ANALYSIS OF THE EXTRACELLULAR PROCESSING OF HIV-1 GP160-DERIVED PEPTIDES USING MONOCLONAL ANTIBODIES SPECIFIC TO H-2D^d MOLECULE COMPLEXED WITH P18-I10 PEPTIDE

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Received October 24, 2001; accepted November 8, 2001

Summary. – The immunodominant peptide of human immunodeficiency virus 1 gp 160 for murine cytotoxic T cells of H-2^d haplotype, has been originally identified as a 15 amino acid residue peptide P18IIIB (RIQRGPGRAFVTIGK) (Takahashi *et al.*, 1988). Further studies have indicated that a more active form of the peptide is generated by removal of the C-terminal dipeptide by angiotensin-I-converting enzyme (ACE), and additional detailed studies have shown that the actual immunodominant peptide is a decamer P18-I10 (RGPGRAFVTI) (Kozlowski *et al.*, 1993). The effect of proteolytic processing on the antigenicity of P18IIIB peptide and its analogs was investigated by functional T cell assays based on the ability of T cell receptor (TCR) to recognize a specific major histocompatibility complex class I (MHC-I)/peptide complex. Recently we described a new monoclonal antibody (MAb) KP15 directed against the MHC-I molecule H-2D^d complexed with the10-mer peptide P18-I10. Using this MAb, the cell surface H-2D^d/P18-I10 complex can be easily detected by flow cytometry (Polakova *et al.*, 2000). Here we examined whether peptides longer than P18-I10 decamer form H-2D^d complexes recognized by KP15 MAb. Further we also analyzed how the ACE processing of P18IIIB-related peptides of different length affects their ability to form complexes with H-2D^d recognized by MAb KP15. These experiments confirmed that the ACE digestion of 15-mer peptide P18IIIB is the most effective in the production of a peptide capable of forming complex with H-2D^d recognized by KP15 MAb. The ACE digestion of longer peptides (16-mer to 19-mer) did not produce a significant quantity of peptides, capable of forming H-2D^d complexes recognizable with by MAb KP15. Peptides shorter than P18IIIB (13-mer to 10-mer), notably the optimally sized P18-I10 peptide lost most of their capacity to form H-2D^d complexes recognized by KP15 MAb. Our results show that the extracellular processing of MHC-I-restricted peptides, which cannot be overlooked in designing peptide-based vaccines, can be also studied by as simple and rapid assay as flow cytometry, provided MAbs specific to a particular MHC-I/peptide complex are available.

Key words: human immunodeficiency virus 1; glycoprotein gp160; exogenous processing, angiotensin-I-converting enzyme

Introduction

Virus-specific cytotoxic T lymphocytes (CTLs; CD8+ T cells) are crucially important in the immune response to many virus infections. CTLs recognize short peptides, usually of 8–10 residues in length, bound to MHC-I molecules. These antigenic peptides are predominantly derived from endogenously synthesized proteins by the action of proteasomes. Therefore the best virus-specific CTL response is achieved by immunization with live attenuated virus (Germain and Margulies, 1993; Rock and Goldberg, 1999). Such vaccination, however, bears an inevitable risk of causing disease. Peptides corresponding to epitopes recognized by CD8+ T cells can be synthesized with high reproducibility and ex-

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Abbreviations: ACE = angiotensin-I-converting enzyme; CTL = cytotoxic T lymphocyte; DMEM = Dulbecco's Modified Eagle's Medium; MAb = monoclonal antibody; MHC-I = major histocompatibility complex class I; TCR = T cell receptor

quisite purity in large quantities. It has been demonstrated that under certain conditions, immunization with such synthetic peptides could not only induce virus-specific CTLs but also protection against subsequent virus challenge (Kast *et al.*, 1991; Uno-Furata *et al.*, 2001). Accordingly, it might be possible to prepare an ideal and safe T cell vaccine based on synthetic peptides (Bona *et al.*, 1998; Ertl and Xiang, 1996; Eisenbach *et al.*, 2000; Ludewig *et al.*, 2000). Synthetic peptides alone cannot stimulate T cell responses, first they must be bound to the particular MHC-I molecules. The cell surface MHC-I molecules are largely unreceptive to exogenous peptides, because they are occupied by antigenic peptides derived from endogenously synthesized proteins. However, the binding of exogenous peptides to the cell surface MHC-I molecules can be greatly enhanced in the presence of serum ß2-microglobulin (Kozlowski *et al.*, 1991; Otten *et al.*, 1992). Serum also contains distinct proteases that can affect the stability of synthetic peptides, ability to bind MHC-I molecules and capacity to induce CTL response. For example, a number of MHC-I-restricted peptides longer than 10 amino acid residues are able to activate T cells. Serum proteases can process such peptides to forms that are more efficiently bound by the MHC-I molecule (Falo *et al.*, 1992; Kozlowski *et al.*, 1992; Sherman *et al.*, 1992). Conversely, serum proteases can greatly decrease, even destroy, MHC-I-restricted presentation of minimal size core peptides. Therefore the understanding of extracellular processing of MHC-I-restricted peptides is important for design of peptide based T cell vaccines.

The envelope glycoprotein gp160 of HIV-1 is an important antigen for studying antigenic peptides presented by MHC-I molecules to CTL. Of particular interest is the model peptide P18IIIB (RIQRGPGRAFVTIGK), a 15-mer from the V3 loop of HIV-1 gp160, which can be presented by H-2D^d MHC-I molecules (Takahashi *et al.*, 1988). For CTL recognition this 15-amino acid residue peptide P18IIIB must be processed by proteolytic enzyme(s) into either a 13-mer containing the C-terminal isoleucine residue or to an active 10-amino acid residue peptide P18-I10 (RGPGRAFVTI) (Kozlowski *et al.*, 1993).

By using functional T cell assays the extracellular processing of P18IIIB peptide in the presence of serum was studied. Using the specific enzyme inhibitor captopril it was found that serum angiotensin-1 converting enzyme activity (ACE), a carboxy dipeptidase, plays an important role in the production of optimally sized peptide (Kozlowski *et al.*, 1993; Lopéz *et al.*, 2000; Nakagawa *et al.*, 2000).

Recently we described new monoclonal antibodies (KP14 and KP15) specific to the MHC-I molecule H-2D^d complexed with the 10-mer peptide P18-I10 (Polakova *et al.*, 2000). Here we extended characterization of the specificity of one of these MAbs, namely KP15, by analyzing the capacity of peptides longer than the P18-I10 decamer to form H-2D^d complexes recognized by MAb KP15. Further we also analyzed how the ACE processing of P18IIIB-related peptides of different length affects their ability to form complexes with H-2D^d. All analyses were evaluated by a simple and rapid flow cytometry with MAb KP15 directed specifically to MHC-I molecule H-2D^d complexed with peptide P18-I10.

Materials and Methods

Cell lines. The following cell lines were used: LKD8 (an H-2D^d transfectant of a TAP-defective mouse embryo cell); SKT4.5 (thymidine kinase-negative L cells, transfected with H-2D^d); B4.2.3 (a T cell hybridoma with specificity to peptide P18-I10 bound to H-2D^d). All cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing heat inactivated 10% fetal calf serum (FCS) and supplemented with non-essential amino acids, 2 mmol/l L-glutamine, 10 mmol/l sodium pyruvate, 100 U/ml penicillin G and 100 µg/ml streptomycin sulfate.

Monoclonal antibodies. The monoclonal antibody 34.5.8 recognizes a peptide-dependent conformational epitope on H-2D^d α1 α2 domains. Monoclonal antibody KP15 (clone KP15/11) specifically binds H-2D^d molecule complexed with an HIV-1 envelope gp160-derived peptide, P18-I10 (RGPGRAFVTI) (Poláková *et al.*, 2000).

Synthetic peptides. Th synthetic peptides listed in Table 1 were prepared by 9-fluoroenylmethoxyl-carbonyl chemistry and purified to >95% purity by HPLC at the Laboratory of Molecular and Structural Biology, NIAID, NIH, Bethesda, MD, USA. The15-mer peptide P18IIIB (RIQRGPGRAFVTIGK) has been demonstrated as an immunodominant CTL epitope in the HIV-1 envelope glycoprotein gp160 recognized by CD8+ CTL in the context of H-2D^d (Takahashi *et al.*, 1988). All peptides, except an H-2D^d-motif peptide (AGPARAAAL), are derived from P18IIIB peptide (elongated, truncated or substituted). A decamer peptide P18-I10 (RGPGRAFVTI) is the minimal sized (core) CTL epitope derived from 15-mer peptide P18IIIB. The peptide names (except originally established names P18IIIB and P18-I10) are simplified to reflect the number of amino acid residues. All synthetic peptides listed in Table 1 were a generous gift from Dr. D.H. Margulies, Laboratory of Immunology, NIAID, NIH, Bethesda, MD, USA).

Loading of cells with peptides. LKD8 cells (H-2D^d) (5 x 10^6 in 1 ml of RPMI medium) were pulsed with synthetic peptides (10 or 100 µmoles) in the presence of 5 µg/ml human β2-microglobulin (Fitzgerald Industries International, Concord, MA, USA) for 2 hrs at 37°C or 12 hrs at 26°C.

Flow cytometry. Approximately 2–5 x 10^{5}cells were incubated with an appropriate dilution of primary MAb for 1 hr at 4°C. After washing with PBS containing 2% BSA and 0.1% sodium azide, the cells were stained with FITC-conjugated goat anti-mouse IgG (1:100 dilution; Dako, Carpinteria, CA, USA) for 40 mins at 4°C. Then the cells washed with PBS were analyzed on a FACScan (Becton Dickinson) flow cytometer in the presence of propidium iodide. Dead cells were gated out based on forward and side scatter, and propidium iodide uptake.

T cell activation assay. B4.2.3 cells (H-2D^d-restricted T cell hybridoma specific to P18-I10 peptide) were activated with presenting cells SKT4.5 (thymidine kinase-deficient) pulsed with P18-I10 peptide. In brief, 1 x 10^4 B4.2.3 cells were stimulated with 2 x 10^3 SKT4.5 cells in the presence of graded concentrations of peptide P18-I10 for 16 hrs at 37°C. Then 1 μCi of [^{3}H]thymidine (ICN Pharmaceuticals, Costa Mesa, CA,USA) was added to the mixture of the cells and incorporation of radiolabel into insoluble material was assessed after a 4 hr incubation at 37°C.

Extracellular processing of peptides by ACE. First the P18IIIB variant peptides (Table 1) at 100 μmol/l concentration were incubated in RPMI medium (without serum) with dipeptidase ACE (Sigma) for 1 hr at 37°C. The final concentration of ACE was 1 mU/ml. LKD8 cells were then added and the mixture was incubated for another hr at 37°C in the presence of 5 μg/ml β2-microglobulin (Fitzgerald International, Inc., Concord, MD, USA). The LKD8 cells, loaded with exogenously processed peptides were stained with mAb KP15/11 and analyzed by flow cytometry. The ACE activity was inhibited with 10 mmol/l captopril (Sigma).

Table 1. Sequences of synthetic peptides, gp160 derived P18IIIB variants, used in this study

Number of aa	Peptide	Sequence
19	P19	riqRGPRRAFVTIgaignm
18	P18	riqRGPRRAFVTIgaign
17	P17	riqRGPRRAFVTIgaig
16	P16	riqRGPRRAFVTIgai
15	P18IIIB	riqRGPRRAFVTIgk
15	P15	riqRGPRRAFVTIga
13	P13	RGPRRAFVTIgai
12	P12	RGPRRAFVTIgk
11	P11	RGPRRAFVTIg
10	P18-I10	RGPRRAFVTI
9	motif	agparaaal

The listed synthetic peptides except H-2D^d motif peptide were derived from the peptide P18IIIB of the HIV-1 strain IIIB. In the sequences, uppercase letters represent the antigenic core peptide P18-I10 sequence. The peptide names are simplified to reflect the length (the number of amino acid residues) of individual peptides. The former peptide names P18IIIB and P18-I10 are not altered for easier comparison of the presented results with those published by others.
aa = amino acid.

Results and Discussion

The H-2D^d/P18-I10 peptide complex is recognized through TCR by activation of specific T cell hybridoma or by flow cytometry with MAb KP15

Recently we described new monoclonal antibodies (KP14 and KP15) directed against the MHC-I molecule H-2D^d complexed with the 10-mer peptide P18-I10. The specificity of these two antibodies is similar to TCR present on B4.2.3 H-2D^d-restricted T cell hybridoma specific to H-2D^d/P18-I10 complex. In initial experiments we compared first the ability of MAb KP15/11 and B4.2.3 cells to recognize H-2D^d/P18-I10 complexes on the surface of SKT4.5 cells (H-2D^d

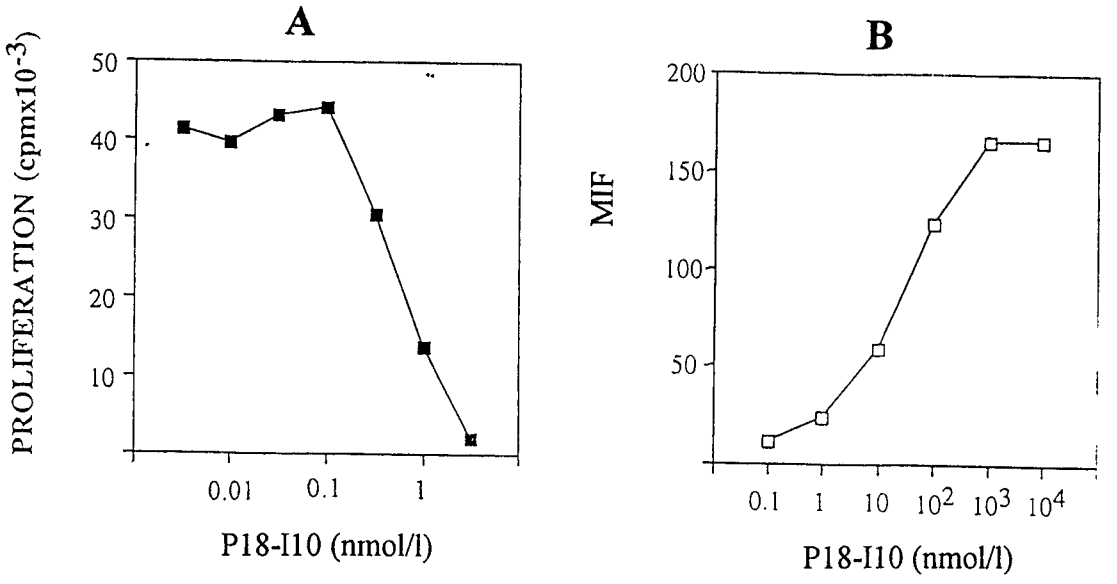

Fig. 1
MAb KP15/11 binding and hybridoma B4.2.3 response to the cell surface H-2D^d molecules pulsed with different concentrations of peptide P18-I10

SKT4.5 cells were loaded with different amounts of peptide P18-I10 and formation of H-2D^d/P18-I10 complexes was analyzed by activation of specific T cell hybridoma B4.2.3. (A) or by staining with MAb KP15/11 specific to H-2D^d/P18-I10 complexes (B). MIF = mean intensity of fluorescence.

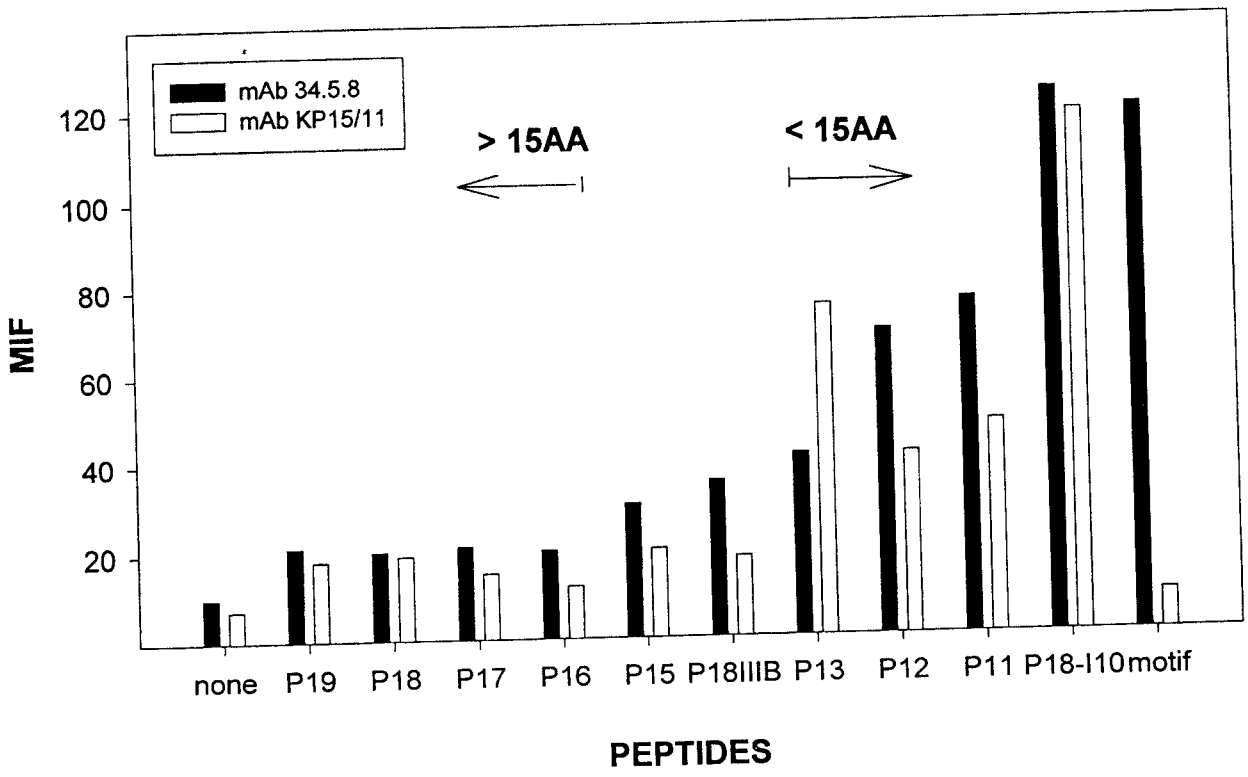

Fig. 2

Distinct recognition of H-2D^d complexed with P18-I10-related peptides longer than 10-mer with mAbs KP15/11 and 34.5.8

LKD8 cells (H-2D^d) were pulsed with indicated peptides longer than decamer P18-I10. The formation of H-2D^d/peptide complexes was followed by binding of MAb 34.5.8 or KP15/11. MIF = mean intensity of fluorescence.

transfected, TK deficient cells) loaded with different quantity of a decamer peptide P18-I10. Fig. 1A shows P18-I10 peptide dose-dependent activation of specific T cell hybridoma B4.2.3, manifested by inhibition of its proliferation. The higher the dose of P18-I10 peptide used to pulse SKT4.5 cells, the higher inhibition of B4.2.3 cell proliferation detected. The maximum stimulation of T cell hybridoma B4.2.3 was induced at 1 nmol/l concentration of P18-I10 peptide. SKT4.5 cells loaded with H-2D^d-motif peptide did not inhibit the proliferation of B4.2.3 cells (data not shown). In Fig. 1B SKT4.5 cells were pulsed with P18-I10 peptide and the amount of MAb KP15/11 bound to the cell surface was detected by flow cytometry. Provided that SKT4.5 cells were pulsed with the motif peptide, no specific binding of mAb KP15/11 to the cell surface was detected by flow cytometry. Our results show that detection of H-2D^d/P18-I10 complex by staining with MAb KP15/11 is less sensitive than the T cell functional assay with B4.2.3 T cell hybridoma. The highest specific fluorescence intensity for MAb KP15/11 binding was achieved by pulsing SKT4.5 cells at 10^3 nmol/l concentration of the peptide. Nevertheless, the sensitivity of flow cytometry in detecting H-2Dd/P18-I10 complex with MAb KP15/11 was sufficient for further experiments regarding analysis of the role of peptide length and ACE processing in the formation of MHC-I/peptide complexes.

Distinct recognition of H-2D^d complexed with P18-I10-related peptides larger than 10-mer with MAbs KP15/11 and 34.5.8

Some peptides longer than the optimal size of 8–10 amino acid residues are also able to produce complexes with MHC-I molecules and thus induce MHC-I conformational epitopes (Cerundolo *et al.*, 1991). To date we characterized the specificity of KP15/11 MAb using only 10-mer peptides. For this purpose the capacity of 10-mer P18-I10 related peptides (modified by a single amino acid substitution at different positions) to form complexes with H-2D^d recognized by MAb KP15/11 was estimated (Polakova *et al.*, 2000).

Now we decided to find out whether peptides longer than decamer P18-I10, namely 11–19-mers, are also able to form

complexes with H-2D^d molecules and whether such complexes can be recognized by MAb KP15/11. To increase the efficiency of peptide loading, TAP-defective LKD8 cells were used, because such cells have on their surface a high number of peptide-receptive MHC-I molecules. First, the cells were exposed to the indicated peptides in the absence of serum to avoid proteolytic degradation and in the presence of 5 μg/ml human β2-microglobulin to improve peptide binding capacity. Thereafter the cells were stained either with MAb KP15/11 (specific to H-2D^d/P18-I10 complex) or with MAb 34.5.8 (specific to conformational epitope of H-2D^d domain induced by any peptide that binds to H-2D^d). Such arrangement of experiments allows detection of H-2D^d complexes formed with P18IIIB-related peptides including peptides longer or shorter than P18IIIB, even if such complexes are not recognized with MAb KP15/11. As shown in Fig. 2, LKD8 cells pulsed with 10-mer peptide P18-I10 were stained equally well with both MAbs (34.5.8 and KP15/11). This is the evidence that P18-I10 peptide not only binds to H-2D^d but also that the resulting H-2D^d/peptide complex is recognized by MAb KP15/11. In contrast, LKD8 cells pulsed with H-2D^d-motif peptide were stained only with MAb 34.5.8, confirming the formation of H-2D^d/motif peptide complex and the specificity of MAb KP15/11 to H-2D^d/P18-I10 peptide complex. Peptides of the size of 15-mer (P18IIIB and P15) or longer had some but very low capacity to form complexes with H-2D^d molecules as indicated by weak interaction with MAb 34.5.8. Likewise these complexes were not stained with MAb KP15/11. In contrast, complexes formed with peptides shorter than 15-mer were stained with both MAbs, 34.5.8 or KP15/11. Here we also confirmed that the core epitope P18-I10 was the most effective in the binding of both MAbs. To our surprise H-2D^d/peptide complexes made with the 13-mer peptide used in this study were stained with MAb KP15/11 more intensively than with shorter 12- or 11-mer peptides. We have no explicit explanation for this finding. Taken together we can conclude that our results obtained by staining with MAb KP15/11 are in good correlation with data published by others where only T cell activation assay was used. (Takeshita *et al.*, 1995). The results with MAb KP15/11 binding to H-2D^d complexed with peptides of different length showed that flow cytometry with MAb KP15/11 is an appropriate method for analysis of extracellular proteolytic ACE processing of P18-I10-related peptides.

15-mer peptides (P18IIIB or P15) after ACE treatment acquire ability to form complexes with H-2D^d molecules recognized by MAb KP15/11

Others have shown that the optimally sized 10-mer peptide P18-I10 can be produced by exposing 15-mer peptide P18IIIB to FCS or to purified ACE dipeptidase (the ACE exposure led to an active form, but presumably the 13-mer. The ACE alone is only a carboxydipepetidase and would not be expected to produce the 10-mer, while the ACE followed by another unidentified serum protease could do this (Kozlowski *et al.*, 1992; Lopez *et al.*, 2000; Nakagawa *et al.*, 2000). Such proteolytic trimming generated biologically active peptides detected by stimulation of appropriate T cells. We analyzed the extracellular processing of 15-mer peptides P18IIIB and P15 and their capacity to form complexes with H-2D^d molecules by flow cytometry using MAb KP15/11. First the 15-mer peptides were incubated for 1 h with different concentrations of ACE at 37°C in serum-free RPMI medium. After addition of LKD8 cells the mixture was incubated for another 1hr at 37°C in the presence of human β2-microglobulin. Thereafter the cells were stained with MAb KP15/11 and analyzed by flow cytometry. The results of these experiments are shown in Fig. 3. The binding of MAb KP15/11 was proportional to the amount of ACE present in the incubation mixture. It has been reported that its specific inhibitor, captopril, can inhibit the ACE activity (Kozlowski *et al.*, 1992, 1993; Nakagawa *et al.*, 2000). As clearly shown in Fig. 3, the highest dose of ACE present in incubation mixture was also inhibited by captopril and the staining of LKD8 cells with MAb KP15/11 was completely abrogated. These results thus prove that the P15-mer peptides (P18IIIB or P15) must be first shortened before they are able to form complexes with H-2D^d, which can be recognized by KP15/11 MAb.

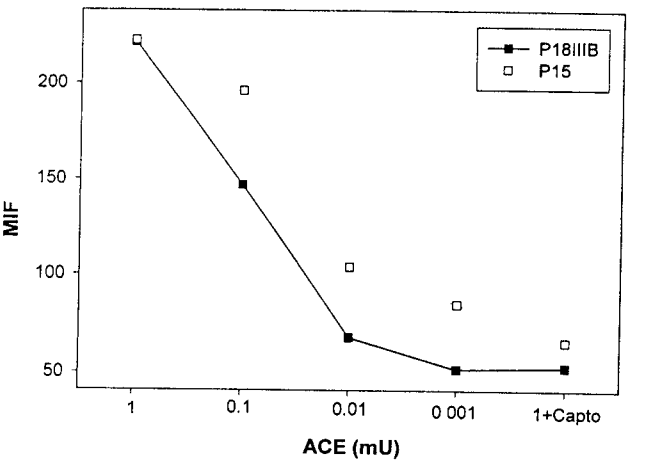

Fig. 3
Exogenous processing of 15-mer peptides P18IIIB and P15 by various concentrations of ACE as evaluated with MAb KP15/11
15-mer peptides P18IIIB and P15 were first incubated with various concentrations of ACE and then loaded to LKD8 (H-2D^d) cells. The generation of shorter peptides able to form complexes with H-2D^d molecules was analyzed with MAb KP15/11. MIF = mean intensity of fluorescence.

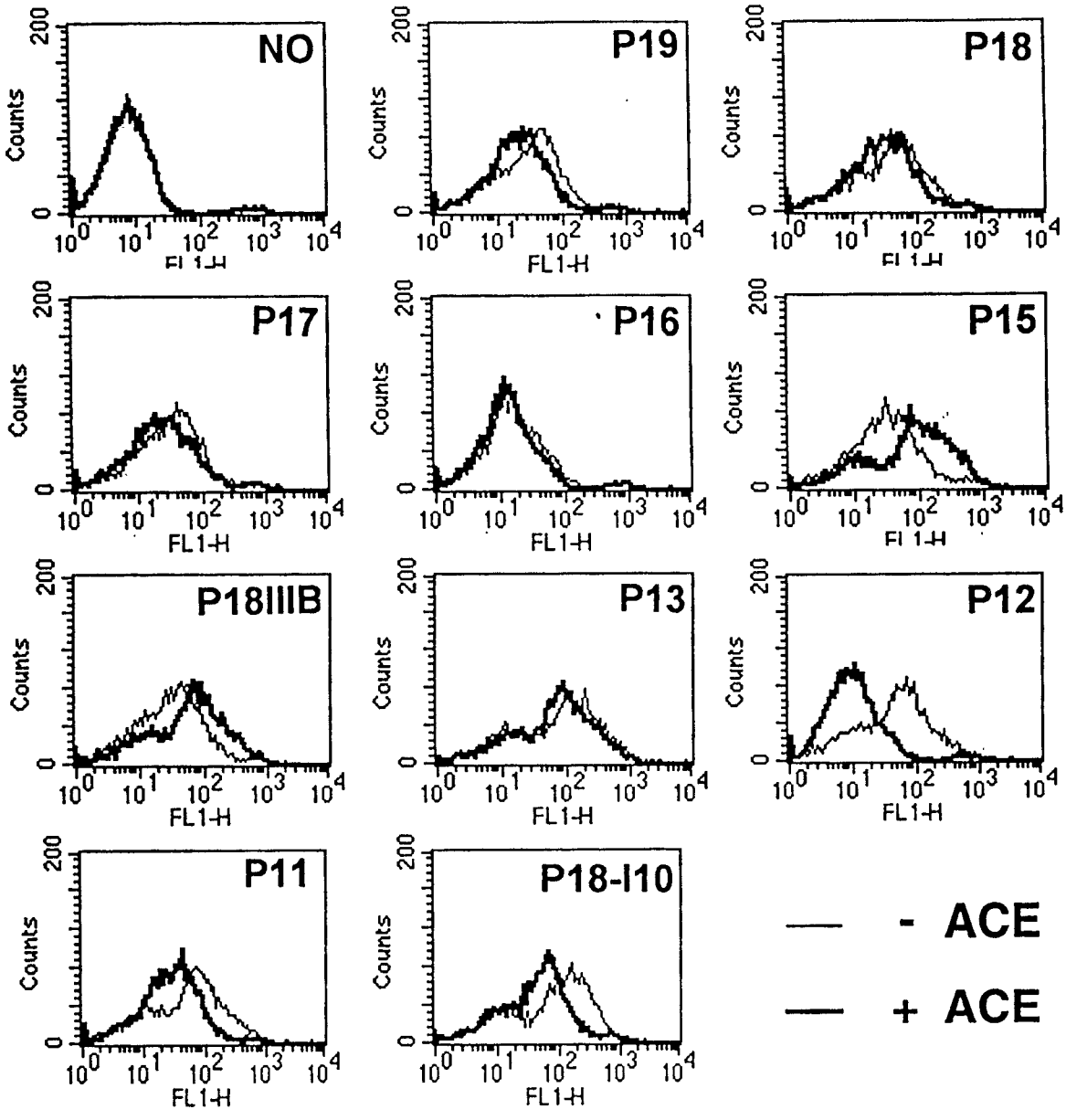

Fig. 4

Flow cytometry analysis of MAb KP15/11 binding to H-2D^d molecules complexed with P18IIIB peptide variants pretreated with ACE

P18IIIB peptide variants of various length, longer or shorter than 15-mer, were first incubated in serum-free RPMI medium with or without ACE protease and then loaded to LKD8 cells (H-2D^d). The efficacy of MAb KP15/11 to binding to H-2D^d/peptide complexes was evaluated by flow cytometry as indicated in histograms. FL1-H = fluorescence intensity; counts = number of cells.

Analysis of MAb KP15/11 binding to H-2D^d molecules complexed with P18IIIB peptide analogs pretreated with ACE

Next we investigated the possibility whether P18IIIB peptide analogs longer or shorter than the 15-mer are able after the ACE treatment to form H-2D^d complexes recognized by MAb KP15/11. In these experiments we used peptide concentration as high as 100 μmol/l to enhance formation of MHC-I/peptide complex with suboptimal peptides. Concentration of ACE was adjusted to 1 mU/ml. All peptides were first pre-incubated with or without ACE in the

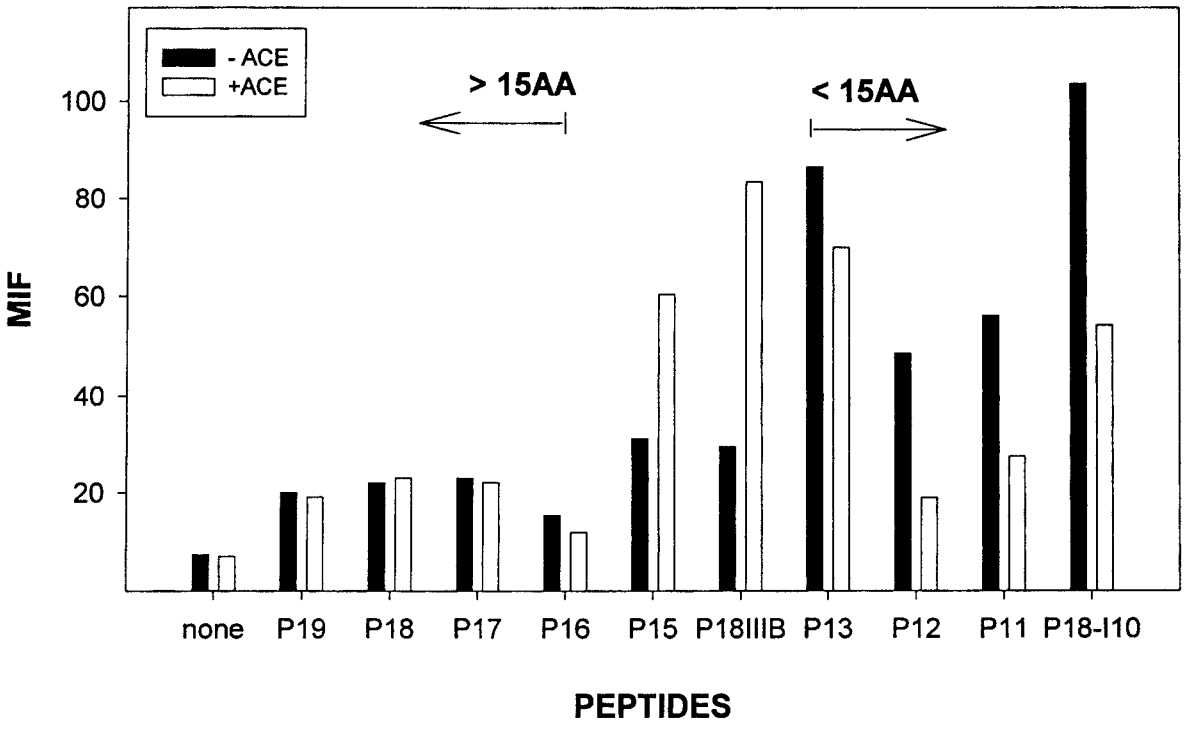

Fig. 5
Statistics of flow cytometry analysis of MAb KP15/11 binding to H-2D^d molecules complexed with P18IIIB peptide variants pretreated with ACE

P18IIIB peptide variants, longer or shorter than 15-mer, were first incubated in serum-free RPMI medium with or without ACE and then loaded to LKD8 cells (H-2D^d). The efficacy of MAb KP15/11 binding to H-2D^d/peptide complexes was evaluated by flow cytometry as indicated by means of chanel fluorescence. MIF = mean fluorescence intensity.

serum-free RPMI medium for 1 h at 37°C and then LKD8 cells were added. The mixture was incubated for another 1hr at 37°C in the presence of human β2-microglobulin. Thereafter the LKD8 cells were stained with MAb KP15/11 and analyzed by flow cytometry. The histograms of a typical experiment are shown in Fig. 4 and their statistical analysis in Fig. 5. The binding of MAb KP15/11 to LKD8 cells was dependent on the length of peptide and on ACE pretreatment. As already shown in the Fig. 2, both 15-mer peptides P18IIIB and P15 had very low capacity to form H-2D^d complexes recognized by MAb KP15/11. At present we cannot rule out the possibility that both 15-mer peptides were slightly contaminated with such a short peptides as P18-I10. On the other hand, 15-mer peptides might be able to form genuine complexes with H-2D^d. Nevertheless, formation of such complexes and/or their recognition by MAb KP15 had very low efficiency. Following the ACE pre-treatment the staining with MAb KP15/11 increased because the 15-mers were processed into shorter peptides, most likely into 13-mers with an appropriate C terminus. The peptides longer than 15-mer, P16-P19, after the ACE pre-treatment did not show any alteration in their ability to form complexes recognized by MAb KP15/11. This finding is consistent with data published by others, where the processing of peptides with ACE or serum, was evaluated by sensitization of target cells towards specific T cells (Kozlowski *et al.*, 1992; Takeshita *et al.*, 1995; Nakagawa *et al.*, 2000). Further we found out that the pre-treatment of peptides shorter than 15-mer with ACE reduced markedly their capacity to produce H-2D^d complexes recognized by MAb KP15/11. Similarly, Kozlowski *et al.* (1995) have demonstrated that the longer versions of the 15-mer peptide p18IIIB are not influenced by ACE processing, while the core peptide P18-I10 looses a T cell stimulatory activity (Kozlowski *et al.*, 1995).

In this study we have shown that the extracellular processing of MHC-I-restricted peptides can be studied by as simple and rapid assay as flow cytometry, provided MAbs specific to a particular MHC-I/peptide complex are available. At present a reliable procedure for construction of hybridomas producing MHC-I/peptide MAbs is available (Porgador *et al.*, 1997; Polakova *et al.*, 2000). More detailed studies allowing statistical evaluation of results will have to be performed with directly labeled MAbs.

Acknowledgements. We are grateful to Dr. D.H. Margulies, Laboratory of Immunology, NIAID, NIH, Bethesda, MD, USA, for his support, generous providing of synthetic peptides and T cell hybridoma, and for helpful comments on the manuscript. This work was partially supported by the grants Nos. 2/6935/20 and 2/7018/20 of the Scientific Grant Agency of Ministry of Education of Slovak Republic and Slovak Academy of Sciences.

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