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Immunology

Direct binding of a synthetic multichain polypeptide to class II major histocompatibility complex molecules on antigen-presenting cells and stimulation of a specific T-cell line require processing of the polypeptide

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ABSTRACT **T-cell activation involves the recognition of foreign antigens as a complex with self-major histocompatibility complex (MHC) proteins on the surface of antigen-presenting cells (APC). Protein antigens usually require uptake by the APC and processing that results in the generation of peptide fragments. The branched synthetic polypeptide (Tyr, Glu)-Ala--Lys was chosen as a model antigen to follow the processing requirements, leading to T-cell activation. It has been demonstrated, by using fixed APC and various inhibitors of proteases, that (Tyr,Glu)-Ala--Lys has to be processed to stimulate a (Tyr,Glu)-Ala--Lys-specific T-cell line of C3H.SW (*H-2*^{b}) origin to proliferate. To determine whether processing of (Tyr,Glu)-Ala--Lys is required to allow its association with the MHC class II molecules, biotin was covalently attached to it. Binding of the biotinylated (Tyr,Glu)-Ala--Lys to MHC class II gene products on the surface of intact normal APC was directly detected by phycoerythrin-streptavidin. The specificity of the binding was confirmed by its inhibition with anti-I-A^b antibodies as well as with excess of nonlabeled (Tyr,Glu)-Ala--Lys. Furthermore, introducing several inhibitors of proteases to the binding assay, we could substantiate that the proteolysis of (Tyr,Glu)-Ala--Lys is required to allow association of the resulting peptidyl T-cell epitopes with the MHC class II molecules themselves. The presence of the biotin moiety in the resulting peptides suggests that the T-cell epitopes of (Tyr,Glu)-Ala--Lys contain the N-terminal portion of the side chains of the branched polypeptide. An apparent K_d of 8.05×10^{-8} M was determined, and optimal binding was detected after 10 hr of incubation with the antigen. The latter phenomenon is not due to slow uptake, since uptake of (Tyr,Glu)-Ala--Lys occurs mainly during the first 30 min of incubation, but rather reflects the events of processing that precede MHC interaction.**

The random synthetic polypeptide antigen poly(LTyr,LGlu)-poly(DLAla)--poly(LLys) [a poly(Lys) backbone with side chains of poly(DLAla) tipped with a random copolymer of Tyr and Glu], abbreviated to (Tyr,Glu)-Ala--Lys, has been the subject of extensive immunological investigations which led, in combination with the use of inbred mouse strains, to the discovery of determinant-specific genetic control of the immune response (1) and to the genetic linkage of the immune response to the major histocompatibility complex (MHC) of the species immunized (2). Hence, the antibody response to (Tyr,Glu)-Ala--Lys was found to be genetically regulated by an autosomal, dominant, quantitative trait located in the *I-A* region of *H-2* in the mouse, and mice bearing the *H-2*^{b} allele are high responders to (Tyr,Glu)-Ala--Lys, whereas *H-2*^{k} mice are low responders (3).

(Tyr,Glu)-Ala--Lys-specific T-cell lines and clones were previously established in our laboratory, and they were shown to proliferate, to provide help to B cells from (Tyr, Glu)-Ala--Lys-immunized mice (4), and to produce lymphokines upon stimulation (5). Nevertheless, the process of presentation of (Tyr,Glu)-Ala--Lys by antigen-presenting cells (APC) for T-cell stimulation is yet to be assessed.

Helper T cells can recognize only antigens that are associated with self MHC proteins on the surface of APC (6). To associate with MHC class II gene products, proteins usually require uptake by the APC and processing in a chloroquine-sensitive endocytic compartment (7). The requirements for processing that determine if a protein antigen is presented in its intact form (8), in a denatured form (9–11), or after partial proteolysis and fragmentation (12, 13) are not fully understood, and they are dependent on (*i*) the structure of the antigen and the relative exposure of the T-cell epitopes for Ia interactions [hence, apamin, a disulfide-bonded 18 amino-acid molecule, requires proteolysis (14), whereas fibrinogen (340 kDa) can be presented without prior processing (8)]; (*ii*) the type of the APC, due to differences in the intracellular processing between macrophages, B cells, and dendritic cells (15, 16); and (*iii*) the fact that T cells with similar MHC specificity show differing requirements for antigen processing and display by APC (17, 18).

Detection of the various processing events was formerly based on the ability of manipulated antigens and APC to stimulate the antigen-specific T cells. However, the influence of such manipulations on the antigen·MHC complex at the surface of APC was never demonstrated directly.

We have recently developed a direct binding assay to MHC class II molecules on the surface of freshly prepared splenic adherent cells for biotinylated peptide derivatives (19). The detected peptide binding was specific in terms of epitope structure and site of interaction on the cells, and it could therefore be applied for studying how interference in processing affects the antigen presentation by the APC.

Here, we describe the requirements for processing of (Tyr,Glu)-Ala--Lys, as a model for polypeptide antigens, both at the level of T-cell stimulation and at the level of direct binding to APC, and we demonstrate that (Tyr,Glu)-Ala--Lys needs partial proteolysis to allow the resulting T-cell epitopes to associate with MHC class II molecules and to be presented to T cells.

MATERIALS AND METHODS

Mice. Mice of the inbred strain C3H.SW (The Jackson Laboratory) were used at the age of 8–12 weeks.

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Abbreviations: MHC, major histocompatibility complex; APC, antigen-presenting cells; BSA, bovine serum albumin; (Tyr,Glu)-Ala--Lys, poly(LTyr,LGlu)-poly(DLAla)--poly(LLys); B-Ag, biotinylated antigen (Tyr,Glu)-Ala--Lys; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane.

Antigen. The synthetic polypeptide (Tyr,Glu)-Ala--Lys was synthesized and characterized as previously described (20).

Monoclonal Anti-I-A Antibodies. The following monoclonal anti-I-A antibodies were utilized: Y3P (anti-I-$A^{b,s}$; ref. 21), MK-D6 (anti-I-A^{d}), and 11-5.2 (anti-I-A^{k}) (Beckton Dickinson).

Establishment of the T-Cell Line. Mice were immunized with (Tyr,Glu)-Ala--Lys in complete Freund's adjuvant (Difco). Two weeks later the T-cell line was established from lymph node cells and maintained in culture as previously described (4).

Proliferative Responses of the T-Cell Line. T cells (10^4 per well) were cultured with 0.5×10^6 irradiated (3000 rad; 1 rad = 0.01 Gy) syngeneic spleen cells in the presence of various concentrations of antigen. Cultures were set in 200 μl of enriched medium, containing 10% fetal calf serum in flat-bottom microtiter plates (Nunc). At the end of 48 hr of incubation [^{3}H]thymidine [0.5 μCi of 5 Ci/mmol (1 Ci = 37 GBq), Nuclear Research Center, Negev, Israel] was added, and 18 hr later cells were harvested onto filter paper and thymidine incorporation was measured by a β counter.

Treatment of APC. To abolish antigen processing by APC, the splenocytes were incubated for 20 min at room temperature in the presence of 1% paraformaldehyde in phosphate-buffered saline (PBS). The cells were then washed three times with a cold solution of 10% fetal calf serum in PBS, incubated for 1 hr in enriched RPMI 1640 medium with 10% fetal calf serum at 37°C, and washed twice more with RPMI 1640 medium (12, 22). Thereafter, the fixed cells were added to the microtiter plates for proliferation assays, as described above.

Inhibition of Processing. The following inhibitors of proteases: antipain, chymostatin, leupeptin, pepstatin, and *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64) (Sigma) were used in various concentrations and were present throughout the proliferation assays.

Biotinylation of (Tyr,Glu)-Ala--Lys. N-terminal biotinylation of (Tyr,Glu)-Ala--Lys was performed at 0°C with excess of biotin-*N*-hydroxysuccinimide (Sigma) in aqueous solution. Excess of unreacted biotin was removed by dialysis.

Direct Binding of (Tyr,Glu)-Ala--Lys to APC. Spleen cells (1×10^8) suspended in RPMI 1640 medium supplemented with 10% fetal calf serum were incubated in Petri dishes (100 × 20 mm, Falcon) for 1 hr at 37°C. Thereafter, nonadherent cells were removed and the plates were washed three times with RPMI 1640 medium and placed on ice. Adherent cells were collected from the plates by using a rubber policeman. Splenic adherent cells (1×10^6 per sample) were incubated with biotinylated (Tyr,Glu)-Ala--Lys in PBS containing 0.1% bovine serum albumin (BSA) at 37°C, followed by incubation with phycoerythrin-streptavidin (Biomeda, Foster City, CA) at 4°C for 30 min. After each incubation the cells were washed twice at 4°C with the above solution. Thereafter, cells were analyzed by flow cytometry using the FACScan (Beckton Dickinson). In each analysis at least 5000 cells were examined.

RESULTS

Requirements for Processing of (Tyr,Glu)-Ala--Lys for Stimulation of the T-Cell Line. A (Tyr,Glu)-Ala--Lys-specific T cell line, TPB3, was established from lymph nodes of (Tyr, Glu)-Ala--Lys-immunized C3H.SW (*H-2*^{b}) mice. (Tyr, Glu)-Ala--Lys was presented to the TPB3 line by paraformaldehyde-fixed splenic APC. Fig. 1 demonstrates that paraformaldehyde-fixed APC failed to stimulate TPB3 cells, in contrast to the efficient stimulation by both nonfixed APC and nonfixed cells that were excessively washed according to a protocol similar to that utilized for cell fixation. The requirements for processing of (Tyr,Glu)-Ala--Lys were further assessed by introducing various inhibitors of proteases to the T cell proliferation assay. Fig. 2 reveals that inhibitors of proteases blocked the antigen-specific proliferative capacity of the line. The inhibition was competitive, since increased antigen concentrations could overcome this blocking (data not shown).

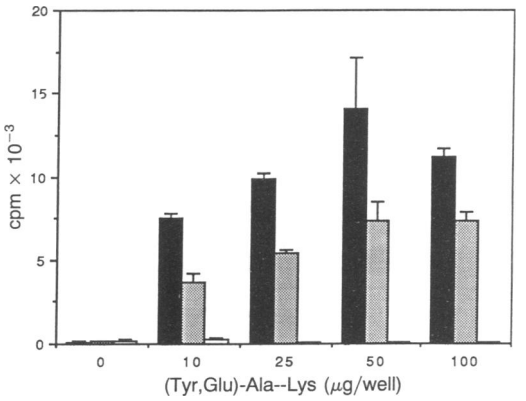

FIG. 1. Effect of fixation of APC on the presentation of (Tyr, Glu)-Ala--Lys to TPB3 T cell line. Splenic cells (0.5×10^6) were fixed with 1% paraformaldehyde and then cultured with TPB3 cells (10^4 per well) and various antigen concentrations. Empty bars, fixed cells; stippled bars, nonfixed splenocytes that were treated similarly to the fixed cells to determine the effect of the excessive washes on their ability to present antigen; solid bars, nontreated splenocytes. Results are expressed as mean cpm of triplicates ± SD.

Direct Binding of (Tyr,Glu)-Ala--Lys to Splenic APC. To get a better insight into the interactions of (Tyr,Glu)-Ala--Lys and the MHC class II proteins on membranes of APC, we directly measured the binding of (Tyr,Glu)-Ala--Lys to the surface of normal live splenic APC. To detect (Tyr,Glu)-Ala--Lys, biotin was covalently bound to its side chains (denoted B-Ag), and APC were incubated with the B-Ag, assuming that binding will be detected by means of phycoerythrin-conjugated streptavidin only if the N termini of the side chains, to which biotin is attached, are presented on the APC membranes. Fig. 3 is a histogram of fluorescence intensities after incubation of live normal C3H.SW APC for 20 hr in the presence of 5 μg (0.75 μM) of B-Ag. Sixty-five percent of the cells were stained above background, with a 74-fold increase in the mean fluorescence intensity. An anti-I-A^b antibody, but not anti-I-A^d or anti-I-A^k antibodies, inhibited this bind-

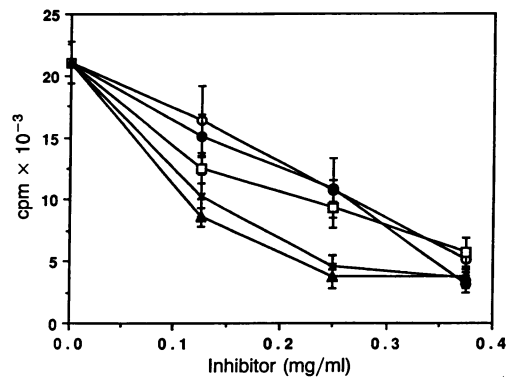

FIG. 2. Inhibition of the proliferative response of TPB3 to (Tyr,Glu)-Ala--Lys by inhibitors of proteases. TPB3 cells (10^4 per well) were incubated in the presence of C3H.SW irradiated spleen cells (0.5×10^6), (Tyr,Glu)-Ala--Lys (50 μg/ml), and the inhibitors: ○, antipain; ●, chymostatin; ×, leupeptin; □, pepstatin; and ▲, E-64. Results are expressed as mean cpm of triplicates ± SD. Baseline proliferation of the line in the absence of antigen was 177 ± 31 cpm.

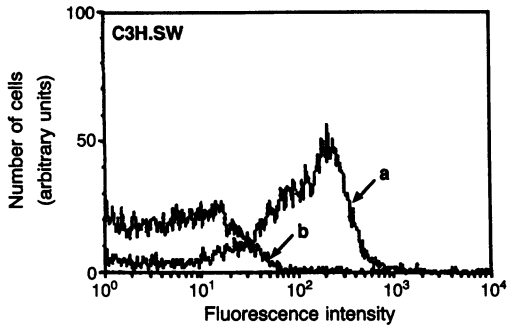

FIG. 3. Binding of B-Ag to the surface of live normal splenic adherent cells of C3H.SW (*H-2*^{b}) mice. Splenic adherent cells (10^6 in 100 μl) were incubated for 20 hr at 37°C with B-Ag (0.75 μM) (trace a) or PBS/BSA alone (trace b), and thereafter stained for 30 min on ice with phycoerythrin-streptavidin.

ing as shown in Table 1, indicating that B-Ag was genuinely bound to MHC class II proteins.

To ascertain that the native (Tyr,Glu)-Ala--Lys and the biotinylated analog bind to the same sites, we coincubated the APC with B-Ag and with an excess of the nonlabeled polypeptide. Table 1 demonstrates that a 5- and a 50-fold excess of native (Tyr,Glu)-Ala--Lys inhibited 59% and 98% of the binding of B-Ag to APC, respectively, suggesting that both analogs interact with the same binding sites.

Fig. 4 demonstrates that the binding of B-Ag to APC of C3H.SW mice is in a dose-dependent manner and achieves a maximum at 5 μg of antigen, since the extent of binding after incubation with 10 μg of B-Ag is not further elevated. The double-reciprocal plot of the equilibrium binding data allows the calculation of an apparent K_d of 8.05×10^{-8} M.

Requirements for Processing of (Tyr,Glu)-Ala--Lys for Presentation by APC. Having established that the binding of B-Ag to MHC class II gene products on intact live APC is similar to the binding of (Tyr,Glu)-Ala--Lys, we could define whether proteolysis was required prior to the interaction with the MHC proteins, by addition of the above-described inhibitors of proteases to the direct binding assay. Fig. 5 shows the disappearance of the binding signal of B-Ag to C3H.SW APC in the presence of all five inhibitors of proteases that were demonstrated in Fig. 2 to inhibit the antigen-specific stimulation of the TPB3 T-cell line. Hence, (Tyr,Glu)-Ala--Lys requires proteolytic processing to allow the interaction of the resulting T-cell epitopes with the MHC class II molecules.

Kinetics of Binding to APC. Using the need for proteolytic cleavage of (Tyr,Glu)-Ala--Lys, we attempted to follow the kinetic pattern of the appearance of the resulting T-cell epitopes·MHC class II complexes on the APC membranes. Fig. 6 illustrates the gradual time-dependent increase in percent of bound cells and in the mean fluorescence intensity after incubation with B-Ag for 0.5–20 hr. Ten hours of incubation led to almost the maximal binding in terms of

Table 1. Binding of B-Ag in the presence of various inhibitors

Inhibitor	% binding above background	% inhibition
None	49	—
Anti-I-A^b (Y3P), 0.1 μM	32	34
Anti-I-A^b (Y3P), 0.3 μM	2	96
Anti-I-A^d (MK-D6), 0.3 μM	54	0
Anti-I-A^k (11-5.2), 0.3 μM	46	6
(Tyr,Glu)-Ala--Lys, 25 μg	20	59
(Tyr,Glu)-Ala--Lys, 250 μg	1	98

B-Ag (5 μg) was incubated with the APC in the presence of various inhibitors for 20 hr. Phycoerythrin-streptavidin was added thereafter and the cells were subjected to flow cytometric analysis. Background binding in the absence of B-Ag was 13%.

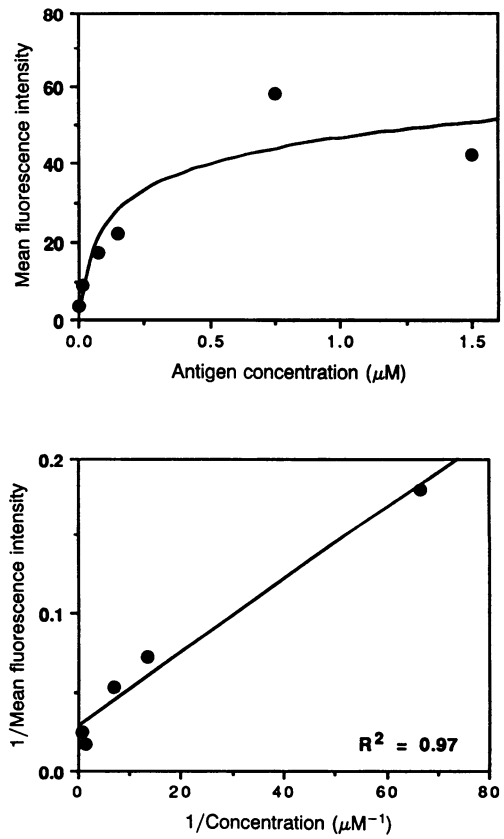

FIG. 4. Equilibrium binding of B-Ag to C3H.SW splenic adherent cells. (*Upper*) Representative binding data for various concentrations of B-Ag after 20 hr of incubation. (*Lower*) Double-reciprocal plot of the net mean fluorescence intensities of the binding signals.

bound cells (32% after 10 hr versus 38% after 20 hr), but the continued incubation resulted in a rightward shift of the histogram, which correlates to higher fluorescence intensity per cell.

Fig. 7 is the fluorescence histogram of cells that were incubated with B-Ag for 30 min, but instead of immediately being stained, they were washed and reincubated in PBS/BSA to the end of 20 hr. Thirty-four percent of cells bound B-Ag after this procedure, in contrast to the 6% binding, detected in Fig. 6, on cells that were incubated with B-Ag for the same time period but were stained with phycoerythrin-streptavidin immediately thereafter.

DISCUSSION

The main findings in this report are that the branched polypeptide (Tyr,Glu)-Ala--Lys requires processing to stimulate its specific T-cell line. Furthermore, processing is essential for the association of the resulting T-cell epitopes with MHC class II molecules and for presentation by the APC.

To study the processing requirements of (Tyr,Glu)-Ala--Lys, we established a (Tyr,Glu)-Ala--Lys-specific T-cell line. The requirements for processing of (Tyr,Glu)-Ala--Lys were monitored in this study according to the ability of fixed APC to stimulate the antigen-specific T-cell line to proliferate in response to the antigen (Fig. 1). Although fixation is a commonly used method in the assessment of processing requirements (11–13, 22, 23), we further delineated the processing events by introducing several inhibitors of proteases to the T-cell proliferation assay (Fig. 2). The ability of these inhibitors to alter T-cell proliferation is by means of interference with proteolytic steps involved in processing and presentation of antigens (24, 25) and is not due to cytotoxicity

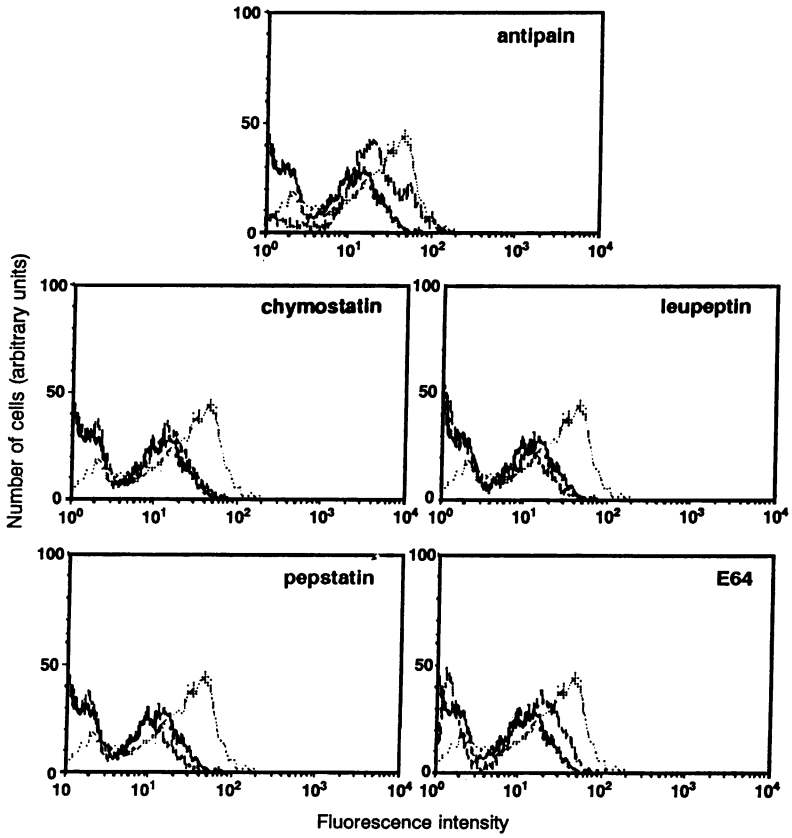

FIG. 5. Inhibition of the binding of B-Ag by inhibitors of proteases. Splenic adherent cells (10^6 per 100 μl) were co-incubated for 20 hr at 37°C with B-Ag (0.15 μM) and several protease inhibitors (0.5 mg/ml). ·····, Staining of cells incubated with B-Ag; IIIII, staining of cells incubated with both B-Ag and the inhibitor; and ——, PBS/BSA alone, for background fluorescence.

to T cells, since none of the five compounds inhibited concanavalin A supernatant-induced T-cell proliferation in the concentrations used for the antigen-induced proliferation experiments. We, therefore, suggest that in order to induce T-cell proliferation, proteolytic processing of (Tyr,Glu)-Ala--Lys is required.

The proteolysis of (Tyr,Glu)-Ala--Lys might be required to allow the interaction of some of the resulting fragments with the MHC class II molecules themselves. Alternatively, (Tyr, Glu)-Ala--Lys could associate with the class II molecules in its native form and the failure to stimulate its specific T-cell line may be due to other reasons (e.g., steric hindrance).

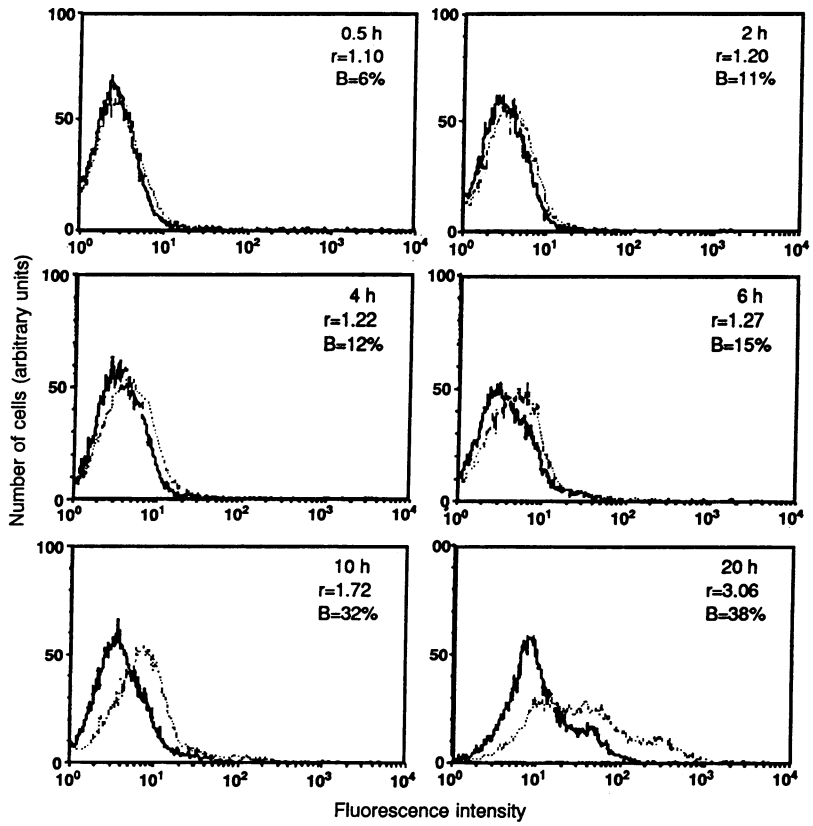

FIG. 6. Kinetics of binding of B-Ag to C3H.SW splenic adherent cells. Splenic adherent cells (10^6 per 100 μl) were incubated for the indicated time periods (h, hours) with 0.75 μM B-Ag (·····) or PBS/BSA alone (——). *r*, Ratio of the mean fluorescence intensities in the presence and absence of B-Ag: *B*, percent binding above background.

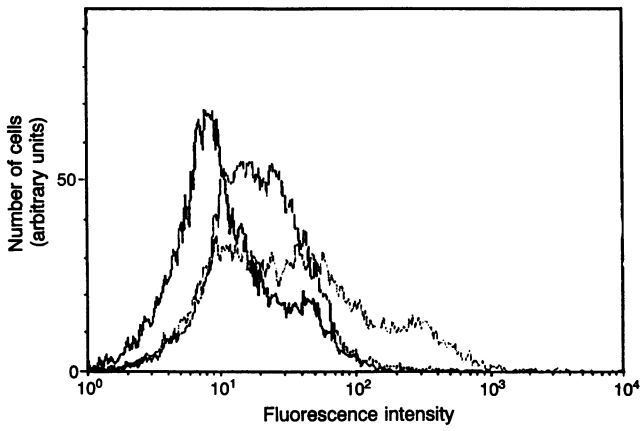

FIG. 7. Uptake of (Tyr,Glu)-Ala--Lys by APC occurs mainly during the first 30 min of incubation. Splenic adherent cells (10^6 per 100 μl) were incubated for 20 hr at 37°C with 0.75 μM B-Ag (·····) or PBS/BSA alone (——), or were pulsed for 30 min with 0.75 μM B-Ag, washed twice, and incubated with PBS/BSA alone to the end of the 20-hr incubation period (▪▪).

The direct binding experiments supported the first suggestion, namely, that proteolysis is needed for the interaction with MHC molecules. The detected binding of B-Ag is specific, since it was inhibited by an anti-I-A^b monoclonal antibody but not by nonrelevant anti-I-A antibodies (Table 1). Moreover, nonlabeled (Tyr,Glu)-Ala--Lys could reduce the binding signal in a dose-dependent manner (Table 1). Thus, the disappearance of the specific binding signal in the presence of several inhibitors of proteases (Fig. 5) indicates that the proteolytic processing of (Tyr,Glu)-Ala--Lys is a crucial step in the presentation of the antigen by APC. In addition, detection by streptavidin of the T-cell epitopes resulting from the proteolytic process implies that these epitopes are still carrying the biotin moiety and are hence located within or near the N termini of the branched polypeptide's side chains.

Consequently, this direct binding assay can be a useful tool in studying the process of antigen presentation in terms of both equilibrium (Fig. 4) and kinetics (Fig. 6) of binding. The apparent K_d calculated from the dose-dependent fluorescence intensities (8.05×10^{-8} M) is one to two orders of magnitude lower than the recently published K_d values for interaction of the hen egg-white lysozyme peptide 52–61 and purified soluble I-A^k in the presence or absence of lysophosphatidylserine, respectively (26, 27). The observed higher affinity is probably due to (*i*) measuring the binding to intact cells in our assay in comparison to determination with purified molecules, and (*ii*) the processing of (Tyr,Glu)-Ala--Lys, which provides the APC with many peptidyl T-cell epitopes that may evidently associate with MHC gene products.

Nevertheless, the appearance of the binding signal of (Tyr,Glu)-Ala--Lys on the APC is slower in comparison with both the lysozyme-(52–61) peptide [maximal APC presentation after a 4-hr pulse (27)] and a peptide that represents residues 195–212 of the human acetylcholine receptor α-subunit [3 hr (ref. 19 and unpublished data)]. The gradual slow appearance of the fluorescent signal after incubation with B-Ag is not due to slow uptake by the APC, since a 30-min pulse with B-Ag at the onset of the 20-hr incubation period resulted in almost maximal binding (Fig. 7). Hence, this delay in the presentation of the multichain polypeptide (Tyr,Glu)-Ala--Lys probably reflects the proteolytic processing that is essential to allow the interaction of the resulting peptidyl T-cell epitopes with MHC class II molecules and the transfer of these complexes to the APC membrane. A similar phenomenon was recently reported, that splenic B cells required 6–8 hr of incubation with native pigeon cytochrome *c* to process and present it to an I-E^k-restricted cytochrome *c*-specific T-cell hybrid for interleukin 2 secretion. In the latter study maximal interleukin 2 levels were detected after 16–24 hr of incubation of the B cells with the antigen (28).

The present study provides the first (to our knowledge) demonstration of molecular aspects involved in the processing of a multichain polypeptide by APC and the association with MHC class II gene products that further lead to T-cell stimulation.

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