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Low abundance of naturally processed T-cell determinants following antigen pulsing of antigen-specific human B-cell lines

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SUMMARY

An attempt was made to recover naturally processed T-cell determinants following antigen pulsing of tetanus toxin-specific human B-cell clones with μg/ml amounts of antigen. Class II major histocompatibility complex (MHC) molecules were isolated from cells pulsed under optimal conditions and the eluted peptides displayed by reverse-phase high-performance liquid chromatography (HPLC). Antigen-pulsed and control-unpulsed cells showed virtually identical optical density $(OD)_{215}$ profiles, although multiple peptides derived from the input antigen could be identified at the radiochemical level. At least four distinct HPLC fractions contained naturally processed versions of the determinant 830–844, detected using the specific T-cell clone Mix 111. Quantification of the most active fraction indicated that approximately 1 pmole of this determinant was recovered from $\sim 5 \times 10^9$ antigen-pulsed cells. Based on the amount of antigen processed following uptake on membrane immunoglobulin, and quantification of the biologically active material recovered, it was estimated that the efficiency of determinant capture was no greater than 1–2%. Further method development and a considerable increase in the number of cells used ($>10^{10}$) would appear to be necessary before naturally processed determinants from exogenously pulsed antigens can be reliably and fully characterized. Finally, a theoretical analysis showed that accurate (± 0·01%) mass information alone could identify a limited number of candidate peptides from known or putative antigens.

INTRODUCTION

Analysis of naturally processed peptides bound to major histocompatibility complex (MHC) has revealed important information about the structure of these peptides and clues about the nature of the cellular processing mechanisms which generate them.[1–3] The peptides analysed so far on class II MHC have mostly been the dominant chemical species and are derived from constitutively processed self-peptides, including MHC molecules themselves, or from abundant serum proteins.[1–3] Little information is available on the abundance and composition of peptides bound to class II MHC following pulsing with authentic exogenous antigens. So far, data have only been reported in the murine system and only for cells pulsed with hen egg lysozyme (HEL).[4–6] Two reports have characterized naturally processed determinants containing the core sequence 52–61 in association with the murine class II MHC molecules A^k.[5,6] T-cell hybridomas were used to follow specific antigenic determinants in chromatographic fractions.[5,6] The large numbers of cells ($\sim 5 \times 10^{10}$) and high concentrations of native protein used in these experiments would not usually be available. Thus for many applications, for example in attempts to isolate putative pathogenic peptides in autoimmune disease models, more efficient uptake and processing of the limited antigen or putative autoantigen available is desirable. In principle, surface immunoglobulin-mediated protein uptake into B cells might dramatically reduce the scale of the experiment required to allow naturally processed peptides to be followed, firstly because of concentrative antigen uptake through specific cell-surface receptors, and secondly as a result of efficient antigen targeting to the peptide-loading compartment.[7–9] Using relatively low numbers of antigen-specific B lymphoblastoid cells and μg/ml levels of antigen, we have tested how readily determinants derived from native antigen can be recovered. We found that naturally processed antigenic peptides eluted from human class II MHC can be followed in first-dimension and in some subsequent second-dimension high-performance liquid chromatography (HPLC) separations using low cell numbers ($\sim 10^9$), optimized antigen-pulsing conditions and specific T cells to identify active fractions. However, the data suggest that considerably larger scale experiments are likely to be required to generate sufficient naturally processed material to allow reproducible second-dimension HPLC and subsequent mass spectrometric analysis. In the absence of sufficient material for direct sequence analysis, we assessed whether, in principle, the presence of a

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unique mass peak in complex fractions from antigen-pulsed versus unpulsed cells would allow unequivocal identification of a peptide from the input antigen. We show that a limited number of candidate peptides is identified when mass measurements accurate to 0·01% or greater are available.

MATERIALS AND METHODS

Cells

Cell clones were generously provided by A. Lanzavecchia (Basel Institute, Switzerland). Epstein–Barr virus (EBV) transformed tetanus toxin-specific cells were grown in RPMI-1640 (Gibco, Paisley, UK) supplemented with 1% non-essential amino acids, 1 mM pyruvate, kanamycin, 50 μM β-mercaptoethanol and 10% fetal calf serum (Advanced Protein Products, Brierly Hill, UK). Ten to twenty litres of clone 4.9 cells were grown in roller bottles (Falcon, Oxford, UK) to densities of $0{\cdot}75–1{\cdot}5 \times 10^6$/ml. Tetanus toxin-specific T-cell clones were maintained as described previously.[10] Clone KS Mix 111 recognizes the previously described P2 peptide comprising residues 830–844 of tetanus toxin[11] bound to DR5 (1104).

Antibodies and reagents

The L243 hybridoma was obtained from ECACC (Salisbury, UK). Antibody was purified from ascites fluid using protein A–Sepharose. Immunoaffinity beads were prepared by covalently coupling L243 to protein A–Sepharose by the dimethyl pimelimidate method[12] at a ratio of 5 mg of antibody/ml Sepharose. Western blotting of class II MHC was performed using the α chain-specific antibody DA6.147.[19]

Immunopurification of class II MHC

After antigen pulsing, $\sim 7{\cdot}5 \times 10^9$ cells were harvested using a Beckman JA-10 rotor at 2000 r.p.m. for 10 min at 4°. The cells were washed once in phosphate-buffered saline (PBS)/bovine serum albumin (BSA) at 0° and resuspended in 40 ml ice-cold Tris-buffered saline (pH 8·0; TBS) and then mixed with an equal volume of double-concentrated lysis buffer.[13] After incubation at 0° for 45 min, the lysates were clarified by centrifugation (Beckman Ti45 rotor, 30 000 r.p.m., 4°, 45 min). The supernatant was incubated with 8 ml of Pansorbin (Calbiochem, La Jolla, CA) overnight at 4°. Class II MHC was isolated by incubating the lysate with 7 ml of L243/protein A–Sepharose adsorbent for at least 2 hr at 4°. The beads were transferred to a column ($20 \times 2{\cdot}5$ cm) and washed with 200 ml TBS/1% Triton X-100 followed by 100 ml 50 mM NH_4Acetate. Class II MHC was eluted from the beads with 1 M propionic acid. The lysates were re-extracted twice more in the same fashion and the combined propionic acid eluates were lyophilized. This material was taken up in 10% acetic acid, treated at 70° for 15 min, cooled, and fractionated on a Centricon 10 filtration system in order to separate class II MHC glycoproteins from low molecular weight-associated peptide material[1,3] released either by the propionic acid elution or by acetic acid. The filtrate was dried and redissolved in 0·1% trifluoroacetic acid (TFA) for further analysis.

HPLC fractionation of class II peptides

Acid-eluted peptides were resolved on reverse-phase columns (VYDAC C18, MICHROM, Reading, UK; $0{\cdot}46 \times 25$ cm) and developed with 0·1% TFA in acetonitrile/water gradients generated by an Applied Biosystems 140B pump (Applied Biosystems), as detailed in the figure legends. The column effluent was monitored with an Applied Biosystems Model 759A UV/Vis Detector fitted with a 12 μl flow cell, and 250-μl fractions were collected directly into teflon 96-well plates with a Gilson autosampler configured as a fraction collector. When selected samples were rechromatographed under basic conditions, a Vydac 'High pH' reverse-phase column (C8; $0{\cdot}46 \times 25$ cm) was used and peptides were eluted with gradients of acetonitrile in water containing 0·1% hexafluoroacetone (Aldrich) adjusted to pH 8·0 with NH_4OH.[14]

Reconstitution of T-cell determinants after HPLC

After resolution of MHC class II peptides by reverse-phase HPLC as described above, 10-μl aliquots from each well of the 96-well plates were transferred to the wells of standard 96-well plates containing 200 μl complete RPMI-1640 growth medium. Alternatively, known amounts of the synthetic peptide 830–844 were added under identical conditions. The plates were then placed in a tissue culture incubator at 37° for 2–3 hr to allow evaporation of volatile components prior to the addition of 1×10^5 mitomycin-treated FC 4.9 cells in 50 μl complete growth medium. Antigen-presenting cells and peptide were left in contact overnight in the incubator prior to the addition of 4×10^4 T cells. After 48 hr the cultures were pulsed with 1 μCi [^{3}H]thymidine for 16 hr and harvested onto glass fibre filter mats using a Scatron harvester. Quantification was by liquid scintillation counting.

Laser desorption–time of flight (LD TOF) mass spectroscopy

HPLC fractions containing active peptide fragments were concentrated 25-fold by dessication and readdition of 10 μl acetonitrile/water 0·1% TFA. One microlitre of the resulting peptide mixture was mixed with 1 μl α-cyano-4-hydoxycinnamic acid and subjected to LD TOF on a Vestec Lasertec Mass Spectrometer, operating at an accelerating voltage of 25 kV and detector voltage of 3·4 kV. Calibration of the machine was achieved using known peptides in the 1000–4000 molecular weight (MW) region.

Accurate mass information identifies limited sets of candidate peptides from known antigens

A computer program was written in order to search the native tetanus toxin sequence for peptide sequences of a certain mass within a given range. The program takes a mass value accurate to within a fixed error range and then finds all the possible sequences consistent with these values. Input of calculated rather than experimentally derived masses allows the degree of accurately necessary for unambiguous identification of the input peptide to be achieved. For example, when the calculated mass of the tetanus toxin peptide 1174–1188 (mass = 1838·0945) was used, 27 peptides between 14 and 17 residues in length were identified if the data were accurate to 0·1% (i.e. 1838·0945 ± 1·84). One of these was the correct sequence. See Table 2 and text for further examples.

RESULTS

Optimization of antigen pulsing

We first tested the various B-cell lines available to see which

expressed the highest level of membrane immunoglobulin specific for tetanus toxin. Clone FC4 [DR 3 (0301); DR5 (1104)] bound up to three times as much antigen as cell lines used in previous studies (data not shown).[15] Following pulsing with radioiodinated antigen, recovery of iodinated peptides on immunoisolated class II MHC[16] was also several times greater for this clone in proportion to the increased number of antigen receptors (Table 1). Consequently, clone FC4 (subclone 4.9) B cells were chosen for antigen-pulsing studies. To optimize the conditions of antigen pulsing, we had to consider the time taken for complexes to appear as well as their turnover rate. Following antigen uptake, loaded class II MHC molecules were first detectable after 30 min of processing.[7,16] As shown in Fig. 1, naturally processed labelled peptides remained stably bound to class II MHC molecules decaying with a half-life of approximately 30 hr, in very good agreement with our earlier estimates of peptide lifetime which followed either the fate of exogenously pulsed peptides or constitutively processed and presented material.[17] Continuous exposure to antigen may not make the most efficient use of available antigen for two reasons. First, the high affinity of membrane immunoglobulin for antigen precludes receptor reutilization.[18] Second, the cells secrete soluble immunoglobulin of the same specificity into the bathing medium which complexes free antigen. We tested two different protocols of antigen pulsing. Cells (1×10^7/ml) were preincubated at 0° with 2 μg/ml antigen for 90 min, and then diluted 10-fold without further addition of antigen and chased at 37° for either 10 or 20 hr (protocol 1). Alternatively, cells (5×10^6/ml) were incubated continuously in the presence of 10 μg/ml antigen at 37° for the same times (protocol 2). The cells were then harvested, fixed briefly and graded numbers tested in a T-cell presentation assay. Cells pulsed under protocol 2 were only twice as potent when graded numbers were titrated as antigen-presenting cells (data not shown), despite the fact that the ratio of input antigen to cells was 10 times greater under this regime, illustrating the problem created by secreted antibody. Nonetheless, to maximize the likelihood of recovering naturally processed tetanus toxin peptides protocol 2 was used.

Isolation of class II MHC-bound peptides from antigen-pulsed cells

Pilot experiments showed that class II MHC molecules immunoisolated on L243 Sepharose beads could be eluted with 1 M propionic acid without deleterious effects on the affinity matrix. Cells were lysed and class II MHC isolated as described in the Materials and Methods. The beads were treated sequentially with NH_4Ac to remove detergent, followed by propionic acid. As shown in Fig. 2, essentially all the class II MHC had been removed from the cell lysate by the third sequential L243 adsorption and propionic acid elution. Routinely, two sequential extractions were pooled for subsequent analysis. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) of the eluted material revealed bands of the expected size for the α and β chains of class II MHC (Fig. 3). Western blotting using the α chain-specific monoclonal antibody DA6.147[19] confirmed the identity of the purified material (data not shown). Approximately 8 mg of class II MHC was recovered per 10^{10} cells.

Table 1. Class II MHC peptide loading correlates with B-cell antigen receptor density

	ALT1.1*	A46	FC4.9
	c.p.m. $\times 10^{-3}$/(sites/cell)		
^{125}I-labelled tetanus toxin binding to cells at 4°†	34·3 (0)	358·8 ($1{\cdot}2 \times 10^4$)	1084·0 ($4{\cdot}02 \times 10^4$)
^{125}I-labelled peptides bound to class II MHC molecules‡	0·1	1·4	5·7

* ALT1.1 are non-specific EBV-transformed human lymphoblastoid cells, whereas A46 and FC4.9 cells are tetanus toxin specific.

† $8{\cdot}9 \times 10^5$ cells were washed twice in PBS/BSA at 4° and resuspended in PBS/BSA containing 2 μg/ml ^{125}I-labelled tetanus toxin ($1{\cdot}2 \times 10^5$ c.p.m./ng) in either the absence or presence of a 20-fold excess of unlabelled tetanus toxin. After 90 min on ice the cells were washed three times by centrifugation in PBS/BSA at 4° and bound antigen quantified by γ-counting. Binding was reduced by 85–90% on the antigen-specific clones. Values in parentheses indicate the number of antigen receptors per cell after subtraction of the binding obtained on the non-specific clone ALT1.1.

‡ $8{\cdot}9 \times 10^6$ cells were harvested, washed and pulsed with ^{125}I-labelled tetanus toxin as described above and, subsequent to washing in PBS/BSA, were chased for 6 hr at 37° in 10 ml complete growth medium. The cells were washed and lysed prior to immunoprecipitation of class II MHC, as described in the Materials and Methods. Class II was eluted from the affinity beads, with 2% SDS, as previously reported,[16] and electrophoresed on a 12·5% SDS polyacrylamide gel. After autoradiography bands corresponding to stable $\alpha\beta$ complexes (63 000 MW) and small peptide material (< 16 000 MW) were excised from the gel and assayed by γ-counting. The values expressed are the summed c.p.m. for both stable $\alpha\beta$ complexes and smaller radiolabelled species; the final values have been divided by 10 to enable comparison with values obtained in the binding studies described above.

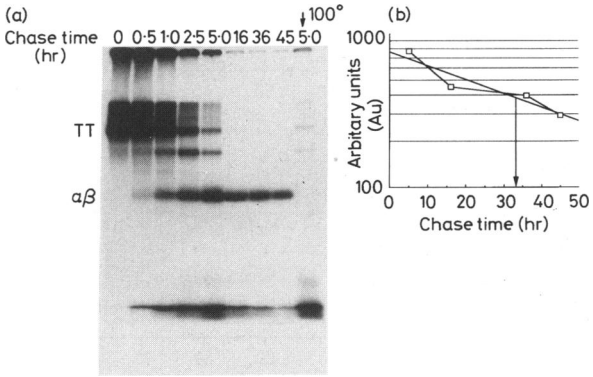

Figure 1. Persistence of naturally processed antigen-derived peptides following antigen pulsing. (a) Clone FC4 cells were incubated with labelled antigen at 0°, washed and chased at 37°. At the times shown an aliquot of cells was removed, class II MHC molecules precipitated and analysed by SDS–PAGE without boiling.[16] Loaded $\alpha\beta$ dimers (~63 000 MW) were first seen after 30 min chase, and persisted for several days. Note, at earlier chase times a background of co-precipitated intact antigen [tetanus toxin (TT) = 150 000 MW] was visible. (b) SDS-stable $\alpha\beta$ dimers were excised and associated radioactivity was quantified by γ-counting and plotted on a semi-log scale against time.

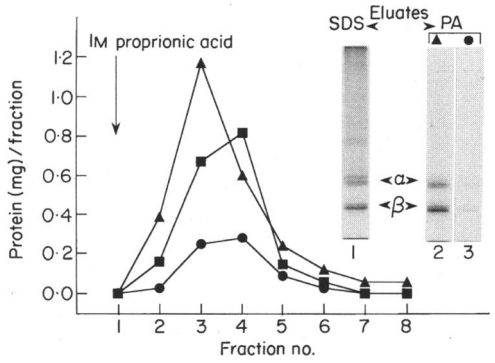

Figure 2. Propionic acid elution of class II MHC from affinity matrices. $7{\cdot}5 \times 10^9$ cells were harvested, washed and lysed as described in the Materials and Methods. After preclearing with Pansorbin, class II MHC was extracted with L243 covalently coupled to protein A–Sepharose. Detergent and non-volatiles were removed by washing in NH_4Ac and class II eluted with 8 × 3-ml aliquots of 1 M propionic acid as described in the Materials and Methods. Affinity extraction of the lysate was performed three times. (first, ▲; second, ■; third, ●) and each extraction monitored by protein assay as shown. Peak fractions were analysed by SDS–PAGE and stained with Coomassie Blue. PA, propionic acid.

HPLC analysis of MHC-associated peptides

To identify tetanus toxin-derived peptides, we first compared the reverse-phase HPLC profiles of peptide mixtures acid-eluted from class II MHC prepared from either antigen-pulsed or unpulsed cells. Material from unpulsed cells appeared as a complex pattern of components absorbing at 215 nm and eluting between 10% and 30% acetonitrile. As shown in Fig. 3,

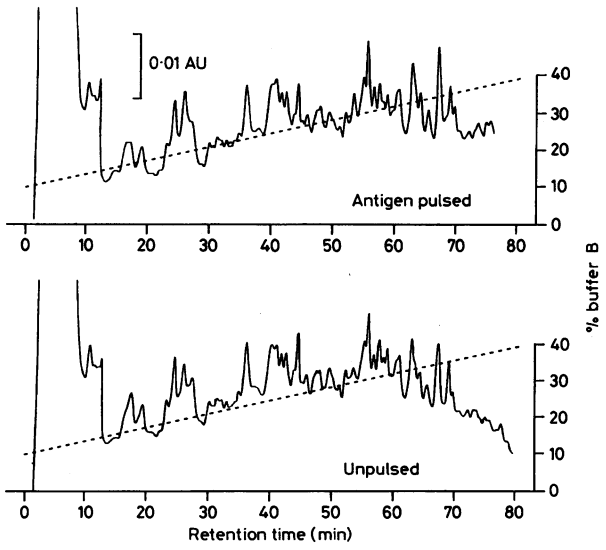

Figure 3. Reverse-phase HPLC profiles of class II MHC-associated peptides recovered from antigen-pulsed and unpulsed cells. $7{\cdot}5 \times 10^9$ FC4 cells were pulsed with 10 mg tetanus toxin in 1000 ml according to protocol 2 (Fig. 2). After immunoisolation of class II MHC molecules (6·4 mg recovered) the associated peptides were acid-eluted and prepared for HPLC analysis as described in Materials and Methods. The gradient was 90% A/10% B, increasing to 50% B over 100 min at a flow rate of 0·5 ml/min. Buffer A was 0·1% TFA, buffer B was acetonitrile/0·1% TFA (8 : 2).

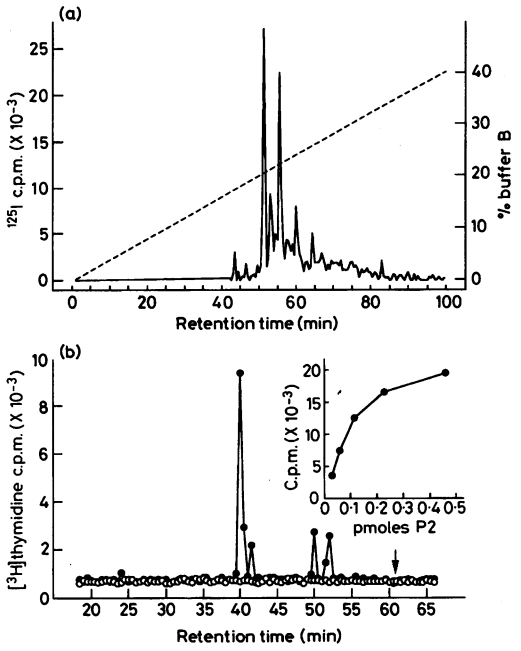

Figure 4. HPLC separation of radiolabelled and biologically active peptides derived from antigen pulsing. (a) 2×10^8 cells were pulsed with ^{125}I-labelled tetanus toxin and the peptides associated with class II MHC molecules displayed by reverse-phase HPLC and fractions collected for γ-counting. (b) $7{\cdot}5 \times 10^9$ cells were pulsed with tetanus toxin and the peptides associated with class II MHC resolved by HPLC and aliquots of individual fractions incubated with mitomycin-treated antigen-presenting cells as described in the Materials and Methods. Alternatively (inset), known amounts of peptide P2 (830–844) were incubated with mitomycin-treated antigen-presenting cells exactly as for HPLC fractions. T cells (clone Mix 111) were then added to aliquots of the peptide-pulsed APC and [^{3}H]thymidine incorporation measured after 48 hr. The retention time of the tetanus toxin-derived index peptide 830–844 is arrowed.

the peptide mixture resolved by HPLC from pulsed cells was virtually indistinguishable from that of unpulsed cells. Thus at the chemical level, no new peaks were evident following pulsing of antigen-specific B cells with tetanus toxin. However, loading of class II MHC in the pulsed cells had clearly occurred, as the cells were potent in antigen presentation assays and, when radiolabelled antigen was used, processed radiolabelled peptides were recovered and could be displayed either on Tris–tricine SDS gels[16] or by HPLC (Fig. 4a). Quantification of the radiolabelled peptides recovered allowed us to estimate the likely abundance of peptides from specific antigen pulsing. Several major radiolabelled species were resolved by HPLC (Fig. 4a). Assuming that the input antigen and the products of natural processing had the same specific radioactivity, the most abundant species (25×10^3 c.p.m.; Fig. 5a) would correspond to approximately 0·1 pmoles of peptide. Accordingly, when scaled up to the level used for pulsing with unlabelled antigen (50-fold increase), it was expected that up to 5 pmoles of the most abundant species from 1×10^{10} pulsed cells would be recovered. As almost 100 nmoles of peptide was recovered from 5 mg of class II MHC in this experiment, it is not surprising that a new species, which most probably constituted less than 0·01% of the total, was not seen in the optical density $(OD)_{215}$ profile.

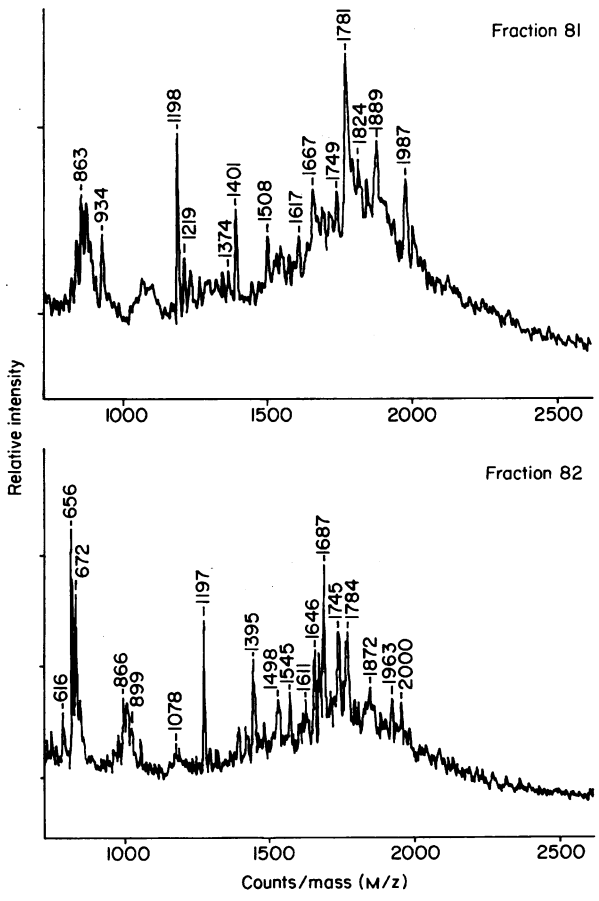

Figure 5. LD TOF mass spectroscopy of first-dimension HPLC fractions. Profiles of typical single fractions from first-dimension HPLC separations. Fractions were concentrated and subjected to LD TOF mass spectrometry as described in the Materials and Methods. Typical spectra for adjacent fractions are shown.

Reconstitution of specific T-cell responses from HPLC-resolved peptide fractions

We next analysed the natural products of antigen processing by screening individual HPLC fractions for their ability to reconstitute the MHC/peptide complexes recognized by specific T-cell clones. In general an aliquot (5%) of each fraction, collected as described in the Materials and Methods, was transferred to growth medium to which mitomycin-treated antigen-presenting cells were added. Each sample was then split depending on the number of T-cell clones to be tested. In initial experiments, proliferative responses for two T-cell clones recognizing different determinants could be reconstituted with HPLC fractions from pulsed cells. Clone KS Mix 111, specific for a determinant mapping between residues 830–844 defined by the P2 peptide[11] in TT, was chosen for further analysis as it gave the most reproducible results. As shown in Fig. 4b, several fractions from pulsed but not from unpulsed cells were able to trigger proliferative responses from this clone. Four per cent of each fraction, corresponding to material from approximately 3×10^8 cells, was sufficient to reconstitute the response. To obtain some idea of the amounts of biologically active peptide present in these first-dimension separations, we constructed a standard curve using known amounts of the 830–844 determinant (P2 peptide) incubated with Mix 111 T cells under the same conditions as the HPLC fractions. As shown in Fig. 4 (inset), we could detect less than 100 fmoles of peptide. Thus the most active fraction, with a retention time of 40 min, would correspond to approximately 80 fmoles of peptide assuming that the naturally processed forms have similar activity to the index peptide 830–844. We base this assumption on the broadly similar potency of peptides containing the minimal determinant for Mix 111 (831–841) but extended by up to three residues at one or other terminus (data not shown) and on other studies which have demonstrated that extensions at the N- or C-termini do not dramatically alter MHC binding or T-cell recognition.[20] Thus, as 4% of each fraction was taken for T-cell assay, we estimated that approximately 2 pmoles of this peptide were present following pulsing of $7{\cdot}5 \times 10^9$ cells.

Further analysis of fractions from first-dimension HPLC separations by LD TOF mass spectrometry revealed many different peptide species, mostly in the 1200–2000 mass range (Fig. 5), suggesting that direct sequencing of these fractions would not be informative. Moreover, LD TOF analysis revealed no reproducible differences between corresponding fractions from antigen-pulsed and unpulsed cells (not shown). This prevented us from adopting the approach of Vignali *et al.*,[6] who detected distinct mass peaks in fractions from HEL-pulsed cells and identified them based on predicted N- and/or C-terminal extensions of the core sequence recognized by the T-cell clone used to monitor the HPLC fractions. We therefore attempted to rechromatograph single biologically active fractions under basic conditions on a pH-stable HPLC column. Fractions were obtained that could still reconstitute a T-cell response, at least in some experiments (data not shown); however, too little 215 nm absorbing material was recovered in these second-dimension separations to permit direct sequence analysis.

Nonetheless, in one experiment we were able to subject an active fraction from pulsed cells and the matched fraction from unpulsed cells to LD TOF analysis. As expected, the complexity of each fraction was reduced compared with first-dimension fractions (Fig. 6). The fraction capable of reconstituting the Mix 111 T-cell response contained a mass peak of 1510·3 that was not seen in the corresponding fraction from non-antigen-pulsed cells (Fig. 6).

Accurate mass measurements define limited peptide sets in known proteins

Since too little material was present to allow further chemical analysis, we tested whether the 1510·3 peak might correspond to the region of the tetanus toxin molecule recognized by T-cell Mix 111 or indeed to any other part of tetanus toxin, by scanning the entire sequence for peptides corresponding to that mass. A computer program was written in order to search the native tetanus toxin sequence for peptide sequences of a certain mass within a given accuracy range. The program takes a mass value accurate to within a fixed percentage error and then finds all the possible sequences consistent with these values. When a mass of 1510·3 was entered with an error of 0·1% (the approximate accuracy of the LD TOF method), 44 peptides in tetanus toxin were identified between 12 and 15 residues in length. One of these included part of the determinant

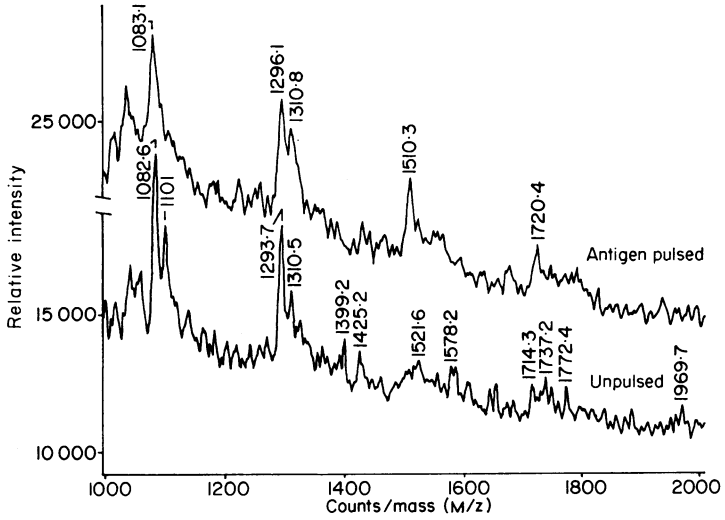

Figure 6. LD TOF mass spectroscopy of biologically active second-dimension HPLC fractions. A fraction able to reconstitute the Mix 111 T-cell response was compared with the corresponding fraction from unpulsed cells by LD TOF analysis. A mass peak at 1510·3 was the only discernible species found to any significant degree in pulsed but not in unpulsed cells.

recognized by T-cell Mix 111, residues 824–836 with a mass of 1509·7798, but this cannot reconstitute the Mix 111 T-cell response (A. Lanzavecchia, personal communication). When an error of 0·2% was programmed, 76 peptides were found, of which two included the Mix 111 determinant, namely 828–840 and 829–841 with masses of 1512·78 and 1512·82. As isoleucine 841 strongly increases binding of this region to the DR5 (1104) MHC molecule[21] and strongly potentiates the Mix 111 T-cell

Table 2. Accuracy of mass measurement and peptide identification

0·1%	0·01%	0·001%	0·0001%
Input GQIGNDPNRDIL			
Mass:13311·4171			
	112 INAIPYLGNSYS 123		
	113 NAIPYLGNSYSL 124		
22 peptides	271 ELFTFGGQDANL 282	271 ELFTFGGQDANL 282	
	388 LLDDTIYNDTE 398		
	922 AIHLVNNESSEV 933	922 AIHLVNNESSEV 933	
	1273 GQIGNDPNRDIL 1284	1273 GQIGNDPNRDIL 1284	1273 GQIGNDPNRDIL 1284
Input QYIKANSKFIGITEL			
Mass:1725·0137			
	16 NDTIIMMEPPYCKGL 30		
	26 YCKGLDIYYKAFK 39		
	98 IKNNVAGEALLDKIIN 113	98 IKNNVAGEALLDKIIN 113	
	145 SGATTKSAMLTNLIIFG 161	145 SGATTKSAMLTNLIIFG 161	
	202 YVPTFDNVIENITSL 216		
	255 SKQEIYMQHTYPIS 268		
	311 SCNDPNIDIDSYKQI 325		
47 peptides	315 PNIDIDSYKQIYQQ 328		
	346 KFQILYNSIMYGFT 359		
	357 GFTEIELGKKFNIKT 371	357 GFTEIELGKKFNIKT 371	
	671 VLLLEYIPEITLPVI 685		
	799 ESSRSFLVNQMINEA 813		
	830 QYIKANSKFIGITEL 844	830 QYIKANSKFIGITEL 844	830 QYIKANSKFIGITEL 844
	831 YIKANSKFIGITELK 845		
	938 AMDIEYNDMFNNFT 951		
	1078 NNNNQYVSIDKFRI 1091		

The mass values indicated, together with a fixed percentage error, were used to search the tetanus toxin sequence. At the 0·1% error level the number of peptides only is indicated. At 0·01% accuracy levels and higher the specific peptides sequences identified in tetanus toxin are listed.

response (A. Lanzavecchia, personal communication), the sequence 829–841 was a strong candidate for the dominant naturally processed sequence. However, it should be stressed that successful detection of active fractions following second-dimension HPLC separations was only achieved in one out of three experiments, underlining the very low abundance of these peptides and the difficulties associated with their recovery.

This analysis raised the question of what degree of accuracy of mass measurement is in fact necessary to unequivocally define a peptide from a known protein sequence without sequence information. It would be useful if accurate mass information could be used to screen selected protein sequences or even protein databases for candidate peptides found on class II MHC molecules in, for example, pathogenic samples but not in controls. As a test case we chose the peptide sequence 830–844 recognized by the Mix 111 clone, which is able to associate with several different class II MHC molecules.[11] This peptide has a calculated mass of 1725·0137. A search of the tetanus toxin sequence at a level of 0·1% error produced 47 peptides 13–17 residues in length. As shown in Table 2, at a level of 0·01% error 16 peptides were found, including both the input sequence mass (830–844) and 831–845. An error of 0·001% yielded four peptides and only when the error was 0·0001% was the single 830–844 sequence found (Table 2). Several other sequence masses were run through the same routine. A similar degree of accuracy was necessary to identify unequivocally the 1273–1284 epitope, which binds to the DRw52a molecule, although being smaller considerably fewer sequences were identified at the 0·01% accuracy level. Two of these six sequences had identical masses due to frame-shifting by one residue, a N-terminal isoleucine (112–123) being replaced by a C-terminal leucine (113–124). Thus accurate mass measurements can define a limited number of possible peptides corresponding to defined sequences in known proteins.

DISCUSSION

Only a few studies has reported recovery of naturally processed class II MHC-associated peptides following pulsing with exogenous foreign antigens, and none to our knowledge in human cells. Our experiments utilized roughly 10-fold fewer cells and 14–100 × less antigen during the 37° pulsing compared with the experiments conducted with HEL in the murine system (10 μg/ml tetanus toxin versus 0·14–1·0 mg/ml HEL).[4–6] We could identify several fractions capable of reconstituting responses by a human T-cell clone in spite of the fact that no differences at the chemical level were observed in the reverse-phase HPLC profiles from pulsed versus unpulsed cells. Even when active fractions were compared by LD TOF with matched fractions from unpulsed cells, no reliable differences were observed. The existing data on murine cells pulsed with HEL have produced somewhat contradictory findings, particularly on the issue of whether exogenous antigen pulsing produces detectable changes at the chemical level in the composition of class II MHC-associated peptides. Two studies have reported that HEL peptides dominated the class II MHC peptide composition, permitting in one study direct sequencing of first-dimension HPLC fractions.[5] When lower (0·14 mg/ml) concentrations of HEL were used there was much less perturbation of class II MHC peptide composition.[6] Nonetheless, several naturally processed forms of the 52–61 determinant could be identified based on distinct mass peaks in fractions from HEL-pulsed cells.[6] However, in the same report Vignali *et al.*[6] noted that several fractions that gave significant T-cell responses yielded no identifiable HEL peptides. We have repeatedly encountered the same problem in antigen-pulsed human cells. Mass spectra of fractions able to reconstitute T-cell responses following first-dimension HPLC separations were not significantly different compared with the same fractions from unpulsed cells, preventing us from characterizing the naturally processed determinants active in these fractions. Second-dimension separations introduced problems with loss of material, but in one experiment did allow an active fraction to be analysed by LD TOF. A peak observed in pulsed but not unpulsed cells had a mass within 0·2% of the sequence 829–841, suggesting that this might constitute the major naturally processed determinant. The discrepancy between the observed and calculated masses is very similar to the mean error found by Vignali *et al.* (0·13 ± 0·12%) in their study.[6] However, further analysis is necessary to confirm the identity of this peak.

Although known T-cell determinants could be detected in HPLC fractions obtained from fewer than 3×10^8 antigen-pulsed cells, our study illustrates the problems likely to be encountered in seeking to characterize the less abundant peptides bound to MHC molecules, including those loaded following pulsing with exogenous antigens or autoantigens. Based on our estimation of the amount of naturally processed P2 determinant, we estimate that the representation of this species is considerably less than the 0·8% occupancy reported by Vignali *et al.*[6] for H-2Ak by peptides which include the 52–61 HEL sequence. As we can estimate the amount of antigen processed in our system, we can also estimate the efficiency of determinant capture. 5×10^9 cells processed 150–300 pmoles of antigen, based on both number of receptors per cell and the production of acid-soluble radioactivity. As in the order of 2 pmoles of the P2 determinant was recovered in the major and minor HPLC fractions, it would appear that for this determinant that the efficiency of processing and capture is roughly 1–2%. This calculation ignores losses during immunoisolation of class II MHC and during preparation of peptide fractions, and so is likely to be an underestimate of the efficiency of capture for this determinant. Other regions of the tetanus toxin molecule may be more or less efficiently captured. At the radiochemical level approximately 0·5% of ^{125}I-label taken up as native antigen is recovered on class II MHC (Table 1).

Receptor-mediated or otherwise augmented antigen uptake is likely to be essential to be able to generate sufficient material to reconstitute T-cell responses to foreign antigens or autoantigens. It seems possible that the successful identification of naturally processed HEL determinants described by several groups may have depended on HEL's positive charge facilitating binding to the negatively charged cell surfaces. We were surprised that membrane immunoglobulin-driven antigen uptake failed to make a significant impact on the peptide composition of class II MHC. However, even if the efficiency of determinant capture is as low as the 2% we estimate, this would still allow a B cell expressing and utilizing 5×10^4 immunoglobulin receptors for antigen capture to display 1000 peptide/MHC complexes, which may be more than enough for T-cell recruitment. Two factors which limited the amount of antigen

captured in our system were the high affinity of the membrane immunoglobulin for antigen, precluding receptor reuse, and the secretion of soluble immunoglobulin of the same specificity into the bathing medium.[18] Presumably the prominence of peptides derived from apolipoprotein B100 reported in several studies[3,22] is due to the abundance of low-density lipoprotein (LDL) in serum and the fact that the LDL receptor can be reutilized hundreds of times.

Several species eluting with different retention times were able to trigger the Mix 111 T-cell clone. Presumably these correspond to the core sequence recognized by T-cell Mix 111 flanked with different amounts of adjoining sequence, i.e. a 'nested set' of peptides exists for this T-cell determinant, analogous to those found for constitutively processed cellular and serum proteins and those derived from HEL pulsing.[1–3,5,6]

In summary, antigen-specific human $CD4^+$ T-cell clones can be used to follow naturally processed determinants following pulsing of relatively low numbers of EBV-transformed B cells with μg/ml amounts of antigen. However, the low abundance of the active peptides and the considerable complexity of the fractions obtained suggest that considerably more material will be necessary before such antigenic or autoantigenic determinants can be fully characterized. None the less, accurate ($\pm$ 0·01%) mass data on peptides present in antigen-pulsed or pathogenic material but absent from controls might allow selected protein databases to be searched to identify candidate peptides even in the absence of sequence information. Those likely to be responsible for biological activity might be narrowed down further by incorporating into such a search programme preferred interaction motifs for particular class II MHC alleles recently identified by systematic peptide side chain scanning.[23]

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