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ORIGINAL ARTICLE

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Production and characterization of a monoclonal antibody for sweat-specific protein and its application for sweat identification

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Abstract Identification of body fluids is a common task in medico-legal practice, but specific markers for sweat have not been identified to date. To develop a method for identification of sweat, we identified a sweat-specific protein and produced monoclonal antibodies by immunizing mice with sweat proteins fractionated by anion-exchange chromatography. Among many sweat-reactive monoclonal antibodies obtained, one monoclonal antibody (G-81) was selected because of its unique specificity. G-81 reacted to sweat but not to other body fluids (e.g. serum, saliva, semen, milk, urine and tears) in ELISA. G-81 specifically stained the eccrine sweat gland and did not stain any other tissue including the apocrine sweat gland. In western blotting, G-81 reacted strongly to a 7 kDa band and faintly to 20, 27 and 33 kDa bands of sweat protein. The N-terminal amino acid sequence (18 amino acids) of G-81-reactive peptides was determined, and an identical sequence was found in an antibiotic peptide dermcidin (110 amino acids) reported recently, suggesting that G-81 recognized a fragment of dermcidin. The G-81-reactive peptide could be detected in 8,192-fold dilutions of sweat by ELISA and could be detected in 200-fold diluted sweat samples (n=26) independent of the protein concentration. The G-81-reactive peptide was very stable and was able to detect sweat stains left for at least 11 weeks at room temperature without substantial loss of reactivity. These facts suggest that G-81 is a very useful tool for sweat identification in medico-legal practice.

Keywords Sweat identification · Sweat-specific peptide · Monoclonal antibody · ELISA · Eccrine gland

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Introduction

Knowledge accumulation and technological progress in DNA polymorphisms have brought innovation in identification of individuals, however, DNA is not able to identify tissues. Messenger RNAs transcribed tissue-specifically and can be utilized for tissue identification, however, it seems to be impractical at present because of their instability, suggesting that antibody-based detection is an indispensable tool for forensic investigations [1, 2]. Therefore, antibody-based identification of body fluids is still a very important and common task in medico-legal practice [3, 4, 5, 6]. In particular, extremely sensitive DNA analysis enables us to perform genetic identification from minute specimens such as fingerprints [7, 8, 9], therefore, a test to identify sweat is particularly important. Sweat seems to be the most difficult to identify among body fluids. It is known that most peptides in sweat are derived from serum proteins, and some of them are common to other body fluids [10]. In numerous studies concerning analysis of sweat peptides [11, 12, 13, 14, 15], only one sweat-specific peptide as a marker for sweat identification has been reported [15], and no monoclonal antibody (mAb) specific for sweat has yet been reported. It seems that this is one reason for the difficulty of sweat identification. Therefore, we aimed to identify sweat-specific peptides and to develop a method for identification of sweat using a specific mAb.

In this paper, we describe production and characterization of a novel mAb specific for sweat, and its application to sweat identification.

Materials and methods

Preparation of body fluids samples

Sweat drops from the faces and arms of healthy volunteers were collected under high temperature conditions and stored at –28°C until use. The pooled sweat was centrifuged at 2,000×g for 10 min, and the supernatant was exhaustively dialyzed against distilled wa-

ter and then lyophilized. Saliva and semen samples were prepared in the same way. The protein concentration of the samples was determined by BCA protein assay (Pierce Chemical, Il.).

Two-dimensional (2-D) electrophoresis

In the first-dimension electrophoresis, 200 µg of sweat, saliva or semen protein or 0.625 µl of plasma was dissolved in 125 µl of a sample preparation solution containing 8 M urea, 1% (±) dithiothreitol (DTT), 0.5% ampholine pH 3.5–10.0 (Amersham Biosciences, NJ) and 2% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate. The sample was successively electrophoresed in Immobiline DryStrip (7 cm, pH 4–7L) (Amersham Biosciences) for 1 h at 500 V and 1,000 V and for 2 h at 8,000 V at 20°C by an IPGphor isoelectric focusing unit (Amersham Biosciences) after 12 h rehydration of the strip. In the second-dimension separation, the strip was equilibrated with 50 mM Tris-HCl buffer (pH 6.8) containing 6 M urea, 30% glycerol, 1% SDS and 0.25% DTT, and then with same buffer containing 4.5% sodium iodoacetoamide instead of DTT. The reduced and alkylated peptides in the strip were separated by SDS-PAGE.

Preparation of immunogen

A sweat sample (40 mg) was fractionated by anion exchange chromatography using a Mono-Q column (HR 10/10) (Amersham Biosciences). Elution was performed with a linear gradient of 20 mM Tris-HCl buffer, pH 8.5, to the same buffer containing 0.5 M NaCl during 50 min at room temperature and at flow rate of 3 ml/min. Each fraction was analyzed by SDS-PAGE with silver staining.

Production of mAbs

Fractionated sweat peptides (ca. 100 µg) emulsified in complete Freund's adjuvant (100 µl) were injected subcutaneously into each hind footpad of female BALB/c mice (aged 7 weeks old). After 9 days, popliteal lymph nodes were used as a source for antibody-producing cells [16]. The myeloma cell line P3U1 was used as a fusion partner. Lymph node cells were mixed with myeloma cells in a ratio of 5:1 and fused by 50% polyethylene glycol (MW 1500). The antibodies to sweat peptides in the culture supernatants were screened by ELISA, and positive hybridomas were cloned by limiting dilution.

ELISA

The wells of microtitre plates were coated with the antigens (diluted body fluids, extracts of sweat stains or chromatography fractions) dissolved in 50 µl of 50 mM carbonate buffer, pH 9.6, at 4°C overnight. All subsequent incubations were made in a volume of 50 µl/well. The plates were blocked with dilution buffer (10 mM Tris-HCl buffer, pH 7.4, containing 0.05% Tween 20, 0.5 M NaCl and 5% skimmed milk) followed by incubation with the primary mAb for 1 h at room temperature. The plates were incubated with biotin-conjugated goat anti-mouse IgG or IgM (CALTAG, Calif.) in dilution buffer for 1 h and then incubated with horseradish peroxidase-conjugated streptavidin (Amersham Biosciences) for 15 min. Color was developed with o-phenylenediamine (0.4 mg/ml) in 0.05 M citric acid phosphate buffer, pH 5.0, containing H_2O_2 (0.006%). Absorbance at 492 nm was measured with a model 550 microplate reader (Nihon Bio-Rad Laboratories, Tokyo, Japan) after addition of 2 N H_2SO_4 (50 µl) to each well.

Western blotting

The polypeptides in body fluids were separated by SDS-PAGE (15% acrylamide with 0.05% bisacrylamide), transferred electrophoretically (1.2 mA/cm^2 for 30 min) onto polyvinylidene difluoride (PVDF) membrane sheets (Nihon Millipore, Tokyo, Japan) with a semi-dry type blotter and then blotted with monoclonal antibodies. The primary mAbs bound to the membrane sheets were detected with biotin-conjugated anti-mouse IgG and IgM, followed by streptavidin-horseradish peroxidase conjugate. Color development was performed with 4-chloro-1-naphthol (0.6 mg/ml) in PBS containing H_2O_2 (0.006%). Peptides separated by 2-D electrophoresis were also transferred onto a membrane as described above.

Immunohistochemical staining

Formalin-fixed paraffin sections were dewaxed, rehydrated and rinsed in 10 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl (PBS). The samples were incubated with 3% H_2O_2 for 5 min at room temperature to inactivate endogenous peroxidase activity and then incubated with 5% skimmed milk in PBS containing 0.5% Tween-20 for 20 min. The sections were incubated with mAbs for 1 h at room temperature in a moist chamber. The sections were incubated with goat anti-mouse immunoglobulins conjugated to a polymer labeled with peroxidase (Envision+, DAKO Japan, Kyoto, Japan) for 30 min. To visualize the reaction products, the sections were incubated with 3-amino-9-ethylcarbazole as a substrate for the peroxidase. The sections were counterstained with hematoxylin.

N-terminal sequencing of sweat-specific peptide

The peptides in sweat were fractionated by anion exchange chromatography with a Mono-Q column (HR 10/10) as described above, and the reactivity of the fractions to G-81 was assayed by ELISA. The most reactive fractions were rechromatographed with the same column and the collected fractions were subjected to cation exchange chromatography with a Mono-S column (HR 5/5, Amersham Biosciences). Elution was performed with a linear gradient of 10 mM sodium phosphate buffer, pH 7.0, to the same buffer containing 0.5 M NaCl, during 50 min at room temperature and at a flow rate of 2 ml/min. The reactive fractions were collected and subjected to gel filtration chromatography with Sephacryl S-100 HR (Highprep 16/60, Amersham Biosciences). The fraction most reactive to G-81 was subjected to N-terminal amino acid sequencing by a protein sequencer (Model 477A, Applied Biosystems Japan, Tokyo, Japan). The PVDF membrane corresponding to the region of the molecular weight 7 kDa and pI 5.1–5.2 in a 2-D blot of sweat was also subjected to N-terminal amino acid sequencing. The resulting sequences were compared with known sequences in the composite protein database provided by the DNA Data Bank of Japan, and computations were performed using the BLAST network service [17].

Preparation and extraction of sweat stains

A filter paper (3MM, Whatman Japan, Tokyo, Japan) was spotted with 10 µl drops of sweat, and the spots were marked with a pencil. The spotted filter paper was allowed to stand for 11 weeks at room temperature. The spots were extracted periodically with 100 µl of PBS, and the extracts were stored in a freezer until analysis by ELISA.

Results

To prepare the immunogen for production of mAbs, pooled sweat was separated into 31 fractions by anion-exchange chromatography with a Mono-Q column (Fig. 1A). These fractions were sorted into 8 groups (A–H) based on SDS-PAGE analysis (Fig. 1B) and mAbs were produced by immunizing mice with each group of fractions. Among many sweat-reactive mAbs obtained, one mAb, G-81 (IgM), which was obtained by immunization with frac-

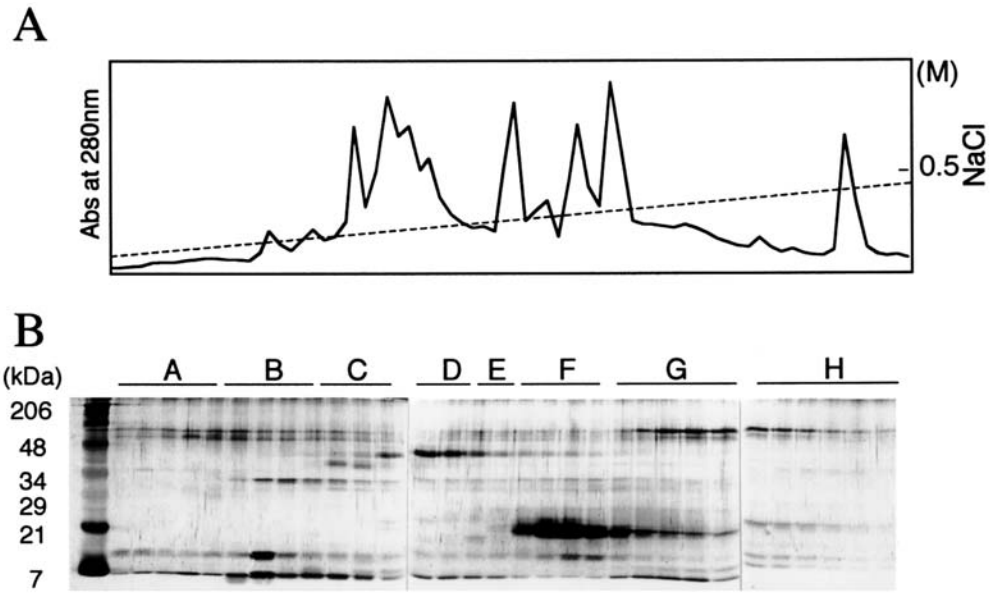

Fig. 1A,B Elution profile and SDS-PAGE analysis of the fractions of anion-exchange chromatography of sweat peptides. The components of sweat were fractionated by anion-exchange chromatography with a Mono-Q column. **A** Elution was performed with a linear gradient of 20 mM Tris-HCl buffer, pH 8.5, to the same buffer containing 0.5 M NaCl. The fractions were analyzed by SDS-PAGE (13%) with silver staining **B**, sorted into 8 groups (A–H) and were used as immunogens for mAb production

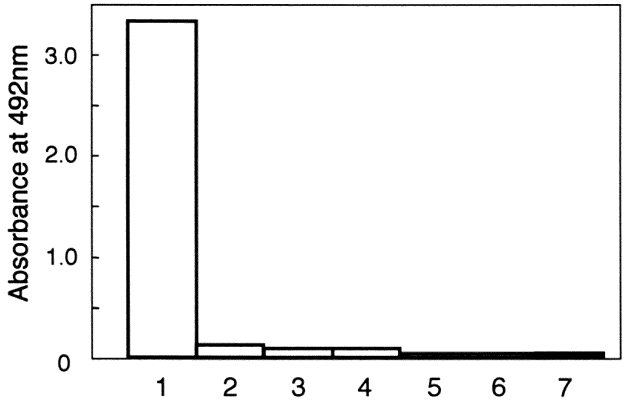

Fig. 2 Analysis of tissue specificity of G-81 by ELISA, 100-fold diluted body fluids in PBS were adsorbed on a microtitre plates and used as solid phase antigens for ELISA, mAb G-81 was used as a primary antibody. Data represent the mean values of 5 samples from different individuals in duplicated measurements (*1* sweat, *2* plasma, *3* semen, *4* saliva, *5* milk, *6* tears, *7* urine)

tion-group C, reacted specifically to sweat and not to other body fluids (e.g. serum, saliva, seminal plasma, milk, urine and tears) in ELISA (Fig. 2). The reactivity of G-81 to sweat of animals was only examined for horse sweat and G-81 did not react (n=3) in ELISA (data not shown). We could not obtain sweat samples of primates, therefore, the species specificity of G-81 remains to be investigated. In immunohistochemical staining of the tissue sections, G-81 reacted only to the sweat gland in the skin (Fig. 3A) but not to other tissues (e.g. mammary gland, testis, prostate, seminal vesicle and salivary gland, results not shown). Detailed immunohistochemical examination showed that G-81 reacted only to dark cells in the eccrine sweat gland and secreted sweat in the ducts (Fig. 3B) but not to the apocrine sweat gland (Fig. 3A). In the western blot analysis, G-81 reacted only to peptides in sweat and not to any other body fluids examined (Fig. 4). The 7 kDa peptide

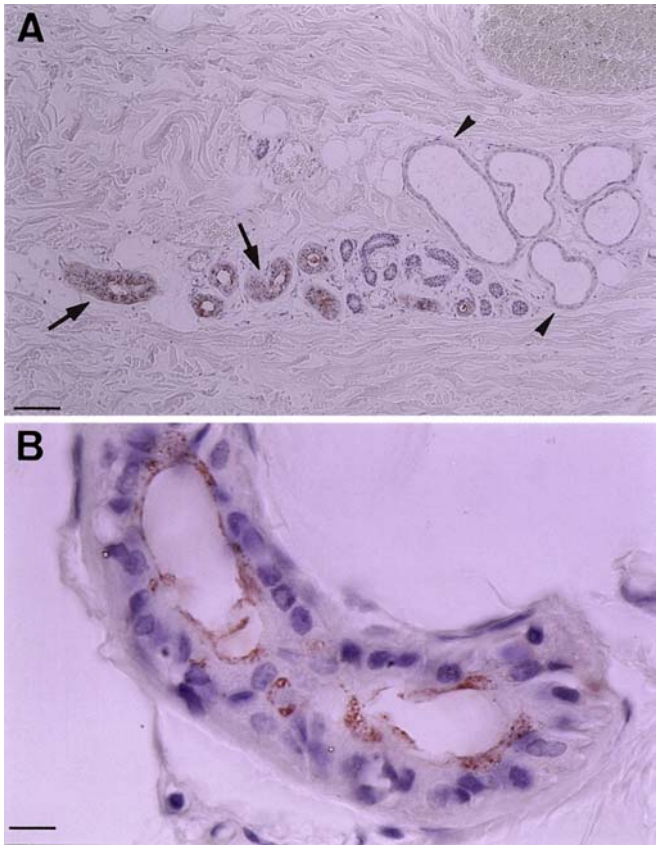

Fig. 3A,B Localization of G-81-reactive peptide in the tissue specimens. Formalin-fixed paraffin-embedded tissue sections were stained with G-81 as a primary antibody and HRP-conjugated anti-mouse Ig polymer as a second antibody. The sections were then stained with hematoxylin. In panel **A**, the arrows and arrowheads indicate the eccrine and apocrine sweat gland, respectively. Only dark cells of the eccrine gland in the skin were stained by G-81 (*Scale bars* 100 μm panel **A**, 10 μm panel **B**)

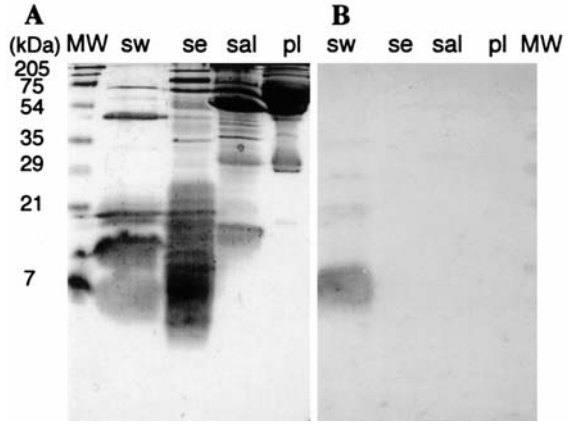

Fig. 4A,B Analysis of tissue-specificity of G-81 by western blotting. The components of body fluids were separated by SDS-PAGE and transferred to a PVDF membrane. The gel was stained with Coomassie brilliant blue (panel **A**), and the membrane was stained with G-81 (panel **B**). mAb G-81 reacted to a prominent 7 kDa band in addition to faint 20, 27 and 33 kDa bands in sweat but not in other body fluids (panel **B**) (*sw* sweat, *se* semen, *sal* saliva, *pl* plasma)

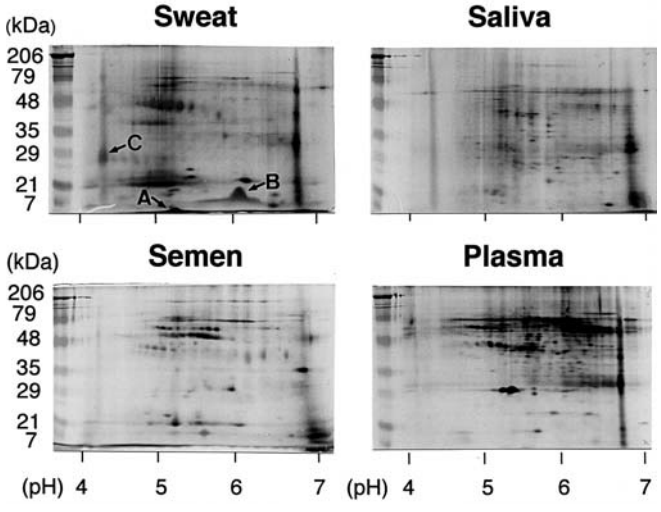

Fig. 5 2-D electrophoresis of body fluids. The components of body fluids were separated by 2-D electrophoresis and stained with silver staining. The first-dimension electrophoresis was performed in Immobiline DryStrips (7 cm, pH 4–7L) and in the second-dimension step was performed by SDS-PAGE on 15% polyacrylamide gels. The arrows (A–C) in the sweat panel indicate candidates for sweat-specific peptides

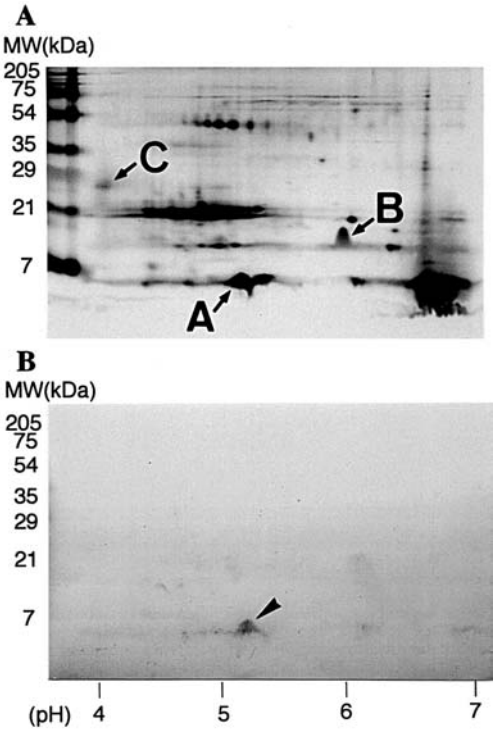

Fig. 6A,B 2-D electrophoresis and 2-D blotting of sweat. The components of sweat were separated by 2-D electrophoresis as described in Fig. 5 except that SDS-PAGE for the second-dimension was performed on 18% polyacrylamide gels. Panel **A** shows silver staining pattern of the gel. The arrows (A–C) in panel **A** indicate candidates for sweat-specific peptides. The sweat peptides in the same 2-D electrophoresed gel were transferred to a membrane and stained with G-81 (panel **B**, *arrowhead* G-81-reactive peptide)

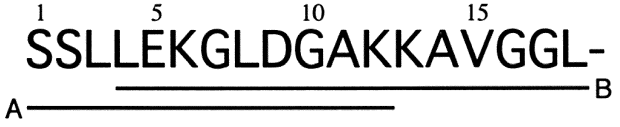

Fig. 7 N-terminal amino acid sequence of two peptides defined by G-81, 7 and 12 kDa peptides which were purified by 2-D blotting and chromatography, respectively, and subjected to N-terminal amino acid sequencing. The N-terminal sequence of 18 amino acids of the peptide was identified by a combination of the sequences from two peptides. The lines indicate the sequences obtained from the peptides purified by 2-D blotting (line **A**) and chromatography (line **B**), respectively

was most prominently stained by G-81 in addition to other faintly stained bands (20, 27, 33 kDa) in sweat (Fig. 4). As shown in Fig. 5, a few peptides (A–C) in the 2-dimensional (2-D) electrophoresis of sweat proteins seemed to be unique for sweat in comparison with those of other body fluids, serum, saliva and seminal plasma. In 2-D western blotting of sweat with G-81 (Fig. 6B), the region (MW, 7 kDa; pI 5.1–5.2) that corresponded to peptide A in Fig. 6A was prominently stained, indicating that G-81 recognized peptide A. For determination of the amino-terminal (N-terminal) amino acid sequence of the G-81-reactive peptide, the membrane corresponding to the region of peptide 1 (MW, 7 kDa; pI 5.1–5.2) in a 2-D blot of sweat was cut out and analyzed by a protein sequencer. Another sample, the 12 kDa peptide reactive to G-81 purified by ion-exchange chromatography (Mono-Q and Mono-S) and gel filtration (Sephacryl S-100), was also subjected to protein sequencing. The amino acid sequences of the samples could be determined as shown in Fig. 7. Each amino acid sequence of the two samples overlapped in 9 amino acid residues, which resulted in 18 residues of N-terminal amino acid sequence of the G-81-reactive peptide that was identical to the sequences of human cachexia-associated protein [18, 19] and survival promoting peptide for neuronal cells [20, 21]. More recently, a peptide having an identical amino

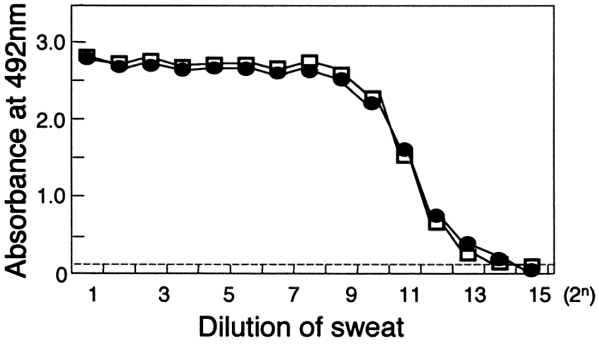

Fig. 8 Detection of G-81-reactive peptides by ELISA. Serial doubling dilutions of sweat samples from two individuals were adsorbed on a microtitre plate and used as solid phase antigens for ELISA which was performed as described in Fig. 2. The broken line is a cut-off value (0.12, mean of blank values plus 2SD). Data represent the mean value of duplicated measurements

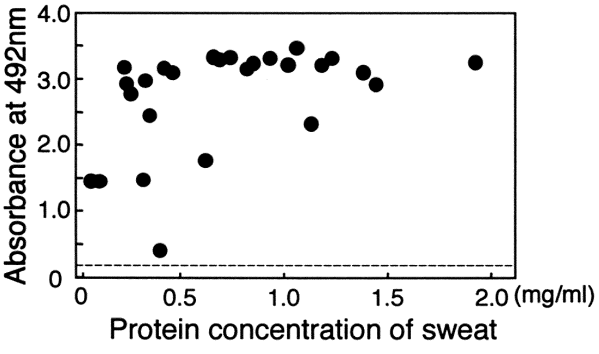

Fig. 9 Relationship between absorbance value in ELISA and protein concentration of sweat. The absorbance values of diluted sweat (×200) samples (n=26) in ELISA using G-81 were plotted to their protein concentrations. ELISA was performed as described in Fig. 2. The broken line is a cut-off value (0.16, mean of blank values plus 2SD). Data represent the mean value of duplicated measurements

acid sequence has been reported, which is a novel antibiotic peptide, dermcidin, secreted by sweat glands [22].

The G-81-reactive peptide in sweat could be detected from sweat samples obtained from two individuals at least to a 8,192-fold dilution by ELISA (Fig. 8). Since it was likely that the detection level of G-81-reactive sweat peptide depended on the protein concentration of sweat, the relationship of the detection level of G-81-reactive peptide and the protein concentration of sweat was examined in 26 sweat samples. As shown in Fig. 9, G-81-reactive peptide could be detected in all sweat samples at 200-fold dilutions independent of the protein concentration (0.07–1.92 mg/ml). To assess the effect of aging on detection of G-81-reactive peptide from sweat stains, the sweat stains on filter papers were kept at room temperature for 11 weeks and were periodically extracted and subjected to ELISA. G-81-reactive peptide could be detected from the stains after 11 weeks storage at room temperature without substantial loss of reactivity (Fig. 10).

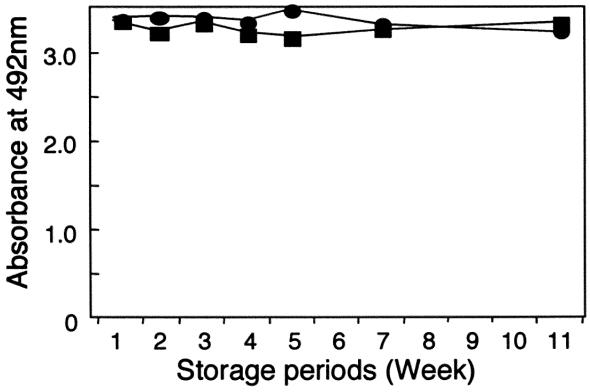

Fig. 10 Detection of the G-81-reactive peptide from aged sweat stains by ELISA. Stains on filter paper made with 10 µl sweat from two individuals were extracted with PBS (100 µl), 50 µl of each extract was subjected to ELISA as described in Fig. 2. Data represent the mean value of duplicated measurements

Discussion

Human sweat consists of eccrine and apocrine sweat secreted from two different sweat glands [23]. Secretion of eccrine sweat is enhanced by a rise of body temperature due to high environmental temperature or exercise, in contrast to that of apocrine sweat, which is induced by tension [23]. The eccrine glands are distributed widely on the body surface in contrast to the apocrine glands, which are restricted to areas such as the abdomen and axilla. Therefore, sweat stains on clothes are considered to consist of sweat derived from both glands, however, it is likely that eccrine sweat is the main component.

Histochemical and immunohistochemical examination of the skin including the sweat gland, supply useful information for wound age determination [24]. Furthermore, morphology of the sweat gland was utilized for post-mortem interval determination [25]. Therefore, in addition to sweat identification, the mAbs specific for sweat may contribute to detection of biochemical and histochemical changes of the sweat gland in the healing process of wound or in the post-mortem interval.

To establish an immunological method for identification of sweat from stains by production of novel mAbs for sweat-specific peptide, we collected sweat from arms and faces produced under high temperature conditions, which seemed to contain mainly eccrine sweat. A few unique spots (A–C) of sweat were observed in 2-D electrophoretic analysis of the collected sweat (Figs. 5 and 6A). However, it was unclear whether they were sweat-specific components or degradation fragments of the components common to other body fluids. Therefore, we did not try to purify the unique peptides in sweat, and roughly divided fractions of sweat by anion-exchange chromatography were used for immunogens (Fig. 1). Fortunately, we obtained a mAb (G-81) that was exactly specific for sweat in ELISA (Fig. 2), immunohistochemical investigations of the tissues (Fig. 3) and western blotting (Fig. 4). It is known that primates secrete sweat to control body temperature [26,

27] and only the horse and donkey other than primates also do. Therefore, the cross-reactivity of G-81 to horse sweat was examined, and the result indicated that G-81 did not cross-react to horse sweat, however, the cross-reactivity to sweat of primates is still to be investigated. In the 2-D western blotting, G-81 reacted to the peptide (MW, 7 kDa; pI 5.1–5.2) corresponding to peptide A, one of the candidate sweat-specific peptides in 2-D electrophoretic analysis of sweat (Fig. 6A). It is likely that peptide C is the sweat-specific acidic glycoprotein described by Takizawa et al. [15]. The N-terminal amino acid sequence (18 amino acid residues) obtained from G-81-reactive 7 and 12 kDa peptides is identical to the parts of previously reported sequences in peptides described as a human cachexia-associated protein and a survival-promorting peptide for neuronal cells [18, 19, 20, 21]. However, another identical sequence was found in the sequence of amino acids 62–79 of a novel, recently reported antibiotic peptide (110 amino acids), dermcidin (DCD), that is secreted by sweat glands [22]. The C-terminal peptide (DCD-1, amino acids 62–109) of DCD, which was found in sweat, was toxic to various microorganisms. Northern blot analysis, in situ hybridization and immunohistochemical staining indicated that DCD was expressed only in sweat glands [22]. All these facts suggest that the G-81-reactive peptide is identical to the C-terminal peptide of DCD, DCD-1.

The sensitivity of ELISA for the G-81-reactive peptide seems to be sufficient for practical use (Fig. 8). Since the G-81-reactive peptide (DCD-1) may play a key role in the innate immune responses of the skin, it seems that the concentration of the G-81-reactive peptide in sweat shows relatively little variation. Indeed, G-81-reactive peptide could be detected at high levels by ELISA from 200-fold diluted sweat samples independent of the protein concentration (Fig. 9). Furthermore, G-81-reactive peptide was stable for at least 11 weeks in stains (Fig. 10), suggesting that it is a suitable marker for identification of sweat in medico-legal practice.

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