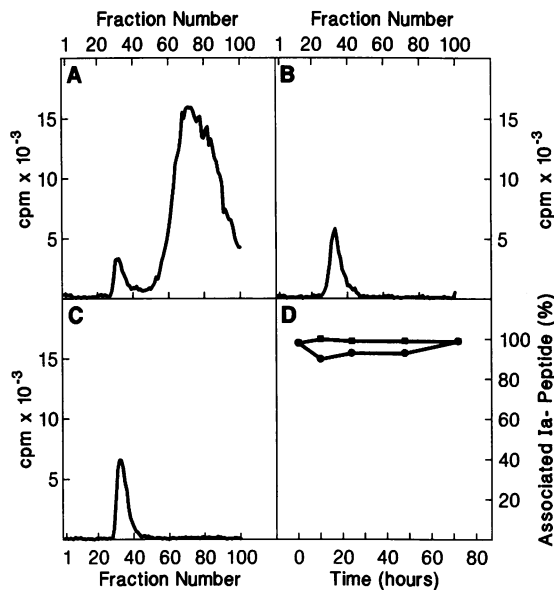


FIG. 5. Dissociation of isolated Ia-peptide complexes is extremely slow. (A) Detergent-solubilized, affinity-purified I-A^k was incubated with ¹²⁵I-labeled Tyr-Glu-HEL-(52-61)-amide, and Ia-peptide complexes (left peak) were separated from free peptide (right peak) by Sephadex G-50 column chromatography. (B) Pooled fractions containing Ia-peptide complexes were analyzed again by chromatography immediately after isolation. (C) Chromatography of Ia-peptide fractions (as in B) after incubation at room temperature for 72 hr. (D) Ia-associated peptide remaining after incubation (as in C) for various periods is expressed as the percentage of the total radioactivity eluting from the column that was contained by fractions 25-50 in the presence (●) or absence (■) of lysophosphatidylserine.

at saturation (6). The remaining may be stably occupied by other peptides or may be denatured by the isolation procedure. We have now shown that the half-life of Ia-peptide complexes measured in fixed cells or in solution is very long, and, in contrast, their half-life in viable cells is relatively short. This implies that in viable cells the turnover of the complexes must play an important role for presentation of foreign peptides.

We first found that cycloheximide inhibited antigen processing by PECs whether these cells were adherent or in suspension (i.e., whether or not a measurable pool of intracellular Ia existed). Under these conditions the uptake and metabolism of antigen remained intact albeit reduced in total amount. The extent of the decline in HEL catabolism was inadequate to explain the virtually complete abrogation of antigen processing in PECs by cycloheximide, implicating a blockade in the later steps of formation or transport of Ia-peptide complexes to the plasma membrane. Since each Ia molecule contains a single peptide binding site (4, 14), previous occupation of Ia with other peptides (15, 16) and a lack of newly synthesized Ia may limit the availability of free Ia capable of forming new Ia-peptide complexes, leading to a decrease in antigen processing. The inhibition by cycloheximide may also result from a loss of metabolic enzymes, transport proteins, or other factors necessary for antigen processing.

Ia recycling may be limited in PECs, (i.e., one Ia molecule may be used to present one or very few peptides during its life time), since the half-life of the I-A-HEL-(46-61) complex was about 5.5 hr and the half-life of Ia was only 9.3 hr. These figures would suggest that each Ia molecule is capable of presenting roughly two different peptides before degradation, and 2-3 hr of exposure to cycloheximide could exhaust the meager degree of Ia recycling present in PECs. Antigen presentation by PECs could, therefore, be relatively dependent on the synthesis of new Ia. Ia recycling could be more

prevalent in PECs than these data would suggest, however, since the incubation of the PECs with HEL-(46-61) was performed at 37°C to enhance association of Ia and peptide, and significant fluid phase uptake of HEL-(46-61) may have occurred. In this case, dissociation of I-A^k-HEL-(46-61) complexes could have been balanced by association of retained HEL-(46-61) with free I-A^k, leading to an underestimate of the dissociation rate of Ia-peptide complexes in PECs.

In contrast, TA3 cells exhibited a more rapid turnover of I-A^k-HEL-(46-61) complexes ($t_{1/2} = 15-53$ min) with an I-A^k half-life (11.3 hr) slightly longer than that in PECs. These data suggest that in TA3 cells one molecule of Ia can perform peptide exchange to present roughly 13-45 peptide molecules before degradation; this may be an underestimate, since a proportion of Ia molecules in these cells may possess an even longer half-life. Thus, the resistance of antigen processing by TA3 cells to cycloheximide may correlate with an increased rate of apparent Ia recycling in these cells.

Some limitations do exist in this analysis. Error may exist in extrapolating functional assays of the decay of peptide antigen presentation to the exact degree of loss of Ia-peptide complexes. Although rapid loss of Ia-peptide complexes has been clearly demonstrated, direct demonstration of the binding of new peptide molecules to the Ia molecule has not yet been provided. These data also address the dissociation rate of only a single species of Ia-peptide complex [I-A^k-HEL-(46-61)] and closely related analogs; the turnover rate of different peptides bound to Ia could vary, leading to somewhat different estimates of the rate of Ia recycling. However, the striking differences in Ia-peptide dissociation observed between the PECs and TA3 cells using the same methodology and T-cell hybridoma support a real difference in Ia physiology between these cell types. In addition, these observations clearly imply the existence of active processes in viable antigen-presenting cells that mediate turnover of Ia-peptide complexes, whether by Ia recycling with peptide exchange (TA3 cells), turnover of Ia molecules by biosynthesis and degradation, or a combination of both processes (PECs). Therefore, the much slower rates of association and dissociation observed with isolated Ia and peptide or fixed cells do not apply to the living cell.

The mechanisms involved in facilitating Ia-peptide turnover by peptide exchange are unknown. Peptide exchange might occur within endosomes; in this case the acidic endosomal environment could facilitate Ia-peptide dissociation and exchange, although this is not supported well by our data. Alternatively, specific proteins or other unknown factors in endosomes or elsewhere in the cell may serve to catalyze or facilitate Ia-peptide dissociation and peptide exchange.

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