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Quantitative Cytometry of MHC Class I Digestion From Living Cells

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Digestion of crude membrane preparations with papain releases the extracellular portion of major histocompatibility complex (MHC) class I molecules. MHC class I molecules are integral membrane glycoprotein complexes formed by the noncovalent association of 2 invariant molecules, the heavy chain and the β_2-microglobulin (β_2-m), to a wide array of peptides. The cleaved soluble moiety retains the antigenic properties of the intact membrane-bound complex. Here we show that MHC class I digestion may be carried out on living cells, and we quantitate the surface expression of MHC complexes by a combined cytometric/high performance liquid chromatographic (HPLC) approach. Papain digestion results in time- and dose-dependent disappearance of membrane MHC-associatedfluorescence as detected by FACS analysis withMHC-specific monoclonal antibodies (mAbs). β_2-m and peptides became detectable by HPLC analysis and western blotting in the digestion buffer and were quantitated by comparison with purified standards. The cytometric assessment of the digestion allows one to simultaneously monitor efficacy and toxicity of the treatment. The procedure we describe allows to selectively retrieve by affinity chromatography MHC from the cell membrane, avoiding any contamination due to intracellular, "immature" MHC molecules. Cytometry 27:77–83, 1997. © 1997 Wiley-Liss, Inc.

Key terms: cytometry; HPLC; MHC molecules; papain; peptides; β_2-microglobulin

$CD8^+$, $\alpha\beta^+$ T lymphocytes recognize peptide antigens in the context of MHC class I molecules. Antigen recognition may result in T-cell activation, with lysis of the class I positive cell, in T-cell programmed death by apoptosis or in T-cell functional paralysis, known as clonal anergy (19).

MHC class I molecules are integral membrane glycoproteins, expressed on most nucleated cells and consisting of a 43–45 kDa polymorphic heavy chain, noncovalently associated with β_2-m (11–12 kDa). The complex is stabilized by the presence of an 8–10 aminoacid peptide that is required both for the correct folding of the heavy chain and for β_2-m to remain associated to the complex at 37°C (1, 13, 28, 15). The dissociation rate of β_2-m reflects the stability of the heterotrimer that also depends on the length and composition of the bound peptide (4, 25).

Papain cleaves the heavy chain at the residue 271, thirteen aminoacids from the transmembrane region (35), thus releasing the heavy chain amino terminal extracellular part, still assembled with the β_2-m and the peptide. Soluble complexes retain the immunogenic properties of membrane-bound receptors (24) and, due to the lack of the hydrophobic membrane spanning region, provided suitable material for MHC crystallisation and X-ray diffraction (3).

Heavy chain/β_2-m/peptide soluble heterotrimers, lacking transmembrane and intracellular domains of the heavy chain, have been purified from biological fluids: these soluble complexes may be generated by alternative splicing, with excision of hydrophobic regions, or may be shed from cell membranes upon cleavage by so far only partially characterized proteases, whose cleavage site, however, must be close to the papain cleavage site (5, 6, 28). Soluble MHC molecules may interfere with T-cell activation (30–33, 40, 14) by competition with membrane-bound MHC molecules for T-cell receptor recognition.

In recent years the hierarchy of the peptide-MHC association, as well as the influence of chaperonins on the

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assembly of the heterotrimer, have been the object of many investigations (15, 2, 20). It would be useful to devise procedures to discriminate and selectively purify ''mature'' cell-bound molecules from their intracellular ''immature,'' putatively chaperonin-associated precursors.

Flow cytometry analysis of cell membrane antigen expression, combined with the use of calibrated microbead standards bearing known amounts of bound antibodies, proved valuable to quantitatively compare the relative membrane expression of different antigens (27, 23, 7, 34, 16, 17, 36, 8). This technique converts the cytometric assessment of the membrane fluorescence in numbers of bound antibodies per cell. The results may be biased in the case of complex antigens like MHC molecules, as different peptides assemble with β_2-m and the heavy chain, and contribute to the overall structure of the complex (12). Here we provide an alternative approach to quantitative cytometry that allows one to convert the membrane fluorescence in absolute amount of MHC molecules per cell.

MATERIALS AND METHODS

Cell Lines

The RMA line is derived from a Rauscher leukaemia virus-induced T-cell lymphoma of B6 origin. The line was kindly provided by Vincenzo Cerundolo and Alain Townsend (John Radcliffe Hospital, Oxford, GB). B16F1 cell line, a melanoma which had spontaneously arisen in B6 mice and stabilized in vitro, was purchased from ATCC (Rockville, MA). The M14 cell line, a human melanoma spontaneously stabilized in vitro, was obtained from S. Ferrini (IST, Genova). The Jurkat cell line, a human acute T-cell leukaemia, was purchased from ATCC (Rockville, MA). Cells were maintained in RPMI 1640 medium supplemented with 10% FCS, and penicillin-streptomycin at 37°C and 5% CO_2.

Antibodies

W6/32 (anti-human MHC class I), BBM.1 (anti human β_2-microglobulin), and 28.8.6S (anti-mouse MHC class I H2 K^bD^b) hybridomas were obtained from ATCC and maintained in RPMI 1640 medium supplemented with 10% FCS and penicillin-streptomycin. Monoclonal antibodies (mAbs) were purified by protein-A affinity chromatography and dialysed in PBS, pH 7.4. MHC class II expression was evaluated with the L243 anti human DR mAb (ATCC) or with the anti-I-A^b mAb (Pharmingen, San Diego, CA). Anti CD95 mAb was purchased from MBL (Nagoya, Japan) and anti CD3 (OK-T3) from Ortho Diagnostic (Raritan, NJ).

Enzymatic Digestion of MHC Class I Molecule and MHC Class I Purification

Cells were extensively washed in PBS, resuspended in 80 mM Na_2HPO_4 (pH 8.0) to a concentration of 10×10^6 cells/ml. Papain (0.5–4 mg/ml), cystein (20 mM), and EDTA (1 mM) (all reagents from Sigma, Saint Louis, MO) were added. After digestion at 37°C, cells were retrieved by centrifugation. Digestion time was different for different cell lines: as treatment efficiency was similar, digestion times were decided mainly based on cell sensitivity to toxicity of the treatment and unless otherwise indicated were 6 h for RMA cells and 2 h for B16, Jurkat, and M14 cells. The supernatant was cleared at 25,000 $\times$ g for 30 min at 4°C and concentrated by ultrafiltration (500 $\times$ g for 2h at 4°C) through an Amicon YM-30 membrane. The retentate, containing papain and solubilized proteins, was further analyzed by HPLC (see below), used for western blotting after precipitation with 10% TCA overnight at 4°C, or further purified by affinity chromatography with the mAb W6/32 coupled to the Affi-Gel Hz resin (Bio-Rad) as described in (18). When indicated, Brefeldin A (5 μg/ml) (Sigma, Saint Louis, MO) was added 1 h before digestion and maintained at the same concentration throughout the digestion.

HPLC Analysis

The HPLC analytical conditions for peptide analysis were set up with a mixture of 40 synthetic peptides (M_r 1–2 kDa) kindly provided by the laboratory of Dr. B. M. Conti-Fine (Department of Biochemistry, University of Minnesota). Lyophilized peptides were resuspended in water to a final concentration of 240 μg/ml for each peptide. Mobile phase was constituted by A:0.1% trifluoracetic acid (TFA) (Fluka Chemie AG, Buchs, Switzerland) and B: acetonitrile:eluent A (80:20, v:v), and flow rate was 0.25 ml/min, monitoring the absorbance at 210 nm. The analysis was carried out using the following gradient: from 0 to 15 min, 10–25% B; from 15 to 40 min, 25–35% B, from 40 to 90 min, 35–100% B. The column was then reconditioned at 10% B.

The HPLC system was a Beckman System Gold (Beckman Instruments Inc., Palo Alto, CA), assembled with 2 dual-piston pumps (model 126) and a variable double-beam UV detector (model 168). The whole apparatus was computer controlled with the System Gold software for storage and handling of data. A Delta Pak column C18, 300 Å pore size (15 cm $\times$ 2.0 mm i.d.) (Waters Associates, Inc., Milford, MA) was employed.

Temporized fractions (0.5 ml) were collected during HPLC analysis in the peptide elution zone, hydrolyzed, and used for aminoacid analysis (10).

Before HPLC analysis, heavy chain/β_2-m/peptide complexes were disrupted, bringing the pH of the retentate from the ultrafiltration to 2.1 by addition of TFA. Quantitation of MHC molecules was made on the base of the area/pmole value obtained analyzing human purified β_2-m (Sigma, Saint Louis, MO).

Western Blotting

SDS-PAGE was performed under reducing conditions on a 15% SDS-gel polyacrilamide. The gels were blotted to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) and subsequently blocked with 3% nonfat dry milk. After washing three times in PBS supplemented with 0.1% Tween 20, gels were blotted for 1 h at room temperature with the mAb BBM.1 (anti-β_2 microglobulin). The blots were washed again 3 times and further incubated with an alkaline phosphate-conjugated goat anti-

mouse antibody (DAKO A/S, Denmark), 1:1000 diluted, for 1 h at room temperature. β_2-m molecules were visualized by addition of alkaline phosphatase substrate solution. As a positive control, 1 µg of purified human β_2-m was blotted.

Flow Cytometry

Cells were labelled by indirect immunofluorescence either before (nondigested sample) or after different times of enzymatic digestion, using W6/32, OKT3, BBM.1, 28.8.6S (1µg/10^6 cells, 30 min at 4°C) or anti-CD95 CH11 (500 ng/10^6 cells, 30 min at 4°C) mAbs. A fluorescein isothiocyanate (FITC)-labelled goat antimouse IgG1 or IgM (Southern Biotechnology Associates, Birmingham, AL) was used as a second-step reagent. Negative control was obtained by treating the cells with the second-step reagent only. Flow cytometric analysis was performed using a fluorescence activated cell sorter (FACSstar^{Plus}, Becton Dickinson, San Jose, CA). Cell vitality after different times of digestion was evaluated according to physical parameters [as described in (11) and (26)]. Statistical analysis was performed using the Kolmogorov-Smirnov Two-Sample Test (39).

RESULTS

To quantitate MHC class I membrane expression by flow cytometry, we first investigated whether papain digestion could be carried out on living cells. The results of this treatment on MHC associated fluorescence of different cell lines are illustrated in Figure 1. Papain digestion was carried out at 37°C, pH 8, on melanoma, thymoma, and leukaemia cells. The analysis was performed on unfixed specimens both before or after 2 h papain treatment, with mAbs recognizing epitopes of either human or murine MHC complexes, followed by staining with FITC-labelled goat antimouse IgG1 Abs as second-step reagents. The negative control (fluorescence background) was obtained by incubation with the second-step reagent only.

Although cells were heterogeneous in terms of species, embryological origin, or MHC expression, the treatment consistently resulted in a decrease of the basal MHC associated fluorescence. The decrease of MHC associated fluorescence upon papain digestion is highly reproducible: when digestion was performed in quadruplicate, standard deviations ranged between 4.48% and 6.24%, while standard errors ranged between 2.24% and 3.06% of the resulting data. Papain digestion significantly affected the amount of MHC associated fluorescence, as assessed by the Kolmogorov-Smirnov two-sample test (Fig. 1). However, it did not influence the expression of other unrelated membrane molecules, like the CD3 or the CD95 (Fas, APO-1) molecules (Fig. 2), constitutively expressed by activated T cells [e.g., see (30)]. The MHC turnover during the enzymatic digestion does not substantially influence the phenomenon, as digestion was unaffected by treatment with Brefeldin A, an agent that interrupts the cell secretory pathway, thus preventing the expression of newly synthesized molecules at a the cell surface while allowing membrane recycling (21, 38) (Fig. 2 C and D). Cell vitality was assessed by staining with vital dies (trypan blue, propidium iodide) or by evaluation of their physical parameters. Only for treatments longer than 2 h did physical characteristics occasionally change, assuming apoptotic features [low Foward SCatter (FSC) and high Side SCatter (SSC); see (11), (26)] (not shown). In the experiments described in this study, however, these parameters were not affected (e.g., see Fig. 2, dot plots).

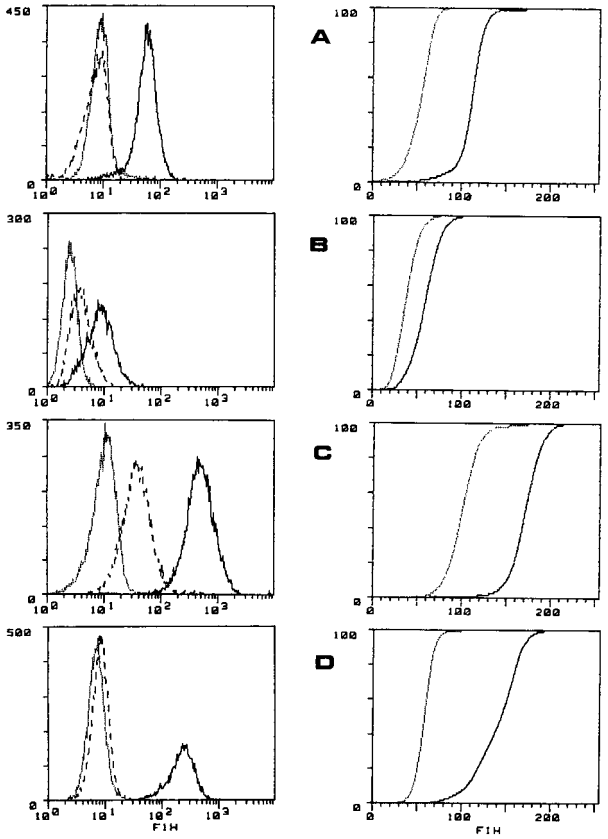

FIG. 1. Papain digestion results in the loss of MHC class I-associated fluorescence. RMA (panel **A**), B16F1 (panel **B**), Jurkat (panel **C**), and M14 (panel **D**) cells were stained by indirect immunofluorescence with saturating amounts of antihuman or murine MHC class I mAbs, followed by saturating amounts of FITC conjugated goat antimouse IgG1 mAb, in the absence (solid line) or in the presence (dashed line) of papain (1 mg/ml). Background fluorescence was obtained with the second-step reagent only (dotted line). The difference between papain-treated and untreated cells was always statistically significant, based on the Kolmogorov-Smirnov algorithms: the D/s(n) values were respectively 66.61 (RMA), 28.55 (B16F1), 66.58 (Jurkat), and 67.96 (M14). X axis: fluorescence intensity (Arbitrary Units). Y axis: cell number.

The MHC-associated fluorescence decreased in a dose- (Fig. 3) and time-dependent (Fig. 4, top) fashion. The calculated correlation coefficient of linear regression (digestion time versus MFI) was always higher than 0.985.

We did not observe any variation in the time kinetic of MHC digestion when mAbs recognizing different epitopes on the MHC complex were used (Fig. 4, bottom).

β_2-m and peptides became detectable by HPLC in the digestion buffer when its pH was brought to 2.1 (Fig. 5, top panel). On the contrary, neither β_2-m nor peptides were detected in the supernatants of cells incubated in the digestion buffer without papain (Fig. 5, top, dotted line).

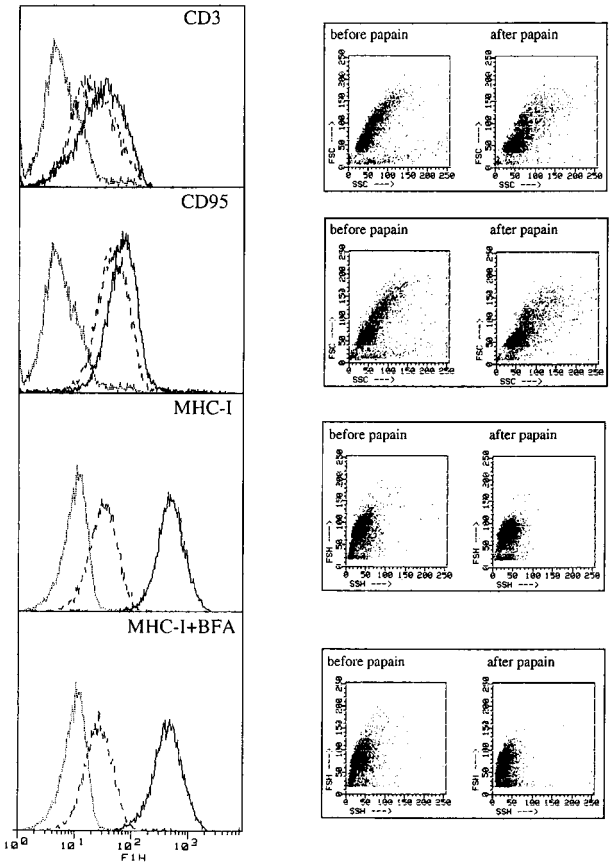

FIG. 2. Papain treatment results in MHC class I digestion but does not affect CD3 or CD95 membrane expression. CD3, CD95, and MHC class I membrane expression was assessed before (solid line) or after (dashed line) papain digestion of Jurkat cells. In the bottom panel, MHC class I digestion was carried out in the presence of Brefeldin A (5 μg/ml). X axis: fluorescence intensity (Arbitrary Units). Y axis: cell number. Dot plots illustrate the physical characteristics (FSC versus SSC) of the cells before or after the papain digestion.

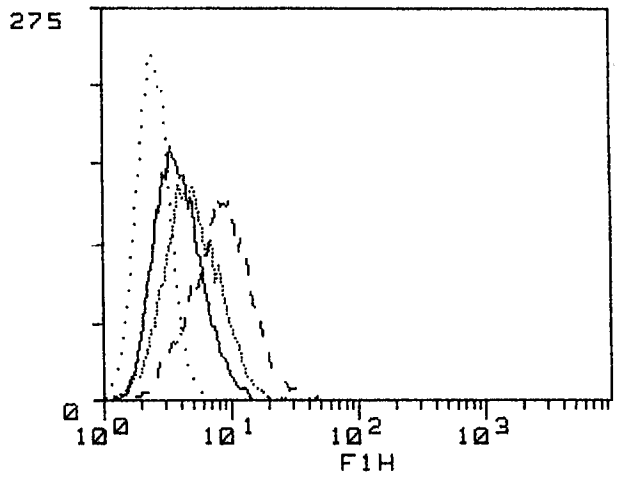

FIG. 3. MHC digestion depends on the concentration of papain. B16-F1 cells were digested in the absence (dashed line) or in the presence of 4 mg/ml (solid line) or of 0.5 mg/ml papain (heavily dotted line). Background fluorescence was obtained with the second-step reagent only (lightly dotted line). X axis: fluorescence intensity (Arbitrary Units). Y axis: cell number.

The above data support the notion that papain releases MHC complexes from the cell membrane, as detected by the decrease of the MHC associated fluorescence evaluated by flow cytometry. Fig. 5 (bottom panels) illustrates the comparison of the HPLC analysis of the digestion buffer with synthetic peptide standards or with purified β_2-m. The availability of purified β_2-m allowed to quantitate the yield of MHC complexes after papain digestion, based on the area/pmol of injected β_2-m. In the experiment shown in Fig. 5, a hundred pmoles of β_2-m (and therefore of MHC soluble complexes) were recovered after digestion of 125 million cells, providing a yield of 0.8 attomoles per cell. As the fluorescence per cell value changed upon digestion from 120 to 64. Each fluorescence unit accounted for 0.0143 attomoles of MHC. Amino acid analysis of the material obtained by collection of the peptide-containing region of the chromatogram confirmed the peptidic nature of the material and provided a further tool for quantification of the purified MHC complexes (not shown).

Solubilized MHC molecules in the papain-treated cell supernatant were purified by affinity chromatography. Fig. 6 (top) shows the western blot of the acid-treated digestion buffer and of the material eluted from the MHC-specific affinity column. β_2-m was present in the digestion buffer and was quantitatively retrieved after affinity chromatography. In Fig. 6 (bottom), total papain released peptides were analyzed either before (solid line) or after (dotted line) affinity chromatography. HPLC profiles of the peptides were strikingly similar.

DISCUSSION

In this report we describe a novel procedure for the quantitative analysis of MHC class I molecules by flow cytometry. MHC class I molecules are peculiar membrane receptors in that they are heterotrimers composed of 2 invariant molecules, the heavy chain and the β_2-m, noncovalently associated to a wide array of different peptides (1, 13, 2, 15, 29). The immunogenicity of spontaneous neoplasms correlates with the amount of MHC class I molecules on the cell surface; their down-regulation favors the escape of tumors from the immune surveillance and will probably influence the results of antineoplastic vaccination procedures. Current approaches for quantitation of membrane molecules by flow cytometry relies on the quantitation of bound antibodies per cell (36, 37, 8). This implies that every membrane molecule bound the antibody with identical affinity. This may not be the case for MHC molecules, as data derived by crystallization and X-ray diffraction of ''empty'' MHC molecules complexed with different synthetic peptides demonstrate that associated peptides contribute to the MHC complex conformation (12). Quantitation by flow cytometry of the total MHC class I and class II membrane expression per cell is therefore a challenge, because MHC complexes stabilized

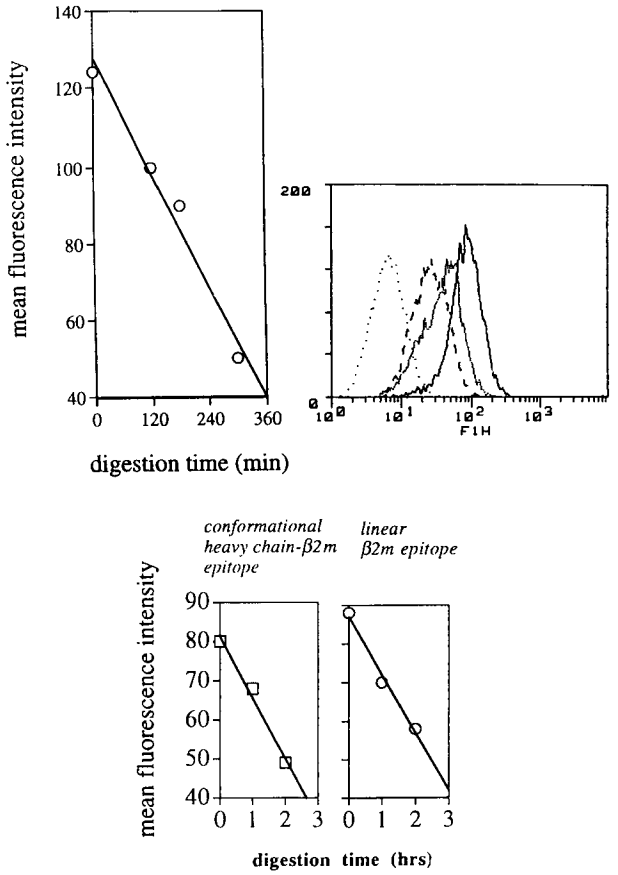

FIG. 4. MHC digestion is time dependent. **Top panels:** B16-F1 cells were stained in the absence (solid line) or in the presence of 2 mg/ml papain for 120′ (heavily dotted line), for 180′ (dashed line), for 300′ (lightly dotted line). The background fluorescence value was obtained with the second-step reagent only. X axis: fluorescence intensity (Arbitrary Units). Y axis: cell number. **Bottom panels:** to assess the time kinetic of the digestion with mAbs specific for different epitopes on the MHC complex, Jurkat cells were stained by indirect immunofluorescence with saturating amounts of the W6/32 mAb, recognizing a conformational epitope of the heavy chain/β_2-m complex (**left panel**) or with the BBM.1 mAb recognizing an epitope on the β_2-m (**right panel**), followed by saturating amounts of FITC conjugated goat antimouse IgG1 mAb. MFI values were assessed after 60′ and 100′ digestion.

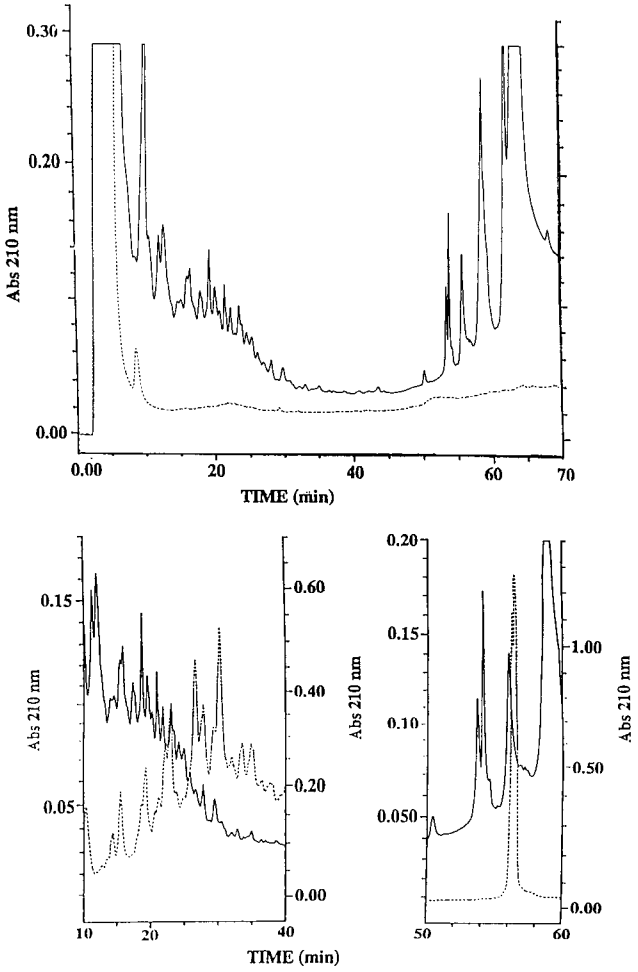

FIG. 5. Papain digestion releases complexes containing β_2-m and peptides from the cell membrane. HPLC analysis was performed on the digestion buffer, either in the presence (solid line) or in the absence (dotted line) of 2 mg/ml papain, as described in the Materials and Methods section (**top panel**). Peptides and the β_2-m retention times were identified by comparison with synthetic peptide mixtures and of purified β_2-m. **Bottom left panel,** comparison of the 10–40 min range digestion buffer (solid line) with a mixture of 41 synthetic peptides 1–2 kDa (dotted line). **Bottom right panel,** comparison of the digestion buffer (solid line) with purified β_2-m standard (dotted line). Standard β_2-m retention time: 56 min.

by different peptides may be differently recognized by MHC-specific mAbs (22; see also 9). The mere assessment of the antibody-bound fluorescence may therefore bias the results, due to the loss of poorly recognized complexes. It would therefore be convenient to be able to correlate the cytometric assessment of the antibody-bound fluorescence with the biochemical quantitation of the amount of membrane MHC molecules.

To address this issue we used a combined cytometric/HPLC procedure that allows one to convert the MHC-associated fluorescence in amount of MHC molecules per cell. We relied on the well-described property of the enzyme papain to cleave the extracellular hydrophilic portion of MHC complexes from crude membrane preparations (35, 24). We speculated that papain digestion of living cells could result in a quantitative decrease of the MHC-associated membrane fluorescence, obtained by staining with specific mAbs. The decrease should, in this scenario, be accompanied by the release of cleaved MHC molecules in the digestion buffer. Our speculation indeed proved correct, and soluble MHC molecules could easily be quantitated by HPLC analysis and comparison with purified β_2-m standards.

Papain is a nonspecific protease that may potentially affect other molecules on the cell membrane and particularly MHC class II molecules and B cell receptors. The lines we used in this study, however, are not of B cell origin; neither are they express class II molecules, as detected by staining with human and murine class II specific mAbs (not shown). Furthermore, papain digestion does not influence the fluorescence associated to MHC-unrelated membrane molecules, like the CD3 complex or the CD95

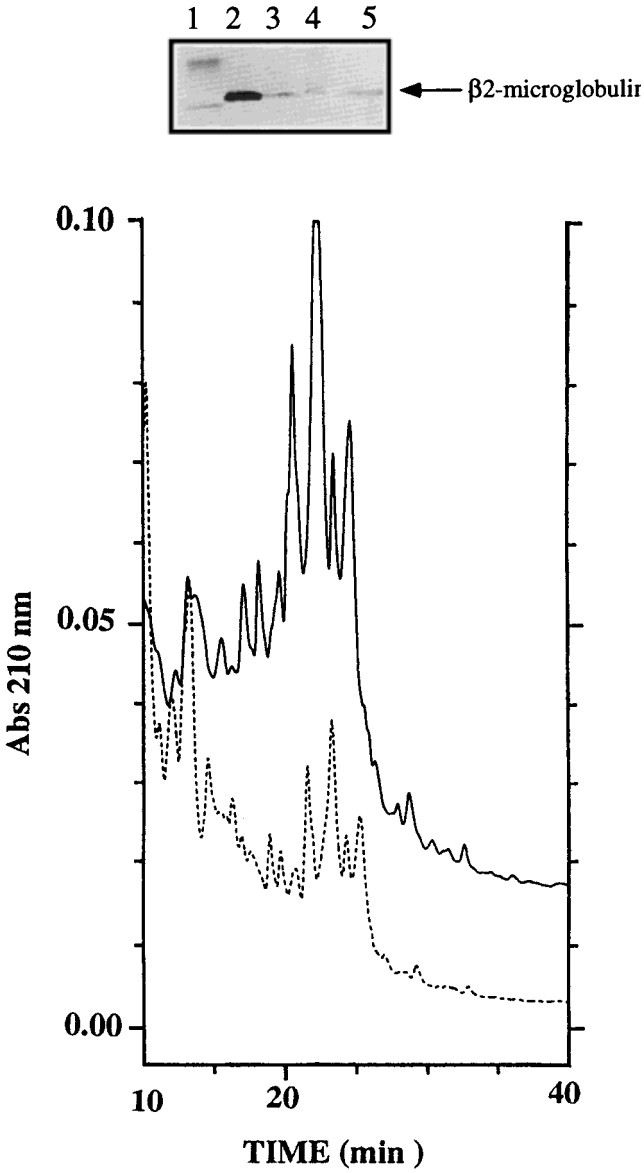

FIG. 6. β_2-m containing complexes can be retrieved by affinity chromatography of the digestion buffer, as detected by western blotting. **Top panel:** the material recovered after papain digestion and acid extraction (**lane 3**) and the flow-through (**lane 4**) or the material eluted at pH 11.5 from an affinity chromatography resin conjugated with the W6/32 mAb (**lane 5**) were run on an SDS polyacrilamide (15%) gel and blotted with the BBM.1 mAb, as described in the Materials and Methods section. **Lane 1:** molecular weight markers (upper, 18.9 kDa; lower, 7.8 kDa). **Lane 2:** purified β_2-microglobulin (standard). **Bottom panel:** comparison between the HPLC profiles of peptides obtained after acid extraction of total papain-released molecules (solid line) or of affinity purified MHC molecules (dotted line).

receptor; in turn, it is not affected by the turnover of MHC class I molecules, as the digestion gives similar results in the absence or in the presence of Brefeldin A, an agent that interferes with the intracellular protein trafficking trough the Golgi complex, preventing the expression of newly synthesized MHC molecules on the cell membrane.

Cleaved MHC complexes can be dissociated in the single components by acid treatment, giving raise to β_2-m and peptides. They are unlikely to derive from material secreted or expelled from ruptured cells, because neither β_2-m nor peptides could be detected in the absence of the enzyme (Fig. 5, dotted line). Finally, cleaved complexes can be retrieved by MHC class-I specific affinity chromatography of the digestion buffer. The affinity-purified complex had an apparent molecular weight of 45 kDa (not shown), compatible with the molecular weight reported for papain cleaved MHC class I molecules (3, 24). Affinity-purified acid-treated complexes still gave origin to β_2-m and peptides (see western blot and HPLC analysis shown in Fig. 6). This also makes it unlikely that further proteolytic digestion of the complex may take place in the experimental conditions we describe.

Quantitation of released β_2-m provides information on the actual MHC expressed on the membrane, independently of the affinity of antibodies for MHC complexes stabilized by different peptides; the ratio between the retrieved MHC molar amount and the loss of MHC-associated fluorescence upon enzymatic digestion provides a novel ''conversion factor'' to transform the qualitative measurement of a fluorescence (arbitrary units) in numbers of membrane MHC molecules per cell.

The procedure we describe is relevant for quantitation of total membrane MHC molecules. It may also potentially be extended to the assessment of the relative peptide occupancy of the total MHC molecule repertoire, using synthetic peptide sequences as standards in association with the purified β_2-m after affinity chromatography purification of the cleaved MHC moieties. Finally, it provides an easy tool to monitor by flow cytometry the enzymatic digestion of cells: it allows one to stop the reaction before cell death occurs and to prevent the release of ''immature,'' putatively chaperonin-associated MHC molecules. After papain solubilization, MHC complexes can be purified by affinity chromatography and provide an optimal substrate for further physicochemical analysis of mature MHC molecule-associated peptides by capillary electrophoresis and mass spectrometry in the absence of any detergent contaminant.

LITERATURE CITED

1. Barber LD, Parham P: Peptide binding to major histocompatibility complex molecules. Annu Rev Cell Biol 9:163–206, 1993.
2. Benham A, Tulp A, Neefjes J: Synthesis and assembly of MHC-peptide complexes. Immunol Today 16:359–362, 1995.
3. Bjorkman PJ, Saper MA, Samraoui B, Bennet WS, Strominger JL, Wiley DC: Structure of the human class I histocompatibility antigen, HLA-A2. Nature 329:506–512, 1987.
4. Cerundolo V, Elliot T, Elvin J, Bastin J, Rammensee HG, Townsend A: The binding affinity and dissociation rates of peptides for class I major histocompatibility complex molecules. Eur J Immunol 21:2069–2075, 1991.
5. Demaria S, Schwab R., Gottesman RS, Bushkin Y: Soluble β_2-microglobulin-free class I heavy chains are released from the surface of activated and leukaemia cells by a metalloprotease. J Biol Chem 269:6689–6694, 1994.
6. Dobbe LME, Stam NJ, Neefjes JJ, Giphart MJ: Biochemical complexity of serum HLA class I molecules. Immunogenetics 27:203–210, 1988.
7. Duperray C, Klein B, Durie BGM, Zhang X, Jourdan M, Poncelet P, Favier F, Vincent C, Brokier J, Lenoir G, Bataille R: Phenotypic analysis of human myeloma cell lines. Blood 73:566–572, 1989.
8. Dux R, Kindler-Rohrborn A, Lennartz K, Rajewsky MF: Calibration of fluorescence intensities to quantify antibody binding surface determi-

nants of cell subpopulations by flow cytometry. Cytometry 12:422–428, 1991.
9. Engelhard VH: Structure of peptides associated with class I and class II MHC molecules. Annu Rev Immunol 12:181–207, 1994.
10. Fermo I, De Vecchi E, Diomede L, Paroni R: Serum amino acids analysis with pre-column derivatization: comparison of the o-phthaldaldehyde and N,N-diethyl-2,4-dinitro-5-fluoraniline methods. J Chromatogr 534:23–36, 1990.
11. Ferrarini M, Heltai S, Toninelli E, Sabbadini MG, Pellicciari C, Manfredi AA: Daudi lymphoma killing triggers the programmed death of cytotoxic Vγ9/Vδ2 T lymphocytes. J Immunol 154:3704–3712, 1995.
12. Fremont DH, Matsumura M, Stura EA, Peterson PA, Wilson IA: Crystal structures of two viral peptides in complex with murine MHC class I H-2K^b. Science 257:919–927, 1992.
13. Germain RN, Margulies DH: The biochemistry and cell biology of antigen processing and presentation. Annu Rev Immunol 11:403–450, 1993.
14. Hausmann R, Zavazava N, Steinmann J, Muller-Ruchholtz W: Interaction of papain digested HLA class I molecules with human alloreactive cytotoxic T lymphocytes (CTL). Clin Exp Immunol 91:183–188, 1993.
15. Heemels MT, Ploegh H: Generation, translocation and presentation of MHC class I restricted peptides. Annu Rev Immunol 64:463–491, 1995.
16. Lavabre-Bertrand T, Duperray C, Brunet C, Poncelet P, Exbrayat C, Bourquard P, Lavabre-Bertrand C, Brochier J, Navarro M, Janossy G: Quantification of CD24 and CD45 antigens in parallel allows a precise determination of B-cell maturation stages: relevance for the study of B-cell neoplasias. Leukemia 8:402–408, 1994.
17. Lavabre-Bertrand T, Exbrayat C, Bourquard P, Lavabre-Bertrand C, Fegueux N, Poncelet P, Rousset T, Taib J, Emberger JM, Navarro M: CD23 antigen density is related to serum gamma globulin level, bone marrow reticulin pattern, and treatment in B chronic lymphocytic leukaemia. Leuk Lymphoma 13:89–94, 1994.
18. Manfredi AA, Yuen MH, Raftery MA, Conti-Tronconi BM: An assay for simultaneous multiple determinations of peptide binding to MHC class II molecules. Analytical Biochem 211:1–7, 1993.
19. Matzinger P: Tolerance, danger, and the extended family. Annu Rev Immunol 12:991–1045, 1994.
20. Melnick J, Argon Y: Molecular chaperones and the biosynthesis of antigen receptors. Immunology Today 16:243–250, 1995.
21. Miller SG, Carnell L, Moore HPH: Post-Golgi membrane traffic: Brefeldin A inhibits export from distal Golgi compartments to the cell surface but not recycling. J Cell Biol 118:267–283, 1992.
22. Murphy DB, Rath S, Pizzo E, Rudensky AY, George A, Larson JK, Janeway CA: Monoclonal antibody detection of a major self peptide MHC class II complex. J Immunol 148:3483–3491, 1992.
23. Oonishi T, Uyesaka N: A new standard fluorescence microsphere for quantitative flow cytometry. J Immunol Methods 84:143–154, 1985.
24. Parham P, Alpert BN, Orr HT, Strominger JL: Carbohydrate moiety of HLA antigens. J Biol Chem 252:7555–7567, 1977.
25. Parker KC, Dibrino M, Hull L, Coligan JE: The β_2-microglobulin dissociation rate is an accurate measure of the stability of MHC class I heterotrimers and depends on which peptide is bound. J Immunol 149:1896–1904, 1992.
26. Pellicciari C, Manfredi AA, Bottone MG, Schaak V, Barni S: A single-step staining procedure for the detection and sorting of unfixed apoptotic thymocytes. Eur J Histochem 37:381–390, 1993.
27. Poncelet P, Carayon P: Cytofluorimetric quantification of cell-surface antigens by indirect immunofluorescence using monoclonal antibodies. J Immunol Methods 85:65–74, 1985.
28. Puppo F, Scudeletti M, Indiveri F, Ferrone S: Serum HLA class I antigens: markers and modulators of an immune response? Immunol Today 16:124–127, 1995.
29. Rammensee HG, Falk K, Rötzschke O: Peptides naturally presented by MHC class I molecules. Annu Rev Immunol 11:213–244, 1993.
30. Rovere P, Clementi E, Ferrarini M, Heltai S, Sciorati C, Sabbadini MG, Rugarli C, Manfredi AA: CD95 engagement releases calcium from intracellular stores of long term activated, apoptosis-prone γδ T cells. J Immunol (1996), in press.
31. Rugarli C, Scorza-Smeraldi R, Sabbadini-Villa MG: Unblocking of T lymphocytes. Lancet i:1297–1298, 1975.
32. Rugarli C, Scorza-Smeraldi R, Sabbadini-Villa MG, Pellò F: HLA antigens in serum from bone marrow harvested for diagnostic purposes. Biomed 25:100–104, 1976.
33. Schneck J, Maloy WL, Coligan JE, Margulies DH: Inhibition of an allospecific T-cell hybridoma by soluble class I proteins and peptides: estimation of the affinity of a T-cell receptor for MHC. Cell 56:47–55, 1989.
34. Shockley R, Lin K, Sung C, Nagy JA, Tompkins RG, Dedrick RL, Dvorak HF, Yarmush ML: A quantitative analysis of tumor specific monoclonal antibody uptake by human melanoma xenografts: effects of antibody Immunological properties and tumor antigen expression levels. Cancer Res 52:357–366, 1992.
35. Springer TA, Strominger JL: Detergent-soluble HLA antigens contain a hydrophilic region at the COOH-terminus and a penultimate hydrophobic region. Proc Natl Acad Sci USA 73:2481–2485, 1976.
36. Thibault G, Bardos P: Compared TCR and CD3ε expression on αβ and γδ T cells. J Immunol 154:3814–3820, 1995.
37. Vogt RF, Cross GD, Henderson O, Philips DL: Model system evaluating fluorescein-labelled microbeads as internal standard to calibrate fluorescence intensity on flow cytometers. Cytometry 10:294–302, 1989.
38. Yewdell J, Bennink JR: Brefeldin A specifically inhibits presentation of protein antigens to cytotoxic T lymphocytes. Science 244:1072–1075, 1989.
39. Young YT: Proof without prejudice: use of the Kolmogorov-Smirnov test for analysis of histograms from flow systems and other sources. J Histochem Cytochem 25:935–941, 1977.
40. Zavazava N, Hausmann R, Muller-Rucholz W: Inhibition of anti-HLA-B7 alloreactive CTL by affinity purified soluble HLA. Transplantation 51:838–842, 1991.