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Kappa marker typing with high-performance liquid chromatography: identification of kappa marker specific tryptic peptide from the kappa light chain of immunoglobulin G

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ABSTRACT

Human serum immunoglobulin G was separated into its heavy and light chains by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred electrophoretically to a polyvinylidenedifluoride membrane. Peptide fragments liberated from the light chain by *in situ* digestion with trypsin were then analyzed by reversed-phase high-performance liquid chromatography (HPLC). On comparing the HPLC patterns of these fragments derived from three major kappa marker (Km) types, two distinct peaks specific for the Km types were detected. Sequencing of the two specific peak peptides confirmed that they were identical to a stretch comprising residues 191–207 of the immunoglobulin kappa light chain, which contains valine/leucine allotypic variation at position 191.

INTRODUCTION

InV-polymorphism in the single constant gene for the kappa light (L) chain, was first reported by Ropartz *et al.* [1] using hemagglutination inhibition reactions: at present InV is called Km (kappa marker). Family and population studies have shown that a single locus controls the expression of all the factors, which are inherited by three codominant alleles: $Km^{1,2}$, Km^3, and the rare Km^1 [1–4]. Chemical studies have shown that kappa chains positive for Km(1) have valine at position 153 and leucine at position 191; those positive for KM(3) have alanine at position 153 and valine at position 191; and those positive for both Km(1) and Km(2) have alanine at position 153 and leucine at position 191 (see Table I) [5–8]. The entire sequence of the Km^3 allele was determined by Hieter *et al.* [9]. Kurth *et al.* [10] amplified the kappa constant gene by

TABLE I

COMPOSITION OF Km ANTIGENS

Km antigen	Residue 153	Residue 191
1	Val	Leu
1,2	Ala	Leu
3	Ala	Val

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the polymerase chain reaction (PCR) and used four allele-specific oligonucleotides to distinguish the alleles in the amplified products.

The detection of Km antigens has usually been performed by the hemagglutination inhibition test with specific antisera; however, it is difficult to obtain anti-Km antisera with high reactivity and specificity. Therefore, we attempted to devise a new Km-typing method, which would not require any Km-specific antiserum as a reagent. We tried to determine the Km types by high-performance liquid chromatographic (HPLC) analysis of tryptic peptides derived from the kappa chain, which contains type specific variation site(s). In the present paper we describe the successful results obtained with this new method.

EXPERIMENTAL

Materials

After their informed consent had been obtained, blood samples were taken from healthy Japanese laboratory workers and students living in the Fukui Prefecture. Separated plasma or serum samples were frozen immediately and kept at −80°C until use. Km typing was performed by the conventional hemagglutination inhibition test with both anti-Km(1) and anti-Km(3) antisera (CLB, Amsterdam, Netherlands). Unfortunately, we were not able to use the rare anti-Km(2) antiserum for serotyping. All other reagents used were of reagent grade or HPLC grade.

Km typing method

Purification of immunoglobulin G. Immunoglobulin G (IgG) was purified from 0.1 ml of serum using Affi-gel Protein A (Bio-Rad, Richmond, CA, USA) according to the manufacturer's instructions. The purity of the IgG fraction obtained was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Preparation of IgG L chain. SDS-PAGE was performed according to the method of Laemmli [11] using a 12.5% polyacrylamide gel measuring 80 × 70 × 2 mm. Prior to electrophoresis, the purified IgG was reduced with 2-mercaptoethanol and S-pyridylethylated with 4-vinylpyridine (Sigma, St. Louis, MO, USA) according to the method of Tempst *et al.* [12]. The separated subunits were transferred to a polyvinylidenedifluoride (PVDF) microsequencing membrane (Bio-Rad) by electroblotting using a KS-8453 apparatus (Marysol, Tokyo, Japan). Transfer was performed in 0.1 *M* sodium 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer, pH 11.0, containing 10% (v/v) ethanol, for 1 h at 0.3 A. Transferred proteins were stained with 0.1% (w/v) Ponceau S (Merck, Darmstadt, Germany) in 1% (w/v) acetic acid and then destained with 1% acetic acid. The light-chain containing region (ca. 25 mm^2) was cut out, transferred to an Eppendorf tube (1.5 ml) containing 1 ml of distilled water, and frozen at −80°C.

In situ tryptic cleavage of electroblotted L chains. This procedure was performed using a modification of a previously described method [12]. The strip stained with Ponceau S was destained by washing with 0.2 m*M* NaOH. After washing with distilled water, the strip was blocked with 0.5% (w/v) polyvinylpyrrolidone, average relative molecular mass (M_r) of 40 000 (PVP-40, Aldrich, Milwaukee, WI, USA), in 0.1 *M* acetic acid for 30 min at 37°C. The strip was washed with distilled water, cut into small pieces of 1 × 1 mm, and placed in an Eppendorf tube (0.5 ml) containing 25 μl of 0.1 *M* NH_4HCO_3 (pH 8.2)–acetonitrile (95:5, v/v). A 1-μg amount of bovine pancreatic trypsin (Type X III, TPCK-treated, Sigma) was added to the tube, and the mixture was thoroughly mixed by sonication and incubated at 37°C for 18 h. After digestion, the reaction mixture was sonicated and centrifuged for 1 min at 4000 *g*. The supernatant was removed and diluted 1:1 with distilled water, and immediately injected into the HPLC apparatus or frozen at −80°C.

HPLC separation of the tryptic peptides. Fresh or thawed solutions containing tryptic cleavage fragments from the L chain were separated on a Microbondasphere C_8 column (150 × 3.9 mm I.D., Waters, Tokyo, Japan) using a Waters Model 600E liquid chromatograph. The following buffer system was employed: solvent I,

0.05% (v/v) trifluoroacetic acid (TFA) in water; solvent II, 2-propanol–acetonitrile (70:30, v/v), containing 0.02% (v/v) TFA. The column was eluted with a linear gradient of solvent II from 0 to 60% over a 40-min period. The flow-rate and column temperature were 0.6 ml/min and 45°C, respectively. Peptides were detected at a wavelength of 214 nm, collected manually based on their absorption profile, and stored at 4°C until further analysis.

Structural analysis of the isolated peptides

Edman degradation of isolated peptides (*ca.* 2–5 μg) was performed on an automated gas phase protein sequencer (Model 477A, Applied Biosystems, Foster City, MI, USA), and the phenylthiohydantoin (PTH) derivatives were identified with an on-line PTH analyzer (Model 120, Applied Biosystems). The amino acids obtained by hydrolyzing each peptide (*ca.* 1–4 μg) in 5.7 *M* HCl at 110°C for 20 h were converted to phenylthiocarbamoyl derivatives using a Waters PICO-TAG work station (Millipore, Tokyo, Japan). The derivatives were separated and quantified using a PICO-TAG amino acid analyzer according to the manufacturer's instructions.

RESULTS AND DISCUSSION

The tryptic peptides of the IgG L chains derived from three plasma samples with different Km types, Km(1+,3−), Km (1+,3+) and Km(1−, 3+), were separated by HPLC. Although the elution profiles of the three samples were very similar to one another (Fig. 1), two peaks (A at a retention time of 29.2 min and B at 30.4 min) were found to be Km-specific: peak A was observed in both Km(1+,3+) and Km(1−,3+), while peak B was present in both Km(1+,3+) and Km(1+,3−). The amino acid composition and sequence analyses of the two peptides isolated separately from peaks A and B showed that the sequence of the peak A peptide was *V*YACEVTHQGLSSPVTK, which is identical to the sequence for positions 191–207 of the kappa-type light polypeptide chain derived from Km(3)-positive individuals, whereas that of the peak B peptide was *L*YACEVTHQGLSSPVTK,

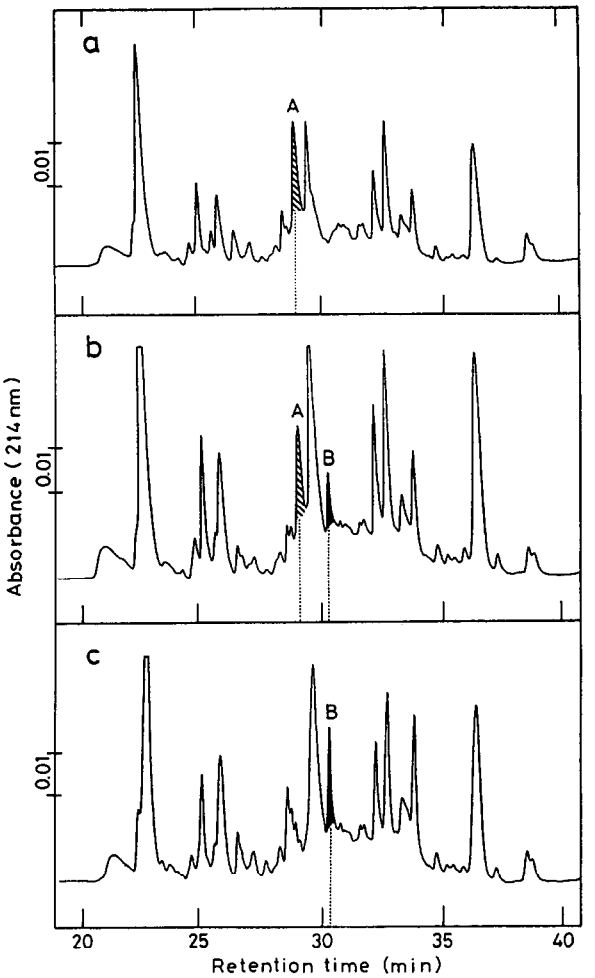

Fig. 1. HPLC patterns of tryptic peptides derived from immunoglobulin G L chain of Km(1−,3+) (a), Km(1+,3+) (b), and Km(1+,3−) (c) plasma samples. Details of the experimental conditions are described in Experimental.

which is also identical for positions 191–207 derived from Km(1)- or Km(1,2)-positive individuals [6,8,13]. This result is shown schematically in Fig. 2. Therefore, if only peak A is present in the HPLC elution profile, the plasma sample is typed as Km(1−, 3+); if both peak A and peak B are present it is typed as Km(1+, 3+), and if only peak B is present as Km(1+, 3−). Next, Km typing of eleven Japanese donors was performed with the new HPLC method. The results were identical with those obtained using the conventional hemagglutination inhibition test, and the donors could be grouped into the

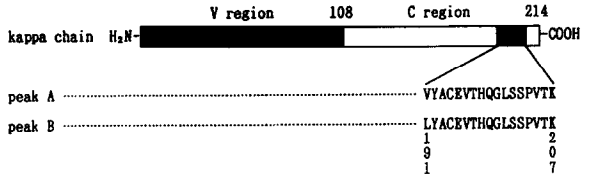

Fig. 2. Schematic representation of positions of Km-specific tryptic peptides from peaks A and B predicted from the primary structure of the immunoglobulin kappa chain established by Milstein *et al.* [6] and Steinberg *et al.* [8]. Amino acids are shown in single-letter code.

three common types [Km(1+, 3−), 2; Km(1+, 3+), 5; Km(1−, 3+), 4]. Both the HPLC profiles and the retention times of peaks A and B, characteristic for the Km type, were stable and reproducible in all cases tested.

Theoretically, in these tryptic digests, there should be another Km-specific peptide for positions 150–169 [VDN*A*(or *V*)LQSGNSQESVTEQDSK] containing the amino acid substitution position 153 responsible for the Km(1, 2)/Km(3)- or Km(1)-antigenic determinant [6,8,13]. In order to find these Km-specific peptides, we performed intensive amino acid sequencing or composition analysis of several major-peak peptides in all the samples. Unfortunately, we could detect neither of these peptides (data not shown). This suggested that the *in-situ* digestion procedure used in this study was not suitable for obtaining these peptides, or that the HPLC conditions were not appropriate for their separation as dominant independent peaks. In order to clarify the reason for this discrepancy between theory and practice, further analysis is needed. Nevertheless, this new HPLC method is thought to have great potential in distinguishing between Km(1) and Km(1, 2) without the use of rare anti-Km(2) antisera.

In routine work SDS-PAGE gives a purer L chain in a shorter time and many plasma samples can be processed simultaneously as compared to gel filtration. About 400–800 μg of IgG, corresponding to 50–100 μl of plasma, are sufficient for Km typing by the HPLC method, and no peptides derived from lambda chains coexisting in the tryptic digests interfere with the Km typing. It is also a great advantage that the HPLC method does not require special rare reagents, equipment, laboratory or technique; moreover, it distinguishes the Km gene products (peptides), but not the Km genes (nucleotides) as reported by Kurth *et al.* [10]. The high sensitivity and resolution of this method will make it applicable for the biochemical analysis of the microheterogeneities shown by polymorphic genetic markers, such as human deoxyribonuclease I (DNase I) [14,15], DNase II [16], ribonuclease (RNase) [17], protein C inhibitor [18], and glycoprotein GP43 [19], which have been found in our laboratory.

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