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Isolation of an HLA-A2.1 extracted human minor histocompatibility peptide*

Purified HLA-A2.1 molecules obtained by affinity chromatography of 6×10^{10} Epstein Barr virus (EBV)-transformed B lymphocytes were used in an attempt to isolate the human HLA-A2.1-restricted minor histocompatibility (H) peptides H-Y and HA-2. Fraction 18 of the high-performance liquid chromatography (HPLC)-separated HLA-A2.1 peptide pool was found to contain the natural HA-2 peptide. An HA-2-specific, HLA-A2.1-restricted cytotoxic T lymphocyte clone lysed HLA-A2.1$^+$ HA-2$^-$ EBV-transformed B lymphocyte cell lines reproducibly and in a concentration-dependent fashion in the presence of fraction 18, but not in the presence of other HPLC fractions. By contrast, H-Y sensitizing activity was not found in any fraction. Amino acid sequencing of peptide fraction 18 revealed a mixture of peptides with maximal length of nine amino acids, in which the presence of Leu at positions 2 and 9 was dominant. Surprisingly, the HA-2 peptide could not be mimicked by any of the peptide mixtures synthesized according to the amino acid sequences found in fraction 18. Our failure to obtain the actual amino acid sequence of the human minor H peptide HA-2 from a peptide pool with the established pattern for binding to HLA-A2.1 may indicate that this CTL defined minor H peptide does not represent an abundant HLA-A2.1 binding peptide.

1 Introduction

Minor histocompatibility (H) antigens represent serious barriers for succesful organ and bone marrow transplantation (BMT) between individuals matched for the major histocompatibility complex (MHC) antigens. Minor H antigens in general fail to induce B cell responses and are characterized by MHC-restricted T cell responses [1, 2]. Due to the lack of available antibodies, thus far little is known concerning the genes encoding human minor H antigens and their polymorphic gene products [3]. Recently, it has become evident that, like virus-specific cytotoxic T lymphocytes (CTL) [4, 5], MHC class I-restricted CTL specific for minor H antigens recognize short protein stretches presented by the restricting MHC class I molecule [6–8]. Another line of investigation revealed that MHC class I molecules bind and present an allele-specific set of self peptides, presumably derived from cellular proteins [9–11]. We set out to isolate CTL-defined human minor H peptides from the pool of peptides naturally presented by HLA-A2.1. In this report we attempted to characterize two HLA-A2.1-restricted human minor H peptides, the male-specific antigen H-Y [12] and a hematopoietic tissue-specific antigen termed HA-2 [2, 13].

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Abbreviations: **BLCL:** B lymphocyte cell line **BMT:** Bone marrow transplantation

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2 Materials and methods

2.1 CTL and B cell cultures

EBV-transformed B cell lines (EBV-BLCL) were expanded in RPMI 1640 supplemented with 10% FCS and antibiotics in 1 l roller-bottle flasks. CTL clone 1R35 specific for the male minor H antigen H-Y in the context of HLA-A2.1 was obtained from PBMC of a female immunized against H-Y as a result of multiple transfusions and unsuccesful grafting of HLA-identical male bone marrow [12]. CTL clone 5H17 specific for the HLA-A2.1-restricted minor HA-2 was obtained from PBMC of a patient shortly after HLA-identical bone marrow transplantation [2]. Both minor H-specific CTL lines were established by repeated *in vitro* restimulation with the original stimulator PBMC. After limiting dilution, clones were maintained by weekly stimulation with allogeneic feeder cells in RPMI 1640 supplemented with 15% pooled human serum and 20 U/ml rIL-2. Immunogenetic data and tissue expression of the HA-2 minor H antigen were previously described [3, 13].

2.2 Purification of HLA-A2.1 and isolation of HLA-A2.1 bound peptides

Pellets of 6×10^{10} EBV-BLCL were incubated for 45 min at 4 °C at 2×10^8 cells/ml in a lysis buffer containing 50 mM Tris, 150 mM NaCl, 0.5% Nonidet P-40, 5 mM EDTA, pH 8 and 0.1 mM PMSF and 1 μg/ml of the protease inhibitors antipain, leupeptin, chymostatin and pepstatin. Centrifugation for 5 min at 500 × g followed by 60 min at 10 000×g

yielded a supernatant which was used to purify HLA-A2.1 by affinity chromatography. The antibodies 7.5.10.1 (anti-HLA class II) and BB7.2 (anti-HLA-A2.1, [14]) were coupled at 5 mg/ml to CNBr-activated Sepharose 4B beads (Pharmacia LKB). The supernatant was sequentially passed, at a flow rate of 8 ml/h, through columns filled with Tris-HCl equilibrated beads (10 ml), anti-HLA class II beads (2 ml) and two columns with anti-HLA-A2.1 beads (7 and 4 ml). Beads were removed from anti-HLA-A2.1 columns and incubated for 15 min at 4 °C in 15 ml 0.1% trifluoroacetic acid (TFA). Supernatants were separated by centrifugation over Centricon 10 (Amicon) filters into a > 10 kDa and a < 10 kDa fraction. The amount of purified HLA-A2 was determined with the BCA protein assay (Pierce) in the > 10 kDa fraction. Purification grade was monitored by silver-stained SDS-PAGE. The low and high molecular weight fractions of HLA-A2.1 were dried by vacuum centrifugation and separated by reverse-phase HPLC using a LiChrospher 60 RP-Select B, 5 µm, 250 × 4 mm column (E. Merck, Darmstadt, FRG). Elution buffers were A, H_2O, B, acetonitrile; and C, 2% TFA in H_2O. Gradients routinely used for separations: 0–30 min linear increase from 5% to 75% B or 0–45 min linear increase from 20% to 50% B, with 5% buffer C being used throughout all gradients, flow rate 1.0 ml/min. Elution was monitored by a continuous flow spectrophotometer at 214 nm. Fractions of 1 or 0.5 ml were collected and dried by vacuum centrifugation.

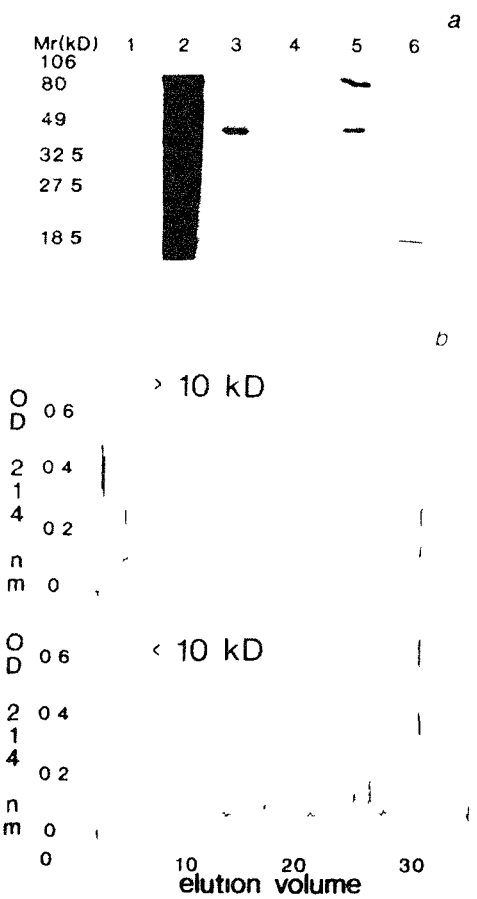

Figure 1. Affinity-purified HLA-A2.1 molecules (a) and HPLC profiles of HLA-A2.1 and of peptides eluted from these molecules (b). (a) SDS-polyacrylamide gel (15%) run under reducing conditions. Lane 1, standard markers with indicated molecular weight (BioRad); lane 2, unseparated lysate; lane 3, affinity-purified HLA-A2.1 > 10 kDa (4.5 µg); lane 4, < 10 kDa material eluted from 4.5 µg HLA-A2.1; lane 5, HPLC fraction 24 of HLA-A2.1; lane 6, HPLC fraction 19 of HLA-A2. (b) Reverse-phase HPLC separation of high (> 10 kDa) and low (< 10 kDa) molecular mass fractions of affinity-purified HLA-A2; the void volume peak representing salt injection and the final peak representing NP40 were found in all HPLC runs. Note that the peak at 19 and 24 ml in the > 10 kDa HLA-A2.1 profile represent β2m and H chain contaminated with some β2m, respectively (a, lanes 5, 6 and b, top profile).

2.3 Peptide sequencing and synthesis

Low molecular weight material eluted from HLA-A2.1 and separated by HPLC was sequenced by automated Edman degradation using a pulsed-liquid protein sequencer 477A equipped with an on-line PTH-amino acid analyzer 120A (Applied Biosystems). Mixtures of synthetic peptides were generated during mixed syntheses using an AMS 422 synthesizer (Abimed Analysen-Technik, FRG) and were purified by reverse-phase HPLC. Four mixtures each contained 64 nonapeptides $X_0LX_1X_2X_3X_4ETL$, $X_0LX_1X_2X_3X_4LTL$, $X_0LX_1X_2X_3X_4ATL$ and $X_0LX_1X_2X_3X_4ITL$, respectively, wherein position X_0 is A, X_1 can carry either F, A, I or E, X_2 is D, G, E or P, X_3 is F or L and X_4 is I or L. Four additional mixtures, each containing 1280 peptides also had all 20 natural amino acids on X_0.

2.4 ^{51}Cr-release assay

^{51}Cr-labeled EBV-transformed B lymphocytes (2.5×10^3) were preincubated in 50 µl with either naturally eluted or synthetic peptides for 30 min at 37 °C. HPLC-purified synthetic peptides were tested in final concentrations between 0.16–5.0 nM. Dried HPLC fractions of the HLA-A2.1-eluted peptides were resuspended in PBS + 50 mM Hepes (usually 250 µl) of which 25 µl (or serial dilutions) were added to the wells. Subsequently 100 µl 15% human serum in RPMI with or without (spontaneous release values) 10^5 effector cells was added to each well and incubated for 4 h at 37 °C. Radioactivity released into the supernatant was determined in a Packard γ-counter. Spontaneous release values for EBV-BLCL were 19–32%.

3 Results

HLA-A2.1 was purified by affinity chromatography [9] from an HLA-A2.1$^+$ EBV-BLCL expressing both H-Y and HA-2 minor H antigens. In total 3.3 mg protein was eluted from the anti-HLA-A2.1 mAb-coated columns. SDS-PAGE and reverse-phase HPLC revealed that this material mainly represented β2-microglobulin (β2m) and H chain (Fig. 1a, b). Quantification of the HPLC β2m peak, by comparison with peak sizes of known amounts purified β2m, indicated that 127 µg or 2.35 nmol HLA-A2.1 was recovered after HPLC. Naturally bound peptides co-purified with HLA-A2.1 were separated from β2m and H chain by acid treatment followed by ultrafiltration (cutoff

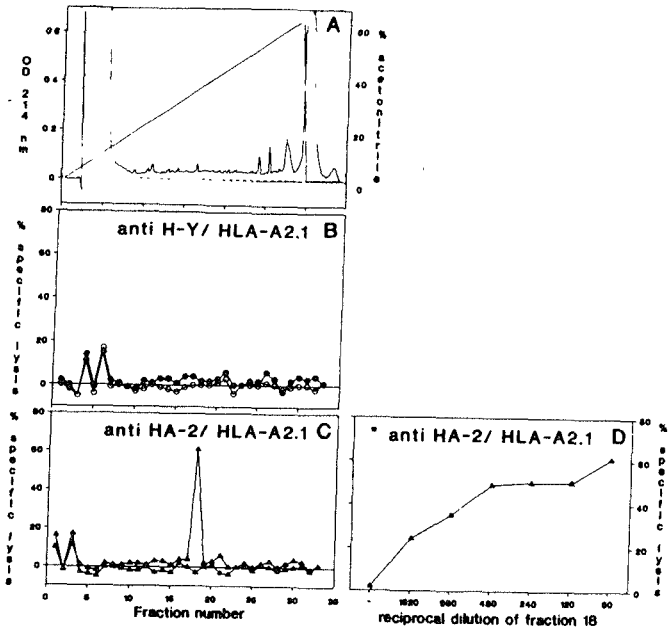

Figure 2. HLA-A2.1 peptide fraction 18 contains minor HA-2. The peptide content from 2.35 nmol HLA-A2.1 affinity-purified from EBV-BLCL of a male, HLA-A2, minor HA-2-expressing individual was separated by reverse-phase HPLC (A). Individual 1-ml fractions were tested for containing minor peptides H-Y and HA-2 by incubating 1/60 of each fraction with HLA-A2.1$^+$, HA-2$^-$, H-Y$^-$ BLCL in the absence (open symbols) or presence (closed symbols) of the HLA-A2-restricted anti-HY CTL clone 1R35 (B) or anti-HA-2 CTL clone. 5H17 (C) at E/T=40. Positive fraction 18 was retested in titrated amounts (1/60–1/1920 of fraction 18; total peptide pool content: 2.35 nmol) for recognition by HLA-A2-restricted anti-HA-2 CTL (D) and was compared to the original HLA-A2$^+$, HA-2$^+$ donor BLCL without added peptide (D, ■).

10 kDa). Low molecular mass molecules (< 10 kDa) were fractionated by reverse-phase HPLC (Fig. 2A). Individual fractions were then tested for recognition by HLA-A2.1-restricted CTL specific for H-Y (Fig. 2B) and for HA-2 (Fig. 2C). None of the fractions rendered HLA-A2.1$^+$ female EBV-BLCL susceptible to lysis by anti-H-Y CTL (Fig. 2B). In contrast, HLA-A2.1$^+$, HA-2$^-$ BLCL incubated with fraction 18 were effectively lysed by anti-HA-2 CTL (Fig. 2C), whereas none of the other fractions could sensitize the target cells for HA-2. Fraction 18 could be diluted more than 2000-fold and still sensitize target cells for HA-2 recognition (Fig. 2D). Comparable HA-2-sensitizing activity was found in HPLC fraction 18 of unseparated HLA-A2.1, but not in the high molecular mass (> 10 kDA) material of filtered HLA-A2.1 (not shown). HA-2 reproducibly eluted in one fraction at a constant position in the HPLC spectrum. Repeated isolation of the peptide content of HLA-A2.1 molecules derived from 4×10^{10} EBV-BLCL yielded identical results (data not shown).

The HA-2-containing fraction 18 was sequenced by Edman degradation and found to contain more than one peptide (Table 1). None of the peptides was more than nine amino acids in length. A dominant Leu was found at positions 2 and 9. To obtain a purer fraction, containing only the HA-2 peptide, a second HLA-A2.1 peptide pool was separated

Table 1. Detected amino acids in minor HA-2 containing fraction 18 at positions 1–9[a)]

1	2	3	4	5	6	7	8	9
–[b)]	L	F[c)]	D	F	I	E	T	L
		A	G	L	L	L		
		I	E			A		
		E	P			I		

a) HPLC peptide fraction 18 obtained from 1.77 nmol purified HLA-A2 was sequenced by Edman degradation. N-terminal peptide yield was 107 pmol decreasing to 20 pmol at the C-terminal position 9. Detection limit was 5 pmol. No significant levels were detected beyond position 9.
b) Significant amounts of all 20 amino acids were found at position 1.
c) Significant amounts of the amino acids indicated were found.

by HPLC using a shallower gradient (20–50% in 45 min with 0.5 ml fractions instead of 5–75% in 30 min with 1 ml fractions). Subsequent cell-mediated lysis analysis indicated two fractions with HA-2 activity. However, sequence analysis of the pooled fractions revealed again a nonapeptide mixture with virtually identical amino acids as found in fraction 18 (see Table 1).

To identify which of the eluted nonapeptides represented the naturally processed HA-2 minor peptide, eight mixtures of nonapeptides covering all peptides possibly present in fraction 18 (see Sect. 2.3) were synthesized, fractionated by HPLC and tested for their ability to induce lysis by anti-HA-2 CTL. However, none of the peptide mixtures could sensitize HA-2$^-$ BLCL for recognition by anti-HA-2 CTL at concentrations as high as 500 pM (data not shown).

4 Discussion

Minor H antigen HA-2 was initially defined by HLA-A2.1-restricted CTL isolated from PBL after MHC-identical, HA-2-mismatched BMT [2]. The HA-2$^+$ phenotype has a frequency of 95% in the HLA-A2.1$^+$ population [3]. In contrast to the previously isolated murine minor H peptides H-Y and H-4^b which are ubiquitously expressed, HA-2 is expressed only by cells of hematopoietic origin [13]. In the situation of MHC-matched, HA-2-mismatched BMT, therefore, these anti HA-2 CTL are not likely to inflict graft-versus-host (GVH) reactions (GVH-target tissues, *e.g.* the skin, does not express HA-2), but might be involved in the so-called graft-versus-leukemia effect [15]. Here, the natural HA-2 peptide presented by HLA-A2.1 to CTL is shown to be present within an HLA-A2.1 peptide fraction containing predominantly nonapeptides with Leu as dominant amino acid at positions 2 and 9. Thus, this finding is compatible with two previous reports indicating that naturally processed HLA-A2.1-bound self peptides are mainly nonapeptides and carry Leu at position 2 and either Leu or Val at the carboxy-terminal position 9 [9, 10].

Unexpectedly, however, none of the synthetic peptide mixtures, covering all nonameric sequences indicated by the observed HLA-A2 1 binding motif (Table 1), could sensitize HA-2$^-$ BLCL for recognition by anti-HA-2 CTL The failure to detect the HA-2 peptide in the peptide mixtures based on the observed A2 1 motif might be due to (1) competition for binding to HLA-A2 1 between the peptides present in each tested mixture, or (2) absence of the HA-2 amino acid sequence among the series of synthetic peptides tested At this stage we cannot rule out the first possibility Additional separate testing will be required to exclude the possibility of competition The second explanation would indicate that the amount of HA-2 peptide present in fraction 18 was insufficient to be detected Should fraction 18 (100 pmol total peptide content based on Edman degradation) contain 20 or more different peptides in equimolar amounts, 1 of which being HA-2, then HA-2 would already be below the detection limit of the PTH amino acid analyzer used (5 pmol) Given the recent estimate that the HLA-A2 1 molecules of a given cell might contain 200 to 1000 distinct nonapeptides [10], this explanation is not unlikely These results stress the enormous sensitivity of a CTL in detecting peptide in the context of an MHC class I molecule Anti-HA-2 CTL could detect as little as 1 in 1920 of the material of fraction 18 which apparently contained insufficient of the HA-2 peptide to be measured by Edman degradation Our observations are in line with previous reports on the isolation of minor H peptides from lysates of whole cells The murine H-2^b-restricted minor H antigens H-Y, H-4^b and Mapki and an HLA-B35-restricted antigen were detectable by the appropriate CTL, but could not be identified as single peptides [5–7] Recent sequence analysis of the self-peptide pool present in HLA-A2 1 and HLA-B27 revealed the presence of a limited number of "dominant" peptides which were present in sufficient amounts to allow sequence analysis [10, 11] Our inability to elucidate the amino acid sequence within the peptide pool derived from 2 35 nmol HLA-A2 1 may indicate that this minor H peptide is not present in comparable amounts to those "dominant" self peptides of which sufficient copies are available to allow identification as single peptides and which determine the peptide-binding profile In fact, it can not be excluded at this point that self peptides, present in quantities undetectable with the current biochemical methods, may not follow the MHC class I allele-specific binding rules based on the composition of the abundant peptides The fact that HLA-A2 1-restricted CTL epitopes exist, defined by synthetic peptides which do not fit the HLA-A2 1 binding profile determined thus far, would be compatible with this hypothesis [16, 17] Starting with larger amounts of purified MHC class I (here 6×10^{10} cells were used, yielding 2 35 nmol pure HLA-A2 1 after HPLC) may allow detection of these minor peptides and determination of MHC class I binding profiles in more detail

Our inability to detect the H-Y, in contrast to the HA-2, peptide thus far could be due to (a) a lower number of H-Y peptides per HLA-A2 1 molecule, (b) inability of H-Y to replace endogenously bound self peptides *in vitro* due to a lower affinity or (c) selective loss of H-Y during purification At this point we cannot exclude any of these possibilities Future purification of H-Y peptides from larger amounts of HLA-A2 1 as suggested earlier may lead to its detection

Although the first human minor H protein still awaits identification, in the mouse polymorphic self proteins located in the cytosol [18] and in mitochondria [19], as well as proteins encoded by retroviral genes [20], can represent minor H proteins, *i e* induce MHC class I-restricted CTL and skin-graft rejection From this, it may be concluded that within any given cell a large source of potential minor H proteins might be present Fortunately, based on the recent understanding of the MHC class I-processing pathway, it may be expected that only a small and selected fraction of the total pool of peptides derived from these proteins will make it to the cell surface, and that again only a selected fraction of these cell surface peptides will manifest itself as minor H peptide Criteria resulting in this peptide selection will include (1) a sufficiently high affinity for binding to one of the available MHC class I molecules, (2) presence of a sufficient number of copies resulting in the minimal number of MHC-peptide complexes at the cell surface required for T cell activation [21], and (3) polymorphism of the peptide and immunogenicity of the MHC-peptide complex formed Should, in contrast to the total number of distinct class I-bound cell surface peptides (estimated 200–1000 for HLA-A2 1), the number of T cell-activating minor H peptides per MHC class I allele be limited to a few or even one, then clinical applications in minor H-mismatched BMT would belong to the future possibilities

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