Original Article

A novel venom protein of the Asian bee (*Apis cerana indica*) with an affinity to human α1-microglobulin

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ABSTRACT

Bee stings are a common health problem throughout the world and can sometimes result in fatal anaphylactic reactions. We have studied Asian bee (*Apis cerana indica*, *Apis cerana nigrocincta* and *Apis dorsata*) venoms and have discovered a novel protein with a molecular size of 50 kDa (p50), as shown by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, which has not been reported in the venom of the Western honey-bee, *Apis mellifera* (AM). The p50 protein showed a unique affinity to human α1-microglobulin (α1-m). As a result, p50 was purified using an affinity column with α1-m. The p50 protein was further purified by an affinity column with a monoclonal antibody raised against p50 in mice. The p50 protein induced an inflammatory reaction following injection into mouse ear; that is, degranulation of mast cells, edema, hyperemia and hyperpermeation of the local capillaries were observed. The reaction was very similar to that seen when phospholipase A_2 of AM, a representative bee venom, was administered by injection. The inflammatory reaction induced by p50 was completely inhibited by mixing p50 with α1-m prior to injection. These results indicate that p50 is a unique venom component of the Asian bee that induces the inflammatory reaction and that human α1-m may be involved as a protective mechanism against bee stings of at least some Asian bee species.

Key words: α1-microglobulin, Asian bee, bee venom, inflammatory reaction.

INTRODUCTION

Allergy to insect stings is an important health problem in many Asian countries.[1,2] Because there are many species in the order of Hymenoptera, it is often difficult to identify the causal species.[3] Even for so-called honey-bees (genus *Apis*), there are four species: *A. mellifera* (AM), *A. cerana*, *A. dorsata* and *A. florea*.[3] *Apis mellifera* is a common Western honey-bee and is found all over the world, but is not always the dominant species in Asian countries. Some *Apis* species, such as *A. cerana indica*, are common in Asian countries and are called Asian bees or Asian honey-bees.

Asian bees have been a main source of honey products for a long time in eastern Asian countries.[4] *Apis cerana* makes hives in a variety of natural cavities ranging from trees to hill sides. The giant honey-bee, *A. dorsata*, makes several large hives in trees, which are called 'bee trees' in Indonesia. The hives have long been objects sought by rural people. Recently, beekeeping of these Asian bees has become an important subject of study from the view-point of both agriculture and forestry with the production of commercially valuable products, such as beeswax and honey. Even after introduction of modern apiculture with AM, beekeeping of these Asian bees still has several advantages, because they have high levels of resistance to parasites and diseases. Although keepers of Asian bees have the occupational risk of sting allergy, we cannot find any research papers describing allergy to Asian bees. Obviously, identification and characterization of venom components in these Asian species will provide clues for a more defined study on etiologic processes as well as for the development of countermeasures to sting allergies and other related reactions.

The main allergenic components in AM venom are reported to be phospholipase A_2 (PLA_2), hyaluronidase and acid phosphatase.[5,6] However, by comparing the

venom of the European honey-bee (*A. mellifera mellifera*) with its Africanized counterpart (*A. mellifera scutellata*), Schumacher *et al.* demonstrated a higher level of PLA$_2$ in the venom of the Africanized variant.[7] This indicates that the composition of venom may differ among different species or even among variants of the same bee species.

In the present study, we investigated Asian bee venoms and identified a new protein with a unique property of binding human α1-microglobulin (α1-m). To our knowledge, this is the first description of a defined protein molecule in Asian bee venoms.

METHODS

Bee venom

Approximately 7000 bees of *A. cerana indica* (Fabricius 1798; ACI) and 4000 bees of both *A. cerana nigrocincta* (ACN; a variant of ACI) and *A. dorsata binghami* (AD) were captured in South Sulawesi, Indonesia. Venom specimens were collected by puncture of the venom sacs. Collected venom samples were transported to Japan in dry ice and were stored at –80°C until use. Four thousand AM bees were purchased from a beekeeper in Japan and venom specimens were collected using the same procedures.

Preparation of antibody

Rabbit antibody raised with PLA$_2$ of AM

Antibody was raised in two Japanese white female rabbits by immunizing with 0.1 mg AM PLA$_2$ (Sigma Chemical Co., St Louis, MO, USA) emulsified with Freund's complete adjuvant (Difco, Detroit, MI, USA). The antigen was intradermally injected on three occasions, weekly, at multiple sites. One month after the last injection, blood was collected from marginal ear veins and the IgG fraction was prepared by ammonium sulfate precipitation. Reactivity of the rabbit IgG raised with PLA$_2$ (rIgG-PLA$_2$) was confirmed by agar gel immunodiffusion (the Ouchterlony test) and immunoblotting.

Monoclonal antibody against p50

Antibody was made according to Shulman *et al.*[8] Briefly, BALB/c mice were injected intraperitoneally with 0.1 mg partially purified p50 protein (see Results) in 0.1 ml Freund's complete adjuvant, followed by the application of antigen in incomplete adjuvant twice, after 4 and 6 week intervals. Spleen cells were fused with a myeloma cell line (SP2/0-Ag) by incubating in 45% polyethylene glycol in Roswell Park Memorial Institute (RPMI)-1640 medium at 37°C for 60 min. After selecting for hybridomas with hypoxanthine, aminopterin and thymidine (HAT) medium, positive clones were identified by enzyme-linked immunosorbent assay (ELISA) using peroxidase-conjugated anti-mouse immunoglobulin antibody (HPR-anti-mIgG; Dako, Glostrup, Denmark). The antibody producing hybridomas were cloned three times and were maintained in RPMI-1640 supplemented with 10% fetal bovine serum and 5% BriClone (Bioresearch, Dublin, Ireland).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting

The SDS-PAGE was performed in 10–20% linear gradient gels by the method of Laemmli.[9] The low molecular weight electrophoresis calibration kit (Pharmacia, Uppsala, Sweden) was used as a marker. Proteins were visualized by silver staining (Silver stain kit; Wako, Osaka, Japan).

For immunoblotting, gel proteins were transferred to poly(vinylidene fluoride) (PVDF) membrane (Atto, Tokyo, Japan). Membranes were incubated with rIgG-PLA$_2$, followed by the corresponding second antibody, for example, anti-rabbit IgG conjugated with alkaline phosphatase (ALP-anti-rIgG; Dako). The membrane was finally stained with 5-bromo-4-chloro-3-indolyl phosphate potassium and nitroblue monotetrazolium chloride (BCIP/NBT; Vector Lab, Burlingame, CA, USA).

To screen serum proteins for bee venom-binding proteins, membranes were incubated with normal human serum or concentrated urine from patients with tubular proteinuria due to chronic cadmium intoxication (Itai-Itai disease; urine of these patients contains high levels of α1-m), followed by the application of a specific antibody against each protein: anti-α1-m, anti-β2-microglobulin (Dako), anti-IgG, anti-IgA, anti-IgD, anti-IgM (Boehringer, Marburg, Germany) and anti-IgE (Dako). The presence of a specifically bound antibody on the membrane was visualized by ALP-anti-rIgG and BCIP/NBT.

Affinity chromatography with rIgG-PLA$_2$

The rIgG-PLA$_2$ (0.1 mg) was coupled to CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions. Bee venom (100 μL from a 1 mg protein/mL stock) from ACI, ACN or AD was applied to the column and was eluted with either 0.1 mol/L acetate

buffer at pH 4.0 or 0.2 mol/L glycine buffer at pH 2.2. The amount of protein in each fraction was determined according to Bradford[10] (Tonein-TP II kit; Otsuka, Tokyo, Japan).

Purification of α1-m from human urine

Human α1-m was purified from 24 h urine specimens of two patients with tubular proteinuria due to cadmium, because patients with Itai-Itai are reported to excrete a large amount of α1-m into their urine.[11] Urine samples were ultrafiltrated and concentrated 10-fold using a Diaflo ultrafiltration membrane (MA 01915, cut-off point 10 000 MW; Amicon, Beverly, MA, USA). A protein fraction precipitable with ammonium sulfate concentrations of 20–80% was collected, dialyzed against phosphate-buffered saline (PBS) pH 7.8 and applied to a column (60 × 2.5 cm) packed with Sephadex G-100 (Pharmacia), as described by Ekström et al.[12] Final purification of α1-m was by affinity chromatography using the antibody to human α1-m (Dako), to give a single band of 30 kDa using SDS-PAGE and silver staining.

Purification of p50

The α1-m (0.1 mg) was coupled to CNBr-activated Sepharose 4B. To enrich p50 by using its property to bind α1-m, venom of ACI (100 μL of a 1 mg protein/mL stock) was applied to the affinity column and was eluted with either acetate buffer or glycine-HCl buffer, as described earlier.

For further purification, a monoclonal antibody against p50 (clone A23) was immobilized by CNBr-activated Sepharose 4B and affinity chromatography on the enriched p50 fraction was performed under the same conditions as described previously.

Biological effects of bee venom proteins

Biological activities of the bee venom protein were assayed according to previous methods,[13,14] with minor modifications. Using female BALB/c mice (age 15 days; bodyweight 25–30 g) without sensitization, 1 μg AM PLA$_2$, purified p50 alone or a 1:1 mixture of p50 and α1-m was injected intradermally into the left ear. Phosphate-buffered saline was injected into the right ear. The injected volume was approximately 10 μL/site. Control animals also received 10 μL PBS. Immediately afterwards, 100 μL of 0.5% Evans blue dye solution was injected into the tail vein. At 20, 30 or 60 min after injection, the right and left ears were compared for blue color and hyperpermeation of local capillaries. Two mice were killed at each observation time and their ears were removed and fixed in 10% formalin and then embedded in paraffin. Tissue sections were examined microscopically after staining with Nissl, Giemsa, or hematoxylin and eosin. Ear thickness was measured by light microscopy (VideoMicroMeter, VM-60; Olympus, Tokyo, Japan) to evaluate hyperpermeation and edema. The extent of degranulation of mast cells was carefully observed and the number of degranulated cells in the total number of cells is expressed as a percentage, as described by Oettgen et al.[15] Results were analyzed statistically by a multiple comparison test (Dunnett's test).

RESULTS

Analysis of bee venom proteins by SDS-PAGE

Venom proteins from ACI, ACN, AD and AM were analyzed by SDS-PAGE. The protein profile of ACI, as visualized by silver staining, was similar to that of ACN, but different from that of AD. There were several protein bands, such as the 25, 40 and 50 kDa bands, detected in the venom of ACI, ACN and AD that were not observed in the venom of AM (Fig. 1).
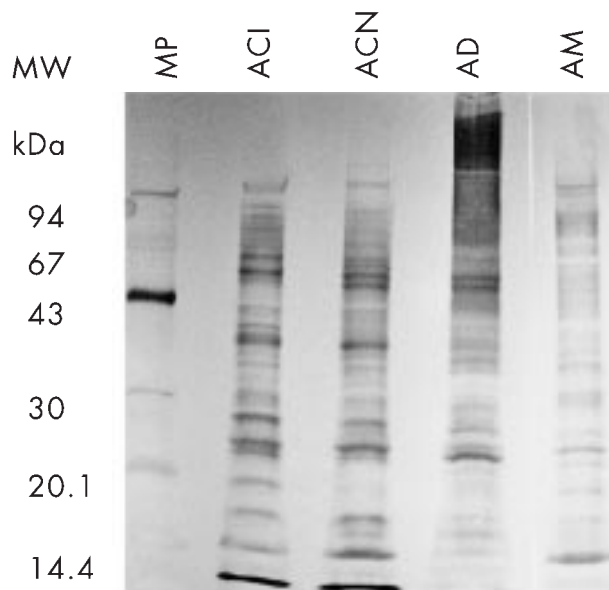

Fig. 1 Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis of bee venom proteins from different species. Venom proteins from the Asian bees Apis cerana indica (ACI), A. cerana nigrocincta (ACN) and A. dorsata (AD) and Western honey-bee, A. mellifera (AM) were electrophoresed on an SDS–polyacrylamide gel and stained with silver. MP, marker protein.

Identification of a protein in Asian bee venom reacting with human α1-m

Because PLA$_2$ is a major component of venom in AM, we first screened immunologically for proteins in Asian bee venoms able to bind rIgG-PLA$_2$. The rIgG-PLA$_2$ antibody formed a clear single precipitin line with PLA$_2$ in the Ouchterlony test. The IgG antibody reacted with PLA$_2$ at 19 kDa with some additional bands in immunoblotting, suggesting that the rIgG-PLA$_2$ mainly contained antibody against PLA$_2$, but also contained trace amounts of antibodies against other venom components, such as melittin micelles.[5] After affinity purification of the venom proteins with the antibody, we detected a dominant band at 50 kDa, tentatively named p50, with some additional bands among these three Asian bee venoms (Fig. 2).

Next, we examined the possible binding of human serum proteins to the venom proteins and found that human α1-m shows affinity to the 50 kDa protein from ACI, ACN and AD (Fig. 3). In the case of AM, human α1-m bound to a 19 kDa protein, which corresponds to the expected molecular size of PLA$_2$ in this species. We decided to purify the 50 kDa protein by the use of this property.

Purification of p50 from ACI venom

Using an affinity column prepared with the α1-m protein, we could obtain the 50 kDa protein in eluates from ACI venom using either glycine or acetate buffer, but with some additional bands (Fig. 4). Clearly, the binding of α1-m to p50 is not specific enough to allow purification of the protein.

The enriched p50 protein fraction was used to raise a monoclonal antibody. Using affinity chromatography and the obtained monoclonal antibody, we were able to obtain a protein fraction that showed a single 50 kDa band by silver staining (Fig. 5).

Biological function of p50

When unsensitized mice were injected intradermally with 1 μg PLA$_2$ from AM, all developed a small nodule with a diameter of 2–3 mm at the site of injection and a blue spot appeared and spread rapidly in the skin of the ear due to increased permeability of local capillaries. A very similar reaction was observed in mice injected with 1 μg purified p50 (Fig. 6). Blue color in the left ears was clearly observed in all mice injected with p50. However, the blue color did not appear in any mice in groups injected with PBS, α1-m or the mixture of p50 and α1-m.
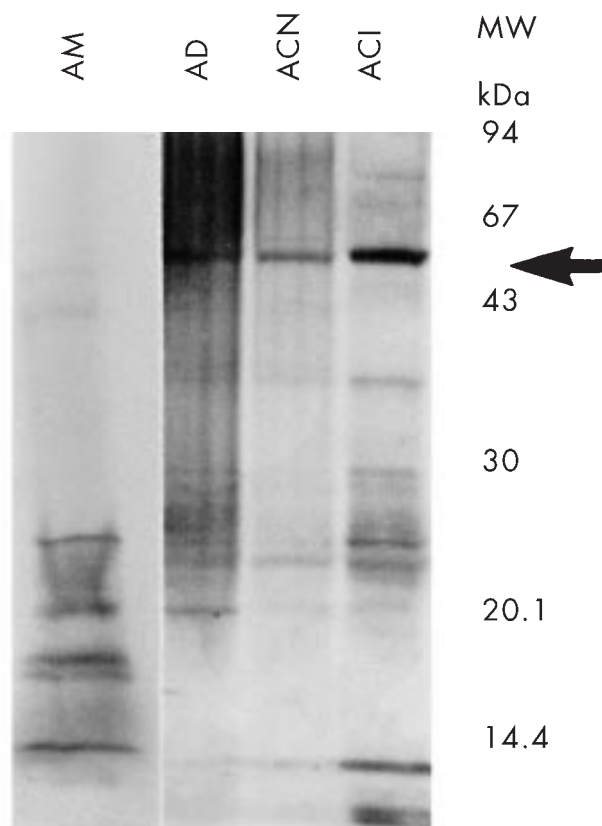

Fig. 2 Venom proteins affinity purified with rabbit IgG-phospholipase A$_2$ (PLA$_2$). Venom proteins of the three Asian bees (*Apis cerana indica* (ACI), *A. cerana nigrocincta* (ACN), *A. dorsata* (AD)) and *A. mellifera* (AM) were purified by affinity chromatography using antibody raised against PLA$_2$ of AM. After elution from the column, the proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and silver stained. A 50 kDa protein, tentatively named p50, was detected in all of the Asian bee species, with some additional bands. In AM, several bands between 12 and 24 kDa were observed.

Ear thickness was measured in all mice by microscopy. Because the variability of the ear thickness data was relatively small and was not significant within the measurement period from 20 to 60 min, the data of six mice (two mice were killed at 20 min, two at 30 min and two at 60 min) were gathered and used to analyze group differences by treatment. As shown in Fig. 7, statistically significant differences were obtained between p50 and PBS and between PLA$_2$ and PBS but not between the mixture of p50 and α1-m and PBS. These results indicate that p50 has biological PLA$_2$ activity and that the biological activity of p50 is blocked by mixing with α1-m.
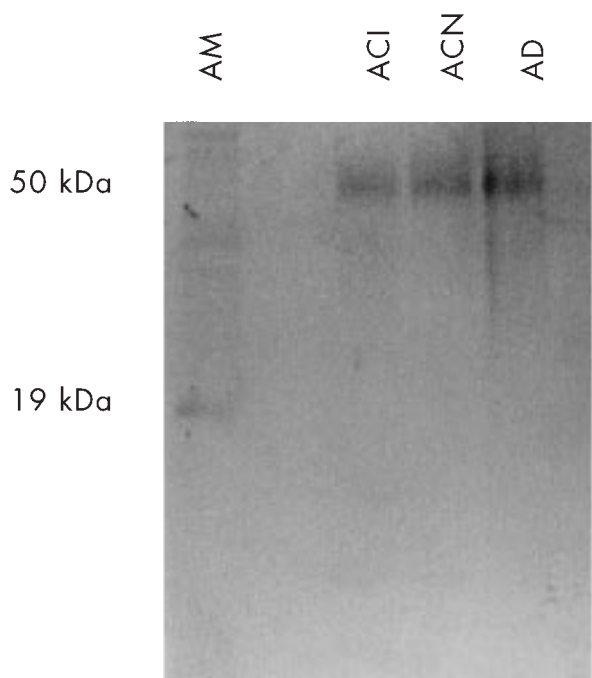
Fig. 3 Binding of human α1-microglobulin (α1-m) to the p50 protein of Asian bee venom. Bee venom proteins enriched by affinity column chromatography with anti-phospholipase (PL)A$_2$ antibody were electrophoresed, transferred onto poly(vinylidene fluoride) (PVDF) membrane and incubated with human α1-m. The bound α1-m was visualized with specific antibody. Human α1-m binds to the 50 kDa protein in Asian bee venom (*Apis cerana indica*, ACI; *A. cerana nigrocincta*, ACN; *A. dorsata*, AD) and to the 19 kDa protein in *A. mellifera* (AM), which corresponds to the molecular size of PLA$_2$, a representative venom protein in this species.

As shown in Fig. 8, the percentage of degranulated mast cells was 53% in the mouse group injected with p50 and was significantly higher than that of the group injected with PBS. However, the percentage of degranulated mast cells was reduced to approximately 18% (i.e. a similar level to the control animals injected with PBS) when p50 was mixed with α1-m protein. Injection of PLA$_2$ resulted in degranulation in 68% of mast cells. These results indicate that the p50 protein identified and purified in the present study represents one of the unique venom proteins inducing inflammatory reactions.

DISCUSSION

In the present study, we collected bee venom by puncture of venom sacs and identified a 50 kDa protein in Asian bee venom, p50, which induced inflammatory reactions. In previously published papers, venom collection by electro-
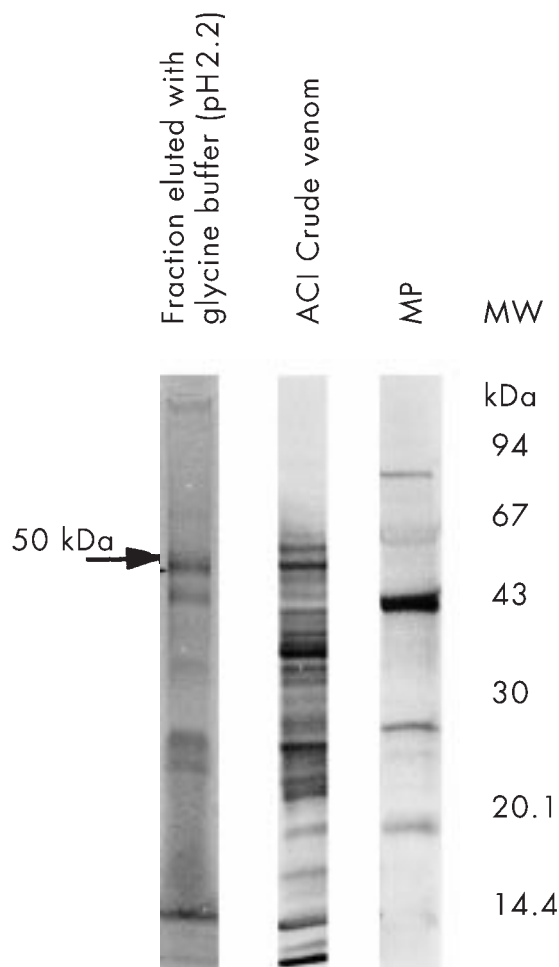

Fig. 4 Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of enriched p50 in the venom of *Apis cerana indica* (ACI). The p50 fraction in the venom was enriched by affinity chromatography using the purified human α1-microglobulin. However, some additional bands were also obtained by this purification process.

stimulation has been the more ideal method for collecting venom specimens for diagnosis and immunotherapy.[5,16] However, we could not use the electrostimulation apparatus to collect venoms in this study, because the bee habitats exist in undisturbed forests away from populated areas. Therefore we cannot exclude the possibility that p50 belongs to bee body proteins. However, it may be reasonable to assume that p50 has a role in allergic reaction at the stinging sites because p50 has the capacity to induce the inflammatory reaction.

Although the molecular size of the PLA$_2$ of AM is 19 kDa, PLA$_2$ in mammalian tissue have varying molecular sizes from 14 kDa (groups I–III) to 85 kDa (group IV).[17] In our preliminary experiments, however, neither PLA$_2$
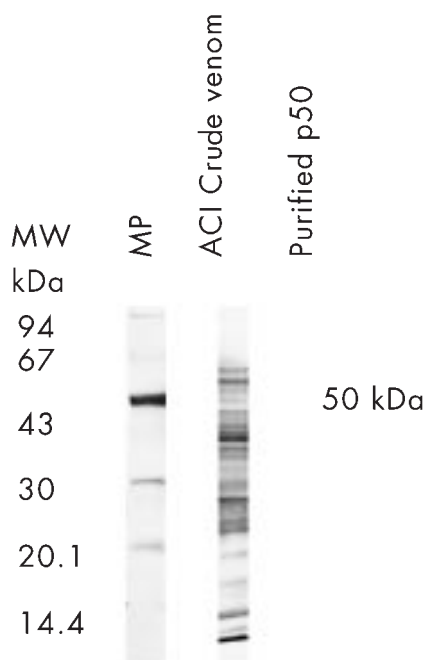
Fig. 5 Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis of purified p50 in the venom of *Apis cerana indica* (ACI). The p50 fraction in the venom was purified by affinity chromatography using monoclonal antibody against partially purified p50. A clear, single band of 50 kDa was clearly visible after silver staining. MP, marker proteins.
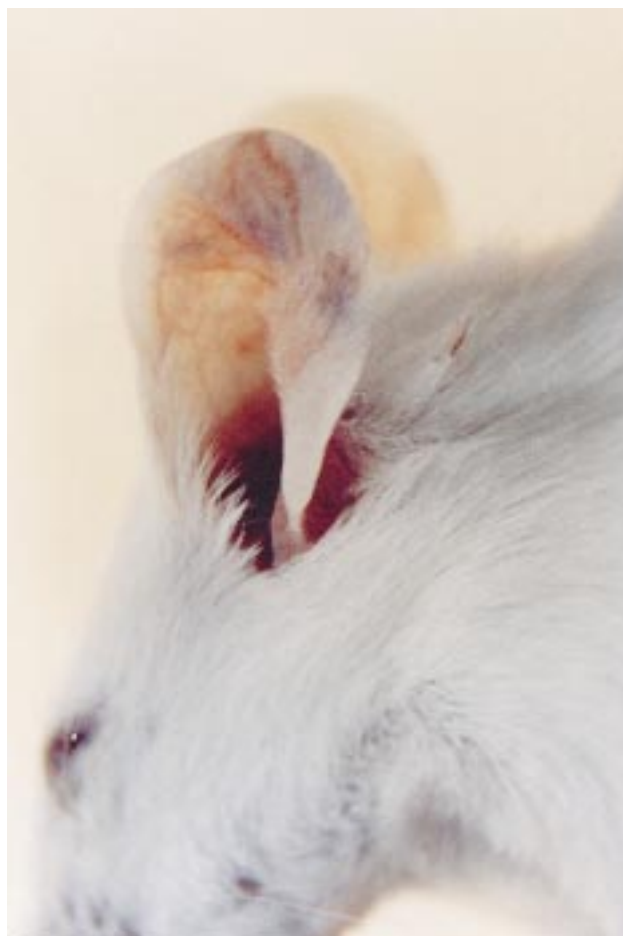

Fig. 6 Hyperpermeation of local capillaries by the injection of purified p50. One μg of p50 protein was intradermally injected in the ears of BALB/c mice, immediately followed by i.v. injection of Evans blue solution. Increased permeability of the local capillaries was demonstrated by a marked blue tinge in the left ear from 30 to 60 min after injection.

nor acid phosphatase activity was detected in the p50 fraction by the methods of Hoffman and Shipman[18] or Hoffman.[19] Further study is needed to determine which epitopes on the protein are recognized by the antibody and to detect the enzyme activities using more sensitive and extensive methods. Because p50 was detected in venom of three Asian bees ACI, ACN and AD, p50 species differences would provide a further interesting theme to be clarified from the viewpoint of insect ecology and evolution.

When p50 was injected into the ear of an unsensitized mouse, local inflammatory reaction, as well degranulation of mast cells, was observed. Chemical mediators, such as histamine, released by this process are considered to trigger the reaction. Degranulation of mast cells is mostly mediated by IgE. However, approximately 15% of subjects allergic to Hymenoptera venom did not release histamine by the IgE-mediated mechanism.[20,21] Mast cell degranulating peptide[22] and PLA$_2$ purified from venom of AM itself were reported to be sufficient for releasing histamine from mast cells.[23] It is very likely, therefore, that p50 in Asian bee venom may directly activate the mast cells through an undetermined mechanism. When

p50 was mixed with α1-m prior to injection, induction of the anaphylactic reaction by p50 was diminished with concomitant inhibition of mast cell degranulation. α1-Microglobulin may inhibit the enzyme activity of p50, which is essential for the activation of the mast cells, although an alternative mechanism cannot be excluded at present. For example, the binding sites on p50 to be recognized by mast cells may be hidden by α1-m.

In our experiment p50 showed an affinity, among human plasma proteins examined, only to α1-m. Because venom proteins affinity purified using α1-m showed several protein bands in SDS–PAGE, more precise studies are needed to determine whether the binding of the α1-m with p50 is specific or non-specific. α1-Microglobulin is reported to belong to the lipocalin
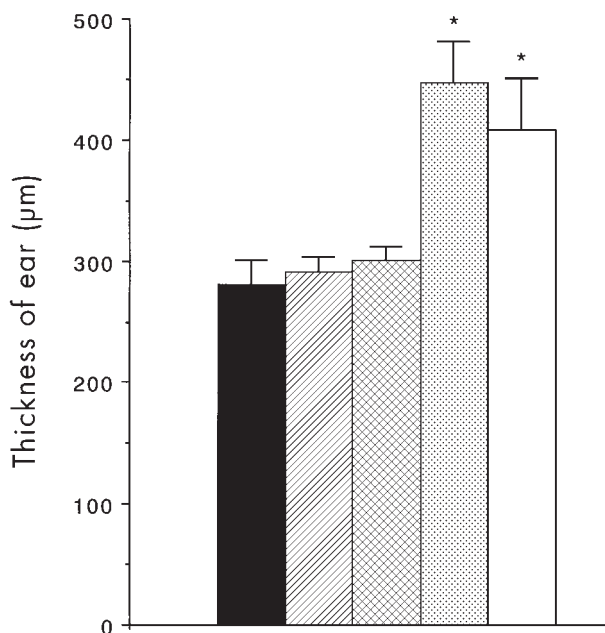
Fig. 7 Thickness of a mouse ear after injection of phospholipase A$_2$ (PLA$_2$; □), p50 (▨), α1-microglobulin (α1-m; ▨), a mixture of p50 and α1-m (▨) or phosphate-buffered saline (PBS; ■). Thickness of the ear was expressed as the mean ± SEM in six mice measured at 20, 30 and 60 min after injection. Statistically significant ($P < 0.05$) differences, indicated by asterisks, were obtained between p50 and PBS and between PLA$_2$ and PBS, but not between the mixture of p50 and α1-m and PBS.

Fig. 8 Degranulation of mast cells after injection of phospholipase A$_2$ (PLA$_2$; □), p50 (▨), α1-microglobulin (α1-m; ▨), a mixture of p50 and α1-m (▨) or phosphate-buffered saline (■). Degranulation was carefully examined for approximately 40 mast cells in each mouse. The extent of mast cell degranulation is expressed as the percentage of degranulated mast cells of the total number of mast cells. The mean ± SEM was calculated for six mice that were killed at 20, 30 and 60 min. The p50 protein is active in the degranulation of mast cells, which was completely inhibited by mixing with human α1-m prior to injection. Statistically significant ($P < 0.01$) differences, indicated by asterisks, were obtained between p50 and PBS, and between PLA$_2$ and PBS, but not between the mixture of p50 and α1-m and PBS.

family.[24,25] Although the biological roles of α1-m *in vivo* are not thoroughly understood, it is considered to be involved in a number of functions including immuno suppression[24] and the transport of small hydrophobic molecules.[26] Amino acid sequences of mouse and human α1-m were found to be highly conserved.[27] In plasma, approximately 50% of the α1-m molecules are linked to other plasma proteins. Human IgA was reported to bind α1-m at an equal molecular ratio.[25] Recently, α1-m was reported to be present not only in human plasma, but also in skin tissue, as demonstrated immunologically.[28] These results and the detoxification of the venom protein by α1-m demonstrated in the present study indicate that α1-m may have a protective role against bee venom in humans.

Although further characterization of p50 is needed to understand its biological and pathological significance thoroughly, our identification of a novel p50 in Asian bee venom and its unique affinity to human α1-m constitute a substantial basis for revealing an etiologic process and developing preventative measures against the anaphylactic reaction to bee stings.
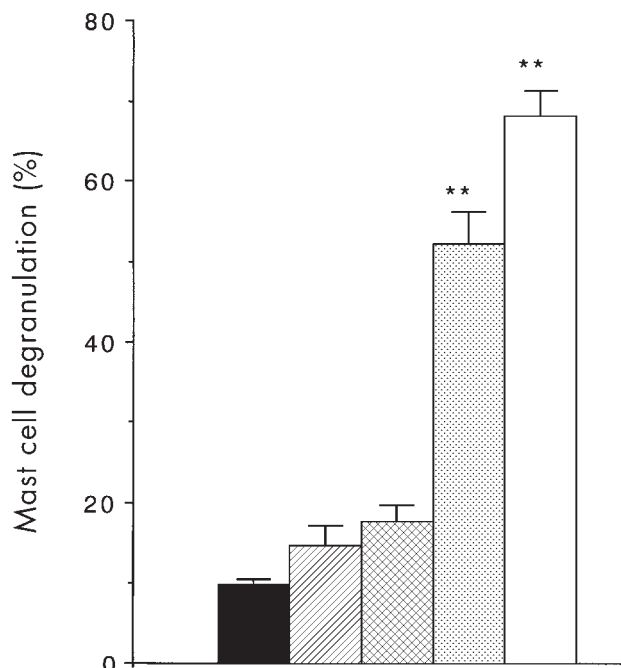
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