Purification and characterization by fast-atom-bombardment mass spectrometry of the polymorphonuclear-leucocyte-elastase-generated $\text{A}\alpha(1-21)$ fragment of fibrinogen from human blood after incubation with calcium ionophore A23187

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The stimulation of human blood with a Ca^{2+} ionophore, A23187, leads to activation of polymorphonuclear leucocytes (PMN) with release of small amounts of catalytically active elastase, as demonstrated by the formation of a characteristic product, the *N*-terminal $\text{A}\alpha(1-21)$ peptide of the $\text{A}\alpha$ subunit of fibrinogen. The identity of the peptide was initially established by radioimmunoassay (r.i.a.) with an antibody raised to $\text{A}\alpha(1-21)$. We now provide independent confirmation of the formation of $\text{A}\alpha(1-21)$ by fast-atom-bombardment-m.s. analysis of the fractions separated chromatographically after spiking of plasma samples with peptide labelled with $[^2\text{H}_8]\text{Phe}$ at position 8. Identity of the peptides was established on the basis of their chromatographic retention time and by the distinct peaks in the mass spectra of these fractions. The relative intensities of the molecular ions of natural and labelled peptides were measured. On the basis of a comparison of the peaks of similar intensities, the concentration of the natural peptide at the time of spiking was close (79%) to the amount obtained by r.i.a. An additional peptide, des-alanyl- $\text{A}\alpha(2-21)$, was also seen. The total amount of material measured by r.i.a. could be accounted for by the sum of these two provides. The addition of label and assay by m.s. has provided an independent physical-chemical method for identifying $\text{A}\alpha(1-21)$ as a characteristic product of PMN elastase release in whole blood, but which is absent in freshly drawn blood.

INTRODUCTION

Polymorphonuclear leucocyte (PMN) elastase is a serine proteinase present only in phagocytes, predominantly in neutrophils [1] and to a lesser extent in monocytes [2]. The gene for this enzyme is expressed only in myeloid precursors in the bone marrow [3], where the enzyme is packaged at extremely high concentrations [4] into azurophilic granules, which are primary lysosomes, of neutrophils and monocytes. It is thought that the primary function of elastase is to kill and degrade infectious and other noxious agents within secondary lysosomes after their phagocytosis by PMN [5]. Elastase, which preferentially cleaves on the carboxy side of aliphatic amino acids, has the capacity to hydrolyse a broad range of naturally occurring soluble and interstitial connective-tissue proteins, including elastin [6]. However, its activity against extracellular substrates is usually tightly regulated by natural inhibitors. Primary among these is α_1 -proteinase inhibitor ($\alpha_1\text{PI}$), a 54 kDa protein present in plasma at high levels over the range 30–50 μM [7]. Healthy individuals have low circulating levels of PMN elastase- $\alpha_1\text{PI}$ complexes in the nanomolar range [8]. These levels are significantly elevated severalfold in individuals suffering from acute inflammatory diseases in which PMN recruitment and activation is occurring [9].

The possibility that elastase can escape full inhibition by natural inhibitors in the extracellular environment and consequently degrade endogenous substrates has been widely speculated upon. The increased susceptibility of individuals with a genetically based deficiency in $\alpha_1\text{PI}$ to the early development of

emphysema, characterized by loss of elastic tissue from lung parenchymal tissue, is well established [10] and has been hypothesized to result from increased elastase activity at sites of PMN activation and enzyme release in the lung [11]. Similarly the emphysema of smokers has also been postulated to result from increased elastase activity due to oxidative inactivation of $\alpha_1\text{PI}$ by cigarette smoke [12]. Cell-biological studies have suggested that PMN on surface matrices can release elastase in response to various stimuli. Under these conditions the elastase is protected from $\alpha_1\text{PI}$ in solution by the microenvironment created by the close contact of the adherent cell and the surface. Here elastase can act on susceptible proteins in the surface matrix, as has been shown *in vitro* [13–15] and *in vivo* [16]. Finally, recent experiments with a specific cephalosporin-based PMN-elastase inhibitor which inhibits lung haemorrhage associated with immune-complex-mediated injury in the rat lung provide pharmacological evidence for PMN-elastase-mediated connective-tissue damage ([17]).

Direct biochemical evidence for extracellular-substrate cleavage by PMN elastase has recently become available. Weitz *et al.* [18] provided evidence that PMN elastase cleaved the $\text{A}\alpha$ chain of fibrinogen between residues Val²¹ and Gly²² to generate an *N*-terminal 21-residue peptide. Weitz *et al.* [18] assayed this material by an indirect r.i.a. procedure. This utilizes an antibody specific for a thrombin-generated $\text{A}\alpha(1-16)$ fibrinopeptide [19], which is used to measure thrombin-cleaved products of larger portions of the fibrinogen molecule, including $\text{A}\alpha(1-21)$. Subsequent studies in our laboratories by Mumford *et al.* [20] have confirmed that PMN elastase generates $\text{A}\alpha(1-21)$ from fibrinogen. Further, they

Abbreviations used: PMN, polymorphonuclear leucocyte; FMLP, *N*-formyl-Met-Leu-Phe; f.a.b.-m.s., fast-atom-bombardment (xenon) m.s.; $\alpha_1\text{PI}$, α_1 -proteinase inhibitor; r.i.a., radioimmunoassay; TFA, trifluoroacetic acid; PiZZ, deficiency of proteinase inhibitor in ZZ homozygotes.

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have generated and utilized a highly specific polyclonal antibody to the C-terminus of A α (1–21) to assay for A α (1–21) in a number of situations. Although A α (1–21) is not detectable in plasma from healthy individuals, in individuals with the inherited deficiency of α_1 -proteinase inhibitor as characterized by the PiZZ phenotype (deficiency of α_1 PI in ZZ homozygotes), and in individuals with normal levels of α_1 PI suffering from emphysema [21], its generation by PMN elastase or by stimulated PMN has been shown in a number of other situations. For example, PMN adhered to a matrix of fibrinogen and subsequently stimulated with *N*-formyl-Met-Leu-Phe (FMLP) generated A α (1–21) [22]. PMN can also be stimulated to generate A α (1–21) in whole blood [23]. This occurs despite the presence of approx. 1000-fold molar excess of α_1 PI in whole blood. Human whole blood stimulated with Ca²⁺ ionophore A23187 to induce the release of elastase from leucocytes generates 1–3 nM concentrations of A α (1–21) [23,24]. Under similar conditions much larger concentrations of PMN-elastase- α_1 PI complexes, in the range of 100 nM are formed [24]. It is therefore likely that some of the high concentration of elastase released by stimulation of the PMN escapes inhibition by α_1 PI for a brief period of time, allowing the hydrolysis of fibrinogen and other substrates by elastase. This possibility is supported by the observation that higher levels of A α (1–21) are formed in A23187-stimulated whole blood from PiZZ individuals [23], indicating the lower inhibitory effect of α_1 PI on PMN-elastase activity in these cases. The availability of this type of direct assay for specific cleavage products generated by PMN elastase released from stimulated cells in biological environments provides a novel quantitative method for monitoring the activity of this enzyme. However, it is highly desirable to confirm further the nature of the product generated by definitive identification of its structure after its chemical isolation from the biological milieu in which it was generated.

In preliminary experiments a series of reverse-phase and ion-exchange chromatography steps gave products which, on sequencing, showed A α (1–21) and the truncated A α (2–21) as low-level components against a high background. In these cases quantification of the peptides could not be achieved. For example, the amounts of amino acid present were more than sufficient to account for the r.i.a. response, but the marginal increase of an amino acid at a given cleavage step appeared less (1/5 to 1/10) than the r.i.a. results. Further, previous results showed that *N*-terminal truncated synthetic peptides were immunoactive. The presence of such active peptides could add to the r.i.a. response, but these, along with unknown background components, would obscure the sequencing data.

In one case, f.a.b.-m.s. analysis of an isolate opened up an alternative to check for the immunoreactive species. Although no distinct peak was seen for intact A α (1–21), a weak peak was present for A α (2–21). Spiking experiments showed the capability to detect these peptides down to 5 pmol in their f.a.b. mass spectra. The capability of detecting these peptides in the 2000 Da mass range at low levels encouraged us to approach the problem with a combination of the chromatographic steps and m.s. 'stable-isotope-dilution' methods. The present case represents a search for all major immunoactive products to the rabbit serum antibody.

EXPERIMENTAL

Materials

The centrifugations of blood were done in a Beckman GPC bench-top centrifuge, and the aqueous ethanol extracts were centrifuged in a du Pont RC-5B centrifuge with a GS-3 rotor.

The h.p.l.c. was run on a Spectra Physics 8800 instrument with an SP 8500 dynamic mixer, an SP 8490 u.v. detector and a SP 4270 integrator. Mass-spectral data were obtained on a Finnigan-MAT TSQ 70B mass spectrometer.

The chemicals used in the fractionations were of reagent grade suitable for h.p.l.c. Ca²⁺ ionophore (A23187) and heparin were obtained from Sigma. House-distilled water was passed through a Millipore four-cartridge Milli-Q system before use. The fibrinopeptide A α (1–21) was synthesized on an Applied Biosystems model 430 instrument, and the fibrinopeptide A α (1–21) labelled with [³H₈]phenylalanine [C₆²H₅C²H₂C²H(N¹H₂)CO₂¹H] (Merck Isotopes, Montreal, Canada) at position 8 on an Applied Biosystems Model 431A instrument. The ³H-labelled phenylalanine was converted into the *t*-butoxycarbonyl derivative with di-*t*-butyl dicarbonate [25]. Both peptides were synthesized on a 0.5 millimolar scale via the basic method of Merrifield [26]. Fractions (10 mg) of the crude labelled peptide was purified on a Vydac C₁₈ 5 μ m 10 mm $\times$ 25 cm peptide/protein column in an aq. 10–70% (v/v) acetonitrile gradient containing 0.1% in trifluoroacetic acid (TFA) over 20 min at a flow of 4 ml/min. The product showed a single peak on a Vydac C₁₈ 5 μ m 4.5 mm $\times$ 25 cm column in an aq. 10–50% (v/v) acetonitrile gradient containing 0.1% TFA. Synthetic unlabelled peptide was similarly chromatographed.

Methods

The purification of the elastase-derived fibrinopeptide A α (1–21) was monitored by a previously described r.i.a. [20]. Briefly, *H*-Cys-Nle-A α (Gly¹⁷-Val²¹-OH) was coupled to BSA using the heterobifunctional coupling reagent *m*-maleimidobenzoyl-*N*-hydroxysulphosuccinimide ester (Pierce). Rabbits were immunized by multiple intradermal injections of immunogen in complete Freund's adjuvant to yield a polyclonal antibody designated as R-20. The assay involved the competitive binding of the probe peptide [¹²⁵I-Tyr]A α (1–21) (15000–20000 c.p.m. with a specific radioactivity routinely of 2000 Ci/mmol). Standard solutions of synthetic A α (1–21) were prepared in Dulbecco's Ca²⁺ + Mg²⁺-free phosphate-buffered saline, with supplemental 0.1% gelatin, 0.01% thimerosal and 1.0 mM EDTA. After incubation overnight with R-20 rabbit polyclonal antibody, separation of bound versus free material was achieved via a charcoal/dextran separation system. The standard curve ranged from 0.02 to 2.0 pmol of A α (1–21) peptide in a final volume of 300 μ l. Standards were made from weighed samples of peptide corrected by amino acid analysis. The limit of detection was 0.03 pmol in the assay. Aliquots (0.1–1%) from the chromatographic fractions were made up to 1 ml in water, and 0.05–0.1 ml portions were assayed.

Stimulation of human blood with ionophore. Heparinized human blood (50 units/ml) was collected from three individuals in seven 50 ml units each (350 ml/donor). The units of 50 ml of blood were stimulated with Ca²⁺ ionophore A23187 (150 μ M) at 37 °C for 30 min as previously described [20]. At the end of the 30 min incubation, the blood from each individual was pooled (350 ml each) and aliquots from each were assayed for A α (1–21). Each pool was then spiked with 0.7 nmol of the phenylalanine-labelled A α (1–21) in 70 μ l of an aqueous solution, and again aliquots were taken for r.i.a. The blood from each donor was centrifuged at 827 *g* at 4 °C for 20 min, and the plasma supernatant was collected.

Fractionation steps. (1) The plasma (505 ml total) was diluted with 3 vol. of ethanol. The resulting suspension was centrifuged at 827 *g* at 4 °C for 20 min to yield a total of 1755 ml of supernatant. The pellets resuspended in 50% (v/v) ethanol in water (1 litre total) and again centrifuged at 4500 *g* at 4 °C for 30 min to give an additional 970 ml of supernatant. The combined

Table 1. Summary of the steps for the isolation of r.i.a.-active blood peptides

Step	Weight	Total peptide (r.i.a.) (nmol)	Specific response (pmol/mg)
1	8.07 g (plasma) (estimated solids)	2.45	0.3
2	7.84 g (flow-through)	0	0
	0.23 g (eluate)	4.0	17
3	4.76 mg (preparative reverse-phase h.p.l.c.)	2.6	550
4	0.3 mg (analytical reverse-phase h.p.l.c.)	2.3	7700
5	< 0.1 mg, peak 1, fractions 16 and 17	0.9	> 9000
	< 0.1 mg, peak 2, fraction 20 (anion-exchange h.p.l.c.)	0.185	> 2000

supernants were concentrated *in vacuo* to 250 ml and an aliquot taken for r.i.a.

(2) The concentrate was passed through a bed of Waters C₁₈ reversed-phase resin (50 g, 55–105 μ m-pore-size packing pre-washed with dichloromethane, methanol and water) and the moist bed was washed with 500 ml of water. An aliquot (1.52 ml) of flow-through plus water wash (760 ml total) was set aside and a portion used for r.i.a. The bulk was concentrated and freeze-dried. The resin was then eluted with aq. 50% ethanol. An aliquot (0.38 ml) of the eluate (380 ml) was taken for r.i.a. and the remainder was concentrated and freeze-dried (0.23 g).

(3) The above freeze-dried material was dissolved in aq. 10% acetonitrile and was chromatographed in two runs using a 2 ml sample loop on a preparative du Pont 5 μ m C₁₈ Zorbax 2.1 $\times$ 25 cm column with an aq. 10–40% acetonitrile gradient, 0.2% in TFA, over 60 min at 10 ml/min. At 60 min the solvent was ramped to 90% acetonitrile from 65–75 min. Aliquots (0.02 ml) of the fractions (20 ml each) were set aside for r.i.a. The h.p.l.c. injection system was thoroughly washed and blank runs were made before loading of plasma fractions.

(4) The freeze-dried r.i.a.-active fractions from the previous step were chromatographed using a 0.1 ml sample loop on an analytical du Pont 5 μ m C₁₈ Zorbax 4.6 mm $\times$ 25 cm column in a similar aqueous gradient over 60 min at 1 ml/min. Aliquots (0.002 ml) of the fractions (1.0 ml) were taken for r.i.a.

(5) The freeze-dried active fractions (0.3 mg) were loaded in a 0.1 ml sample loop in the chromatographic buffer and run on an Altex/Applied Science 3 μ m Adsorbosphere NH₂ 4.6 mm $\times$ 15 cm column in an isocratic 0.1 M-ammonium acetate buffer adjusted to pH 5.3 with acetic acid over 60 min at a flow of 0.2 ml/min. Aliquots (0.001 ml) of the fractions (0.4 ml) were taken for r.i.a. The r.i.a.-active fractions were freeze-dried. The tubes were shaken with four drops of aq. 50% methanol, centrifuged, and the solutions transferred into four 30 mm-long acid-washed micro-tubes (autosampler vial inserts; Alltech/Applied Science). The solutions were dried under a slow N₂ stream. The transfer process was repeated. The products from these steps were used for the m.s. studies.

Fractions from step 5 (Table 1) were examined by f.a.b.-m.s. on a Finnigan-MAT TSQ-70B mass spectrometer. Fractions 15 (inactive control), 17, and 20 were dissolved in 3 μ l of water, and

0.5 μ l aliquots used for m.s., and fraction 16 was dissolved in 6 μ l of water and 0.5 μ l aliquots were used for m.s. The m.s. samples were loaded by adding 0.5 μ l of the aqueous fraction concentrate to 1 μ l of a dithioerythritol/dithiothreitol (1:4, w/w) matrix. Spectra were recorded from *m/z* 1900 to 2100 every 2 s in profile mode.

RESULTS

Synthetic fibrinopeptide A α (1–21) (I, Fig. 1) had been prepared earlier for use as a standard for calibration for the direct r.i.a. for A α (1–21). The synthesis of the stable-isotope-labelled peptide (II, Fig. 1) provided a reference that was well separated from the natural peptide by m.s., even in the case of comparison of very weak peak envelopes. The label was placed well into the chain to mark any truncated peptides that also might be generated during, or subsequent to, the incubation step. Peptide labelled with [³H]₈phenylalanine at position 8 replacing the normal peptide led to a product with an 8-mass-unit shift in its molecular ion. Samples of 50 ng of labelled and unlabelled peptide could be seen by f.a.b.-m.s. as peak profiles centred at *m/z* 2046 (*M*+H)⁺ and 2054 (*M*+H)⁺ respectively (Fig. 2). Samples down to 5 ng showed distinct peaks at signal-to-noise ratio of 2–5 (results not shown).

Detection of peptide labelled with [³H]₈phenylalanine after spiking into blood

The blood samples were incubated with Ca²⁺ ionophore A23187 to induce release of elastase from the leucocytes. An r.i.a. run on aliquots at the end of the incubation showed an average concentration of A α (1–21) of 2.1 $\pm$ 0.7 nM for three donors. At the end of this incubation period the labelled peptide was added to give a calculated increase of 2 nM in peptide concentration (2.1 nmol to 1.05 litres of whole blood). The amount chosen for spiking was such as to provide a concentration at the level detected by r.i.a. (1–3 nM) in normal individuals found in previous studies. The amount was based on the weight of the peptide as a salt-free anhydrous compound corrected by amino acid analysis. Aliquots were taken for r.i.a. immediately after the addition of the spike. The r.i.a. showed 3.5 $\pm$ 0.7 nM or an increase of 1.4 $\pm$ 1.1 nM. The variation within this group reflects individual variations of elastase and elastase-inhibitor levels. This increase was consistent with earlier spiking experiments using unlabelled peptide which had showed a 60–70% r.i.a. response to added unlabelled peptide. The attenuation of the increase, as well as some variability of the response, may be due to plasma factors in the individual donors that could interfere with the r.i.a. response.

Fractionation steps

The results of the fractionation steps are summarized in Table 1. The spiked blood was immediately chilled and centrifuged. The plasma was diluted with 3 vol. of cold ethanol, and an aliquot of the aqueous-ethanol supernatant was taken for r.i.a.

Ala-Asp-Ser-Gly-Glu-Gly-Asp-Phe-Leu-Ala-Glu-Gly-Gly-
Gly-Val-Arg-Gly-Pro-Arg-Val-Val

I

Chemical *M*, 2045.25

Ala-Asp-Ser-Gly-Glu-Gly-Asp-[³H]₈Phe-Leu-Ala-Glu-Gly-
Gly-Gly-Val-Arg-Gly-Pro-Arg-Val-Val

II

Chemical *M*, 2053.30

Fig. 1. Peptide sequences of natural and labelled fibrinogen A α (1–21)

Fig. 2. (a) F.a.b.-m.s. of normal synthetic fibrinogen peptide A α (1–21) (50 ng) over m/z 2000–2100 and (b) f.a.b.-m.s. of [$^3\text{H}_6$]phenylalanine-labelled synthetic fibrinogen peptide A α (1–21) (50 ng) over m/z 2000–2100

Fig. 3. Anion-exchange h.p.l.c. of r.i.a.-active product

The r.i.a.-active product from analytical reverse-phase h.p.l.c. was run on a 3 μm Adsorbosphere NH_2 column. The flow rate was 0.2 ml/min, and fractions were collected at 2 min intervals. The u.v. absorbance is shown as a continuous line. The r.i.a. response in percentage inhibition is shown as a broken line with $\circ$ symbols. The main r.i.a. response is seen in peak 1 (fractions 16 and 17) and in peak 2 (fraction 20).

(Table 1, Step 1). Concentration of the aqueous-ethanol fraction gave a cloudy aqueous solution. When this was passed through the bed of a preparative reverse-phase resin, the r.i.a.-active product was strongly held. The aqueous flow-through showed no r.i.a. response for the peptide. The peptide was recovered from the bed by elution with aq. 50% ethanol. This r.i.a.-active product was then run on the preparative reverse-phase column in two runs. Fractions of 20 ml were taken at 2 min intervals. The r.i.a.-active fractions from these two runs (13–15, main run, and 17–18, small second run) were charged on to the analytical reversed-phase column in two runs. Fractions of 1 ml were taken at 1 min intervals using an aq. 10–40% acetonitrile linear gradient run over 60 min. The r.i.a. response was mainly in fractions at times 32 and 33 min with 10–20% tailing into fraction 34 in both runs. Synthetic normal A α (1–21) showed the same retention time as plasma fractions by r.i.a. Blank runs subsequent to the synthetic peptide control runs showed no r.i.a. activity when assayed at 5 times the sample volume used for the controls. Finally (Table 1, Step 5), the resulting active fractions were run on an anion-exchange column in an ammonium acetate

buffer (Fig. 3). The r.i.a.-active fractions, 16, 17 and 20, as well as an adjacent r.i.a.-inactive fraction, 15, from this step, were used for the m.s. Fractions 16 and 17 (600 and 300 pmol respectively by r.i.a.) represented the major r.i.a. product and are designated as peak 1. Fraction 20 (200 pmol by r.i.a.), which in this run was fairly well separated from the main peak, was designated as peak 2. Although a weak r.i.a. response was seen elsewhere, the calibration curve was regarded to be only reliable in the 20–80% binding range of the r.i.a. Low inhibition of binding (80–100% range) has not provided a reliable indication of peptide.

F.a.b.-m.s.

H.p.l.c. peak 1. Fraction 16 (0.5 μl of 6 μl) showed *pseudo*-molecular ions for normal A α (1–21) peptide centred at m/z 2046 ($M^+ + \text{H}$) and for labelled peptide at 2054 ($M^+ + \text{H}$), Fig. 4). Similarly, fraction 17 (0.5 ml of 3 ml) showed m/z 2046 and 2054 along with peaks at 2068 ($M^+ + \text{Na}$) and 2076 ($M^+ + \text{Na}$). The intensities of the $M^+ + \text{H}$ and $M^+ + \text{Na}$ ions were comparable. In contrast, fraction 15, which gave a weak r.i.a. response, showed only weak peaks above noise at m/z 1954/1955 ($M^+ + \text{H}$) and 1976/1977 ($M^+ + \text{Na}$) for some unknown molecular species of 1953/1954 Da (results not shown).

H.p.l.c. peak 2. Fraction 20 (0.5 μl of 3 μl) showed no m/z 2046 nor 2054, but rather m/z 1975, which was consistent with the des-alanine peptide A α (2–21). No peak was seen in the region m/z 1983, which would correspond to the [$^3\text{H}_6$]phenylalanine-labelled A α (2–21) peptide. Some evidence for this peptide was seen previously, first weakly in Edman sequencing and subsequently in a mass spectrum of a previous isolate. The question arose as to whether the A α (2–21) peptide was generated in the original blood sample during the incubation or was produced during the isolation. The absence of labelled A α (2–21) indicated that the A α (2–21) peptide must be generated before the spiking step and was not an artifact of the work-up procedure.

Estimation of the peptide concentrations in blood

The spiking was done at the end of the incubation with ionophore A23187 to raise the concentration of peptide by approx. 2 nM. Neither chromatographic fractionation nor r.i.a. would discriminate between labelled and unlabelled peptides. In contrast, the peaks for the natural and $^3\text{H}_6$ -labelled peptide are clearly distinguished by f.a.b.-m.s. (Fig. 2). The peak ratio of the two in the mass spectrum of the isolate (Fig. 4) should reflect the ratio of the two at the time of spiking.

Fig. 4. Partial f.a.b. mass spectrum of anion-exchange fraction 16 from spiked plasma

The curve shows the average of scans 15–45 over the mass range m/z 1900–2100. Peaks at m/z 2046 and 2054 correspond to the protonated normal fibrinogen peptide A α (1–21) and to the protonated labelled peptide respectively. Additional weak peaks can be seen barely above noise for $(M^+ + Na)$ and $(M^+ + H - H_2O)$.

The f.a.b.-m.s. peak envelopes for natural A α (1–21) and labelled A α (1–21) were of similar intensities. Comparison of f.a.b.-m.s. peak areas using an averaged baseline gave a ratio of 1.18 for m/z 2046 (natural) to 1 for m/z 2054 (labelled) on the following basis. The pseudomolecular ions were observed for both normal A α (1–21) (m/z 2046) and labelled A α (1–21) (m/z 2054), and the ratio of the intensities of the ions were determined manually by comparison of peak areas corrected for the average baseline. In this procedure the cursor on the mass-spectrometer monitor was placed on the mass of interest and the intensity in arbitrary units was noted. This was done at points before, during and after the peak envelope of interest. The points before and after provided a baseline which was then subtracted from the peak envelope. By using this method the ratio of the peak envelope for m/z 2044–2048 for the endogenous peptide to the peak envelope of 2051–2057 for the labelled peptide was 1.18. The r.i.a. data after the addition of label to blood showed an increase of 1.4 nM in measured peptide. By using this estimate of label, the concentration of natural A α (1–21) peptide would then be $1.18 \times 1.4 = 1.65$ nM at the end of their ionophore incubation period before spiking. This by itself is in reasonable agreement (79%) with the activity of the 2.1 nM observed by r.i.a. Further, an estimate can be made of the amount of A α (2–21) in the second, but weaker, r.i.a.-active peak (fraction 20). The r.i.a. indicated 0.185 nmol in total in this peak, whereas 0.9 nmol was detected in peak 1 (fractions 16–17, Table 2). The unlabelled portion of peak 1 is $0.9 \text{ nmol} \times 1.18 / (1.18 + 1) = 0.487$ nmol. The unspiked blood sample should then contain the peak 1 and peak 2 components in the ratio of 0.49:0.18. This assumes the similar response in the r.i.a. for the two components²¹ as well as similar m.s. response to the two peptides. From these peptides a total of 3.68 nM ($1.4 + 1.65 + 0.63$ nM) or 105% of the concentration found by r.i.a. (3.5 nM) is accounted for after spiking. Since the errors in the measurement of the low-intensity peaks and in earlier work on the r.i.a. are in the range of 10% each (based on repeated assays for a given sample), the estimate is approximate, but nevertheless consistent with these two components as the source of the r.i.a. response.

Quantification of the m.s. to fraction 16 was done by comparison of the response to an aliquot of fraction 16 with that spiked with a known amount of synthetic unlabelled peptide. An

aliquot of fraction 16 (0.5 μ l of the 6 μ l sample) with added synthetic unlabelled peptide (0.5 μ l of a 20 μ g/ml aqueous solution; 5 pmol) was analysed by f.a.b.-m.s. In this case an increase in the peak envelope centred at m/z 2046 to that at m/z 2054 gave a ratio of 1.81. This increase from 1.18 would correspond to about 18.5 ng of natural A α (1–21) along with this 10 ng addition. Assuming the f.a.b.-m.s. response was the same for the labelled and unlabelled peptide, the labelled peptide was present at 15.9 ng in the m.s. sample. The fraction-16 aliquot would then contain 18.6 ng of m/z 2046 and 15.8 ng of m/z 2054, which would total 34.6 ng or 17 pmol. R.i.a. of a portion (1/100) of a similar aliquot of 16 (0.5 μ l of 6 μ l in the microtube) gave 19 pmol. The two aliquots give results in good agreement.

DISCUSSION

In these samples the mass-spectral monitoring was concentrated on the molecular-ion region of interest. A narrow scan range (generally m/z 1900–2100 rather than m/z 0–3000) was used to improve sensitivity. The amount of label used in the present experiment was designed to provide a concentration of labelled A α (1–21) comparable with the level indicated by the r.i.a. on natural immunoreactive material, so that a reliable comparison of signal intensities could be made. Earlier work showed suppression ($2\text{--}5\times$) of the f.a.b.-m.s. signal by plasma residues in the matrix when unlabelled synthetic peptide was added to an adjacent inactive fraction in comparison with that signal from synthetic peptide in the usual dithiothreitol/dithioerythritol matrix alone. Therefore, for all mass spectral comparisons, the samples are for peptides in similar matrices so that signal responses could be compared directly. A large number of peaks of unknown structures were seen at a mass range below m/z 1000 in scans made in other runs, but these have not been examined further. The isolation and detection problems in the present case were compounded by the release of intracellular enzymes from the ionophore-treatment step. The present m.s. approach has provided a window in the upper-mass range for seeing the larger peptides.

The truncated A α (2–21) may have been produced by action of either elastase or another enzyme from a group of peptidases [27], e.g., an aminopeptidase, which would be released in the iono-

phore treatment of the leucocytes and would be active during the incubation period. A related peptide, A α (2-16), had been detected by h.p.l.c. along with A α (1-16) from the cleavage of human fibrinogen with bovine thrombin [28]. In the present study the r.i.a.-active peptides were generated under physiological conditions of whole blood in association with a different enzyme. Even in purified fractions we could not reliably detect A α (1-21) and A α (2-21) by h.p.l.c. alone. In the blood-sample fraction, no mass-spectral evidence was obtained for r.i.a.-active peptides which might have further deletions from the *N*-terminal region. In the present work phosphorylated peptides [Ser-3 A α (1-21) or Ser-3 A α (2-21)] were not detected in the f.a.b.-m.s. samples of the r.i.a.-active peaks. Since in these experiments we were working close to the limit of detection, we would not necessarily see these if they were in the range of 10% of the total in these particular blood samples. Nevertheless, the m.s. method better defined the species suggested by the r.i.a.

Related peptides from fibrinogen have been run by f.a.b.-m.s., including fibrinogen A α (1-16), fibrinopeptide A [29,30] and fibrinogen A α (1-15) [31]. In these cases the availability of synthetic material did not cause the problems of sample size constraint or of loss of sensitivity because of the higher molecular mass in the products in the above plasma fractions. Nevertheless, these applications point to the utility of m.s. to detect such peptides. Enkephalins and related peptides in the 500-1000 Da range have been determined by r.i.a. and m.s. [32], and the utility of m.s. in reading through a complex mixture has been noted by Fridland & Desiderio [33].

This m.s. approach coupled with high-resolution chromatography provided a window to see low levels of peptides in mixtures. Considering the possibilities for errors in the handling of small quantities and volumes, and in the r.i.a. method itself, we can say with reasonable assurance that the r.i.a. assay is accounted for by the two fibrinogen-derived peptides. In this isolation the majority of immunoreactive peptide is the A α (1-21). Further, the absence of labelled A α (2-21) peptide suggests that the unlabelled A α (2-21) peptide is generated before spiking. Its presence should not invalidate the r.i.a. measurement, inasmuch as variations in the *N*-terminal region are reported to have little affect on the r.i.a. response. Finally, the f.a.b.-m.s. technique allowed the detection of peptides in the 2000 Da range on the nanogram scale in mixtures of 1-5% purity, not just in a simplified model case, but in a product of a natural biological system.

We thank Ms. Myra C. Walden and Ms. Lorena L. Bennet for assistance in the preparation of this manuscript.

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Received 29 April 1991/24 July 1991; accepted 29 July 1991