

Major histocompatibility complex class I presentation of exogenous and endogenous protein-derived peptides by a transfected human monocyte cell line

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SUMMARY

Monocyte/macrophages are professional antigen-presenting cells of the cellular immune system, serving to generate peptides for major histocompatibility complex (MHC) class II-restricted recognition by CD4⁺ T-lymphocyte effector cells. Antigen presentation by these cells involves the internalization of extracellular proteins and their fragmentation within vacuolar compartments. The resulting peptides become associated with MHC class II molecules. The final destination of exogenous peptide antigens, however, is not absolute in monocytes. Processed peptides, derived from exogenous proteins, can also associate with MHC class I molecules. To study simultaneous presentation of peptides derived from exogenous and endogenous proteins by human leucocyte antigen (HLA) class I molecules, we isolated the peptides from a human immunodeficiency virus *nef* transfected U937 monocytic cell line. The HLA class I-bound peptides were separated by reverse phase-high performance liquid chromatography. Comparison of the peptide sequence data with protein databases revealed that the peptides derived from extracellular, as well as intracellular, proteins, suggesting that monocytes have a more generalized MHC class I antigen-processing pathway than previously documented.

INTRODUCTION

Immune recognition of extracellular and intracellular pathogens makes use of topographically distinct and specialized antigen-processing pathways for delivery of peptides to major histocompatibility complex (MHC) class I and class II molecules. Proteins synthesized and processed within the cytosol are presented by MHC class I molecules, while exogenous proteins, internalized and degraded in vacuolar spaces, are presented by MHC class II molecules. These distinct MHC class I and class II antigen-processing pathways are supported by extensive experimental data,¹ including structural analysis of peptides bound to MHC molecules.² The majority of MHC peptide-sequencing data, however, is based on the use

of B cells as the source of MHC molecules and their bound peptides. Recent data have shown that monocytes/macrophages may serve as accessory cells for MHC class I-restricted responses and are able to process and present exogenous particulate antigens to CD8⁺ T cells on MHC class I molecules,^{3–6} in apparent disagreement with the models of MHC class I and class II antigen-processing.

In this report we have utilized the human U937 monocytic cell line to study the origins of naturally processed and presented human leucocyte antigen (HLA) class I bound peptides in monocytes.⁷ As a control for endogenous antigen processing, the U937 cells were transfected with a minigene coding for the human immunodeficiency virus (HIV) *nef* protein. The HLA class I molecules were isolated from *nef*-transfected U937 cells and bound peptides were eluted and separated by reverse phase high performance liquid chromatography (HPLC) for single fraction sequencing using Edman degradation. The sequence data revealed two peptides with sequence similarity to the intracellular *nef* protein precursor. Other peptides, found in high copy number, matching to the *dbl* proto-oncogene, V-CAM, signal peptides of β_2 microglobulin, and exogenous bovine serum albumin, were also bound to the HLA class I molecules of U937 *nef*-transfected cells. These latter findings provide direct evidence that monocytes present exogenous soluble proteins via MHC class I molecules.

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Abbreviations: CTL, cytotoxic T lymphocyte; HPLC, high performance liquid chromatography; PMSF, phenylmethylsulphonyl fluoride; TLCK, tosyllysylchloro-ketone; TPCK, tosylprolylchloro-ketone.

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MATERIALS AND METHODS

Cell lines and culture conditions

The human monocytic leukemia cell U937, stably transfected with the HIV-1 *nef* gene from lymphadenopathy-associated virus (LAV) was a kind gift of Dr Tsunetsugu-Yokota, and has been previously described.⁸ Hygromycin-resistant control U937 cell transfectants were prepared as previously described.⁹ Cells were cultured in roller bottles using complete medium containing RPMI-1640 media supplemented with 10% fetal calf serum, 1% glutamine, and 0.5% gentamicin (all supplied by Gibco, Grand Island, NY) and hygromycin (200 units/ml) at an initial density of 0.5×10^6 cells/ml.

The murine hybridomas producing the monoclonal antibody (mAb) W6/32,¹⁰ anti-HLA-A,B,C (obtained from American Type Culture Collection) or B1.23.2,¹¹ anti-HLA-B,C (a kind gift of Dr Alessandro Sette; Cytel San Diego, CA) were cultured in IgG-free media.

Reagents

The mAb W6/32 and B.1.23.2 were obtained from hybridoma culture supernatants and purified by protein A affinity chromatography with reagents supplied by Bio-Rad (Richmond, CA). Cyanogen bromide-activated Sepharose 4B, purified normal mouse IgG, Aprotinin, tosyllysylchloroketone (TLCK), tosylprolylchloroketone (TPCK) and iodoacetamide were obtained from Sigma Co. (St Louis, MO), Pepstatin A, leupeptin and phenylmethylsulphonyl fluoride (PMSF) were obtained from Boehringer Mannheim (San Diego, CA). All HPLC solvents, TFA, Nonidet-40 and the Bicinchonnic Acid protein assay kit were obtained from Pierce (Rockford, IL). *Tran*[³⁵S]-label 1mCi/mmol was obtained from ICN Biomedicals, Inc. (Costa Mesa, CA). All other reagents were of the highest commercially available quality.

Immunofluorescence cytofluorometry analysis of HLA class I and CD4 expression

The expression of HLA class I A and B locus molecules was determined by immunofluorescence flow cytometry using mAb specific for HLA-A3 (GAP.A3), HLA-Bw6 (SFR8.B6), HLA-B,C (B1.23.2) and W6/32, as previously described.¹² The expression of CD4 was analysed using phycoerythrin-labelled anti-CD4 mAb from Becton-Dickinson (Mt. View, CA).

Immunoprecipitation analysis of Class I expression

U937-*nef* or control cells (5.0×10^6 total cells) were metabolically labelled with [³⁵S]methionine for analysis of HLA class I molecule expression as previously described.¹²

Polymerase chain reaction analysis of *nef* expression

Expression of HIV-*nef* mRNA was determined by reverse polymerase chain reaction (PCR). Total RNA was obtained from cells and reverse-transcribed into cDNA. The cDNAs were analysed for the presence of *b* actin and *nef* message using specific primers (LAVNEF1: 5'-ATG CTG CTT GTG CCT GGC TA-3'), LAVNEF2: 5'-TGA ATT ACG CCT TCC AGT CC-3', positions 8506–8658¹²). Amplification products were analysed by agarose gel electrophoresis.

Immunoaffinity purification of class I molecules and elution of bound peptides

U937-*nef* transfected cells were cultured to a final yield of

2×10^{10} cells in selection media. HLA class I molecules and bound peptides were isolated as previously described¹² with the following modifications: the precleared detergent lysate was sequentially immunoprecipitated with anti-HLA-B,C mAb (B1.23.2) coupled to Sepharose-4B followed by immunoprecipitation with mAb W6/32 coupled to Sepharose-4B, enabling the separation of HLA-B,C molecules from HLA-A molecules. The yield of HLA-A and B,C molecules was approximately 280 μ g and 320 μ g, respectively. HLA-A or HLA-B,C molecules were incubated for 3 min at 100° in dilute trifluoroacetic acid (TFA), then centrifuged through a 3000 MW ultrafiltration cartridge (Micron 3, Amicon, Danvers, MA). The low molecular weight (< 3000) fraction was concentrated and stored at -70° until separation by reverse-phase chromatography. In parallel, 2 ml of the complete growth medium was separated into high and low molecular weight fractions by ultrafiltration. The low molecular fraction was lyophilized to dryness and reconstituted in dilute TFA. The concentration of bovine serum albumin (68 000 MW) in the complete growth medium was estimated after sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Blue staining by comparison to known quantities of authentic bovine serum albumin run in parallel.

HPLC purification and sequencing of eluted peptides

The low molecular weight, TFA eluates of MHC molecules were separated by reverse-phase HPLC using a C18 column (Vydac, 2.1 \times 250 mm) and Waters Associates (Waltham, MA) equipment. The gradient was created using a solution (A) of H₂O/0.1% TFA in combination with solution (B) of 80% acetonitrile/0.1% TFA at a flow-rate of 0.200 ml/min. The low molecular weight fractions were reconstituted in solution A and applied to the column. One minute fractions were collected from a linear 2-hr gradient running from 2% to 80% solution B. From the absorbance trace at 215 nm of the eluate, the single fractions containing absorbance peaks were selected for microsequencing by N-terminal Edman degradation using a Hewlett-Packard G1000A protein sequencer (Palo Alto, CA). The low molecular fraction of the complete growth medium was loaded onto the C18 sequencing column and washed with 5% acetonitrile/0.1% TFA and sequenced as a pool.

RESULTS

Expression of HIF-*nef*, CD4 and HLA class I molecules in *nef* transfectants

To confirm the presence of the *nef* gene in U937 transfectants, total RNA from U937 *nef* transfectants and control cells was purified and reverse transcribed into cDNA. The populations of cDNA were examined for *nef* message using the polymerase chain reaction and HIV-*nef*-specific primers. Analysis of the amplification products showed that only the *nef*-transfected cell line expressed the expected 150 bp *nef* product (data not shown).

One of the consequences of *nef* expression in U937 cells is the down-regulation of CD4 expression.⁸ To determine whether *nef* expression in U937 cells affected the expression of HLA class I molecules as well, we performed immunofluorescence flow cytometry and immunoprotein assays to determine the relative level of HLA class I molecules expressed in

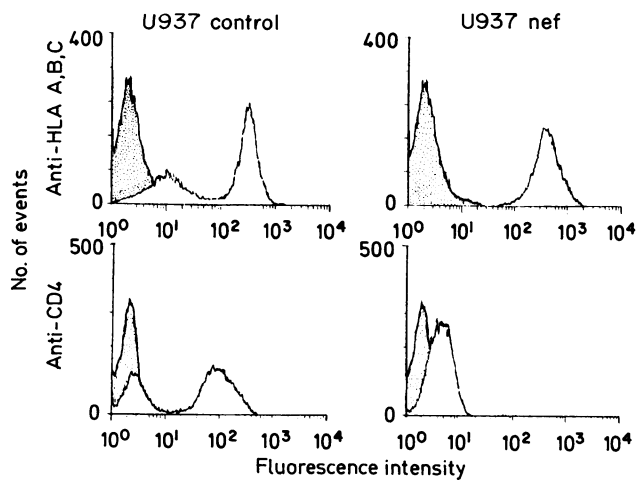


Figure 1. Immunofluorescence cytometry analysis of CD4 and HLA class I expression on U937 control and U937-*nef*-transfected cells. Stippled area, irrelevant mAb; non-stippled area, specific mAb (anti HLA-A,B,C or anti-CD4 mAb).

U937-*nef* transfectants and U937 control cells. Immunofluorescence flow cytometry, using the anti-HLA-A,B,C-specific mAb (W6/32) showed no change in the modal fluorescent intensity (Fig. 1). U937 control cells, however, had a significant number of weakly positive cells. The *nef*-transfected population showed a unimodal distribution in fluorescence intensity. The cell surface expression of CD4 was determined in parallel with HLA class I molecules. As previously reported,⁸ the U937-*nef*-transfected cells showed a significant reduction in the level of CD4 expressed at the cell surface as compared to the U937 control cells (Fig. 1). Immunoprecipitation analysis using either W6/32 or anti HLA-B,C mAb showed little or no difference in the total amount of HLA class I molecules synthesized by the U937-*nef* or U937 control cells (data not shown).

Structure of HLA class I bound peptides from U937-*nef* and control cells

Low molecular weight material, eluted from HLA-A or B,C heterodimers, was released by acid-heat denaturation, purified by ultrafiltration and fractionated by reverse-phase HPLC. The absorbance traces at 215 nm, for each separation, are shown in Fig. 2. To characterize structurally the HLA class I bound peptides of U937 cells, samples from selected fractions were analysed by sequential Edman degradation. The sequence of the peptides eluted from the HLA class I molecules obtained from U937-*nef* cells is shown in Table 1.

Although the material in the fractions was not homogeneous, we were able to identify, in many instances, the amino acid sequence of major and minor peptide sequences present in each fraction. Major and minor sequences were assignable, on the basis of the picomolar yield of each (PTH) amino acid derivative in the cycle. The assignment was corroborated by the repetitive yield of each sequence. The repetitive yields for all reported sequences ranged from 89% to 95%, with an average value of 93%. The overall repetitive yield suggests that the reported lengths of the peptides correspond to their actual

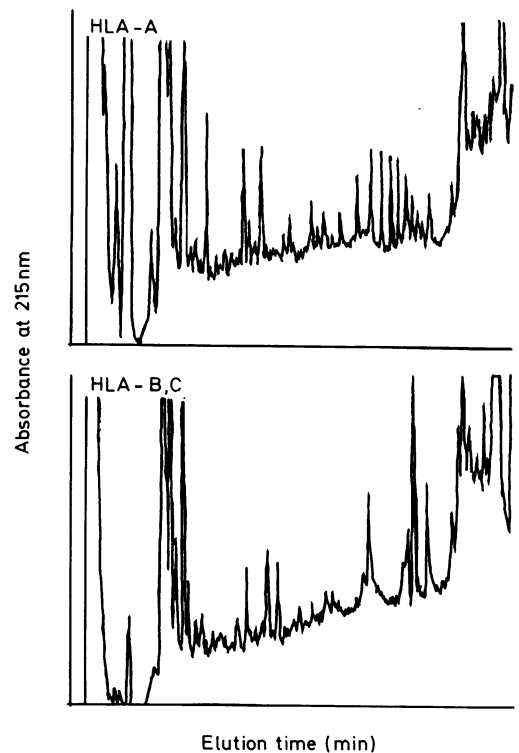


Figure 2. Reverse-phase HPLC of immunoaffinity purified HLA-A and HLA-B,C-bound peptides. Low molecular weight material, from HLA class I molecules, was applied to a C18 column and eluted with a 2-hr long gradient ranging from 0.1% to 64% acetonitrile.

lengths. Although sequences were assigned only when the yield of two or more PTH amino acid derivatives in a cycle differed by more than 35%, it is not excluded that some of the sequences are composites or contain errors. In some fractions it was not possible to assign major or minor sequences due to similar concentrations of differing peptides. Such fractions are indicated by 'composite sequence' in Table 1. In other instances PTH amino acid derivatives did not correspond to the PTH standards. Under these circumstances the residue is reported as X.

As a control, the concentration of peptidic material (greater than nine amino acid residues long) within the growth medium was determined by sequencing. Two major peptides, unrelated to albumin, with the following N-terminal sequences, DPID-PEAAYK (180 pmol/ml) and ITHKPVIGHAR (125 pmol/ml) were found. Other peptidic material was present at concentrations below 10 pmol/ml.

Precursor proteins of MHC class I bound peptides

The sequences of HLA-A and B,C bound peptides, obtained from U937-*nef* cells were compared to known sequences contained within the EMBL and Swissprot Databases. The database searches for peptide sequence homology to known protein precursors revealed that six of the obtained sequences showed 100% similarity to known precursor proteins.

Within the pool of peptides bound to the HLA-A3, we were able to identify two peptides with 100% similarity to known precursors. Fraction 41 contained the peptide SVKHPGGRK,

Table 1. HLA class I-bound peptides of U937-*nef* cells

Fraction	Sequence	Yield (pmol)	Match (residues)
<i>HLA-A locus peptides</i>			
28	NPYTDPTXGAT	2.3	—
31	DVKAPSAKY	1.1	—
34	KLYKNPAKY	3.3	—
36	AVYXXANXKL	2.1	—
37	DVYERXEXM	< 1.0	—
40	<i>Composite sequence</i>	13.8	—
41	SVKHPGGRK	3.2	E2F related transcription factor (85–93)
45	KLYLXTANK	3.1	—
51	DWYENVVLKK	4.7	—
52	ATYPPMRXKQ	3.1	—
56	VYPFDRLEI	4.1	VCAM (144–152)
	<u>ALYEPLVWY</u>		
59	DYLDVXLAY	1.3	—
63	KLFDYXXWTY	2.2	—
67	GLYDVFEAD	2.3	—
70	KLAPIWLDY	2.9	—
72	<i>Composite sequence</i>	4.3	—
74	<i>Composite sequence</i>	2.1	—
76	GLFERSARX	2.1	—
78	DTPNVLIVIXL	5.7	—
79	SSLAFHHKAR	5.2	8/10 HIV-1 <i>nef</i> (188–198)
	<u>D P V</u>		
80	<i>Composite sequence</i>	1.0	—
<i>HLA-B,C locus peptides</i>			
39	DPDRNYLYK	4.1	—
46	LQYEFDVILSP	12.8	<i>dbl</i> -proto-oncogene (444–452)
48	<i>Composite sequence</i>	2.4	—
53	<i>Composite sequence</i>	2.8	—
60	DEYGVARTYLDLFEME	2.7	—
61	YPYNXINXDN	< 1.0	—
70	DPVESNYXY	5.6	—
	<u>SEYRVKEXK</u>	1.2	'CMV-Like' Ref. 11
82	DEFEFPWV	2.5	—
	<u>YPLTFGWXY</u>	< 1.0	8/9 HIV <i>nef</i> (134–142)
83	LQXQPFDEHVKLV	13.6	Bovine serum albumin (55–67)
89	GLFEQVRQDNQ	2.4	—
93	IQRTPKIQVYTR	22.3	β_2 Microglobulin Signal peptide (1–12)
95	NELTEFAKTXVAD	8.3	Bovine serum albumin (68–80)
	<u>KQR-DV- - -RA</u>		
97	GLVDPEVFAY	< 1.0	—

Standard IUPAC amino acid abbreviations are used. When multiple PTH amino acids were present in consecutive cycles and no sequence was identifiable, composite sequence is indicated. Minor sequences are underlined. X denotes an unidentifiably PTH-amino acid derivative. Homologies to precursor proteins are all 100% unless preceded by a fraction. Numbers in parenthesis indicate the position of the corresponding amino acid residue matches.

which matched to the human E2F-related transcription factor (residues 85–93). The second match found among the HLA-A3 bound peptides corresponded to the extracellular domain of a member of the integrin family of proteins (VCAM, residues 144–152), and lastly, the peptide SSLAFHHKAR matched at

eight out of 10 residues to the deduced amino acid sequence of *nef* within LAV (SRLAFHHVAR, residues 188–197).¹³ The sequences of the peptides eluted from the HLA-A3 molecules generally carried the expected binding motif of HLA-A3 bound peptides.^{14,15}

From the peptides bound to the HLA-B,C molecules (HLA-B18 and B51), 12 sequences were obtained, and yielded four complete matches to database precursors. The peptide LQYEFDVILSP, originating from fraction 46 of the MHC-bound peptides of U937-*nef* cells, corresponded to residues 444–452 of the *dbl* proto-oncogene.¹⁶ Two peptides from fractions 83 and 95, matched to portions of bovine serum albumin, the fourth match, for peptide IQRTPKIQVYTR (fraction 93), indicated that this molecule formed part of the signal peptide of β_2 microglobulin (residues 1–10).

Two additional matches were found in the sequences of peptides isolated from the HLA-B,C molecules of U937-*nef* cells. In fraction 70, present as a minor species, a previously identified peptide, bound to HLA class I molecules of U937 cells, was found.¹¹ The sequence of this peptide, SEYRVKEYR, corresponds, at seven out of nine residues, to a protein of the HXLFI transmembrane glycoprotein of cytomegalovirus AD 169 and has been previously isolated from U937 cells.¹⁷

The peptide YPLTFGWXY in fraction 82 of the peptide pool eluted from HLA-B,C molecules also found as a minor species, corresponds to a portion of a previously identified HLA-B18-restricted CTI epitope of the *nef* protein (YPLTFGWXY, *nef* 132–147).^{18,19} This peptide was found at a concentration that approaches the detection limit of our Edman sequencing chemistry and thus, is a 'low confidence' sequence. The peptide, however, shares the proline residue at position two with three other peptides isolated from the pool of HLA-B,C molecules and yielded no signal during the eighth cycle of Edman chemistry. This finding is consistent with cysteine occupying position eight of the peptide.

DISCUSSION

The data presented in this report illustrate the origins of naturally processed self and non-self peptides which are presented by MHC class I molecules of a human monocyte cell line. Our data show that peptides presented by the HLA class I molecules of monocytes may originate from exogenous as well as endogenous protein precursors. Thus, U937 monocytes differ from other cells (mostly of B lineage) studied so far which present MHC class I-bound self peptides derived from endogenous protein precursors.²

From the naturally-processed pool of HLA-B,C-bound peptides of U937 cells, a peptide matching to a portion of the *dbl* proto-oncogene was characterized. This finding is at odds with the pattern of expression of the *dbl* mRNA, which has been reported to be limited to tumours of neuroectodermal origin,^{20,21} and to normal brain, adrenal and gonadal tissue.¹⁶ The possibility that the *dbl* peptide is derived from some other, as yet uncharacterized, precursor protein, however, cannot be ruled out. In preliminary experiments, the synthetic *dbl* peptide was found to elicit CTL-reactivity in the context of HLA-B51 (Z. Liu, unpublished results).

The *dbl* oncogene was originally isolated by transfection of NIH/3T3 cells with DNA of a human B cell lymphoma²² and shows limited similarity to the *bcr* proto-oncogene.²³ The

predicted protein structure of *dbl* suggests that it is a hydrophilic cytoplasmic phosphoprotein with no presecretory signal peptide. The *dbl* protein is thus similar to interleukin-1 β , which also serves as a precursor for MHC class I-bound peptides in differentiated U937 cells.¹² The pathway whereby these precursor proteins gain entry to the endogenous antigen-processing pathway may involve transport across a membrane followed by vesicular degradation or, more simply, degradation by the normal proteosomal machinery and transport by the TAP proteins to the endoplasmic reticulum.

During assembly of MHC class I molecules in the endoplasmic reticulum, signal peptides, cleaved from maturing proteins, have access to the antigen-binding groove. The signal peptide of β_2 microglobulin, comprising a major species of self-peptide bound to the HLA class I molecules of U937 cells, was presumably generated in this manner.

The peptide, YPLTFGWXY, found as a minor sequence in fraction 82 of the peptides eluted from HLA-B,C molecules matched to a portion of the HIV *nef* protein (*nef* 134–142) expressed by the transfected U937 cells used in these studies. This sequence is also contained within a previously characterized HLA-B18-restricted CTL epitope of the *nef* protein characterized by Culmann *et al.*^{18,19} The generation of this peptide from the pool of intracellular protein precursors is most probably dependent on proteosomal degradation and TAP transport to the MHC class I peptide loading compartment and demonstrates that U937 cells exhibit conventional endogenous antigen processing.

A second peptide, SSLAFHHKAR, bound to the HLA-A3 molecules, showed partial homology to residues 188–197 of the *nef* protein. The reported sequence of LAV *nef* (residues 188–197) is SRLAFHHVAR¹³ and is mismatched at positions two (serine for arginine) and eight (lysine for valine) from the U937 peptide. It is unlikely that the sequence we found is in error at position two, as peptides with arginine in position 3 are usually associated with HLA-B27,²⁴ an allele absent from U937 cells. The high yield of this peptide and its sequence suggest that this fragment may have been generated from a protein precursor other than *nef*. Structural homology between HIV proteins and self proteins has been proposed as a possible pathogenic mechanism for the development of autoimmunity during HIV infection.²⁵ On the other hand, it should be noted that the sequence of this U937-self peptide corresponds to the deduced amino acid sequence of *nef* found in other isolates of HIV.²⁶

In contrast to the finding that MHC class I-bound peptides derived from precursor proteins of cytosolic origin, is the observation that U937 cells also present peptides derived from the soluble extracellular proteins such as bovine serum albumin. These findings provide structural confirmation of the recent data provided by Harding *et al.*^{4,5} on the existence of an alternate MHC class I antigen-processing pathway active in monocyte/macrophages.

In the alternate class I processing pathway, extracellular antigens are degraded in the vacuolar compartments associated with phagocytosis. The resulting peptides have access to MHC class I molecules that have already exited the endoplasmic reticulum/Golgi compartments. Alternatively, these peptides may gain entry to the cytosol to merge with the conventional endogenous antigen-processing pathway.⁶ The loading of peptides onto class I molecules may take place by replacement

of low-affinity peptides already bound or by simple occupancy of empty MHC class I molecules.

It is unlikely that the albumin-derived peptides characterized here were derived from peptides present in the extracellular medium and loaded at the surface of U937 cells. The yield of bovine serum albumin-derived peptides found bound to the MHC class I molecules of U937 cells was high, accounting for roughly one-fifth of the mass of the peptides isolated from the HLA-B,C pool. Hence, to achieve loading of these peptides, a high proportion of empty cell surface MHC class I molecules and a commensurate concentration of the precisely 'tailored' peptides would be required. Such precisely 'tailored' peptides were not found in the extracellular compartment.

β_2 microglobulin signal peptides and bovine serum albumin-derived peptides accounted for significant and approximately equal fractions (10% or 20 pmol each) of the peptides isolated from the HLA class I molecules of U937 *nef* cells. Using these data, we were able to calculate the relative efficiencies of MHC class I presentation of endogenous proteins versus proteins of exogenous origins. Comparison of the relative concentrations of β_2 microglobulin (approximately 0.3 μ M, based on 2×10^5 MHC class I molecules per cell) versus bovine serum albumin (approximately 70.0 μ M) revealed that a 200-fold excess of exogenous albumin protein was required to achieve the same MHC occupancy as the β_2 microglobulin signal peptides. These calculations suggest that abundant exogenous proteins are represented in the population of MHC class I-bound peptides.

The conclusions of this study, that monocyte/macrophages have the capacity to present soluble exogenous proteins on MHC class I molecules, deviate from recent findings that only particulate exogenous antigens supply peptides which may bind to MHC class I molecules.⁵ These studies, however, have used T-cell clones which detect specific CTL epitopes within the soluble antigens tested. The failure to detect the presentation of these antigens on MHC class I molecules, however, can be attributed to destruction of the epitopes during vacuolar processing. A corollary of this hypothesis is that the peptide fragments derived from bovine serum albumin found in these studies are protected from degradative processes encountered within the lysosomes. If such 'privileged' regions generally exist, then it is simple to understand the mechanism whereby soluble protein antigens can also induce CD8⁺ T-cell responses.

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