

S. Spitzauer^a
 Ch. Schweiger^a
 J. Anrather^c
 Ch. Ebner^b
 O. Scheiner^b
 D. Kraft^b
 H. Rumpold^a

^a Institute of Clinical Chemistry
 and Laboratory Diagnostics,

^b Institute of General
 and Experimental Pathology, and

^c Clinic of Veterinary Medicine,
 Department of Internal Medicine,
 University of Vienna, Austria

Characterisation of Dog Allergens by Means of Immunoblotting

Abstract

Sera from 75 patients with clinical type I allergy against dogs were investigated by means of immunoblotting using extracts prepared from dog hair/dander (CAN XI D) and saliva. In addition, selected sera were tested on extracts made of hair, skin, salivary glands (parotis and submandibularis), serum and liver. A 69-kD IgE-binding protein was identified in all extracts tested with an incidence of approximately 40% and shown to be dog albumin by means of inhibition experiments. In 96% of patients' sera IgE antibodies reactive with a 19-kD and/or a 23-kD protein of the hair/dander extract (CAN XI D) were observed. IgE binding to a 23-kD band was also detected in the hair and saliva extracts, but not in skin, salivary gland, serum and liver extracts. A 19-kD IgE-binding protein was strongly expressed in skin and to a lesser degree in saliva, but not in hair, serum and liver. Preincubation of patients sera with the hair extract and subsequent probing with the hair/dander extract (CAN XI D) inhibited IgE binding to the 23 kD protein whereas preincubation with the skin extract abolished IgE binding to the 19-kD protein. Using the hair/dander extract as inhibitor, IgE binding to the 19- and 23-kD proteins of saliva was abrogated. Thus it is concluded that the 23-kD protein is preferentially expressed in hair and saliva whereas the 19-kD protein is found in saliva and skin. Furthermore these two proteins are likely to represent immunologically independent major allergens.

Key Words

Allergy
 IgE
 Immunoblot

Introduction

Dog hair and dander extracts are complex mixtures containing various proteins. Several studies have been carried out to examine the allergenic composition of dog hair/dander extracts using gel filtration, crossed immunoelectrophoresis and crossed radioimmuno-electrophoresis. Loewenstein's general studies of mammalian allergens [1] revealed several dog allergens, Uhlin et al. [2] demonstrated 24 antigens, whereas Ford et al. [3] found 28 al-

lergens in dog hair/dander extracts. Some of these allergens have been identified as serum components, like albumin (Ag3) and γ -globulin (Ag23) [2]. By means of gel filtration, two components around 28 kD were characterised by Yman et al. [4] in dog dander extracts. Recently, a major allergen (Ag13) was purified by Schou et al. [5] revealing a molecular weight (MW) of 25 kD on SDS-PAGE and 22 kD by HPLC gel filtration. This allergen accounts for approximately 50% of the allergenic activity of dog hair and dander extracts.

By use of SDS-PAGE combined with immunoblotting Boutin et al. [6] described dog allergens of 69, 43, 24 and 22 kD [6]. Most of the components described so far have not been identified in dog hair/dander only, but also in saliva, urine and serum.

In the present study we attempt to further characterise the origin of dog allergens by means of immunoblotting employing extracts made of dog hair/dander, skin, hair, saliva, salivary glands, serum and liver.

Material and Methods

Sera

Sera from 75 patients, 36 male and 39 female, aged 6–63 years, were selected for this study according to case history, positive skin prick tests and positive radioallergosorbent tests (RAST) with dog allergens. In addition all patients had a positive RAST and/or skin prick test with other allergens like tree pollen, grass/rye pollen, mite, cat and horse dander, guinea pig epithelia (detailed serologic information about patients shown in figures 1–6 is given in table 1). Sera from 22 healthy persons, with a negative case history of any type I allergy and negative skin prick test and RAST to dog allergens were pooled and used as a negative control serum pool. After collection, sera were stored at -20°C until used.

Allergen Extracts

Hair/Dander Extract (Can XI D). Powdered dog hair and dander from five different breeds (Alsatian, boxer, collie, dachshound and poodle) was obtained from Allergon (Engelholm, Sweden) and extracted essentially according to Ipsen and Loewenstein [7] with modifications [8]: 10% powdered dog hair and dander were extracted in 10 mmol potassium dihydrogen phosphate, 2 mmol EDTA, 10 mmol sodium diethyldithiocarbamate, 3 mmol sodium azide, pH 7, under continuous shaking for 20 h at 4°C . To the extraction mixture 20 g/l polyvinylpyrrolidone were added to avoid possible interactions of protein material in the extracts (allergens) with aromatic compounds possibly present in dog hair/dander extract. Particles were removed by centrifugation (40,000 g for 1 h at 4°C). The supernatant was extensively (72 h) dialysed against 10 mmol potassium dihydrogen phosphate, pH 7, lyophilised and stored at -20°C . Before use, extracts were dissolved in distilled water and diluted 3:1 in a 4-fold concentrated Laemmli sample buffer [9].

Hair Extract. Washed dog hair from several different breeds and cross-breeds was obtained from a dog-grooming parlour. Dogs were shampooed and trimmed. Hair was washed again and cut into 2-mm pieces and extracted as described above.

The extracts were dissolved in distilled water and diluted 3:1 in a 4-fold concentrated Laemmli sample buffer and boiled for 10 min.

Skin Extract. Dog skin was obtained from several different breeds and cross-breeds. Tissues were homogenised under liquid nitrogen, dissolved 3:1 in 4-fold concentrated Laemmli sample buffer and boiled for 10 min.

Saliva Extract. Saliva from several different breeds and cross-breeds was diluted 3:1 in a 4-fold concentrated Laemmli sample buffer and boiled for 10 min.

Dog Serum. Sera from several different breeds and cross-breeds were diluted 3:1 in a 4-fold concentrated Laemmli sample buffer and boiled for 10 min.

Dog Salivary Glands. Salivary glands (parotis, submandibularis) were obtained from two different cross-breeds, homogenised under liquid nitrogen, dissolved 3:1 in 4-fold concentrated Laemmli sample buffer and boiled for 10 min.

Dog Liver. Dog liver was homogenised under liquid nitrogen, dissolved 3:1 in 4-fold concentrated Laemmli sample buffer and boiled for 10 min.

The protein concentration of all extracts was determined by the Peterson modification [10] of the Lowry method and adjusted to about 1.5 mg/ml for SDS-PAGE. Each batch of all extracts was characterised with respect to protein concentration and protein pattern by SDS electrophoresis and Coomassie blue staining. The reproducibility of IgE-binding patterns was tested with selected patients' sera by immunoblotting.

SDS-PAGE

Samples were diluted in a 4-fold Laemmli sample buffer (0.0625 M Tris, 2.3% SDS, 10% glycerol, 0.025% bromphenol blue, either with or without 5% 2-mercaptoethanol and heated at 95°C for 10 min. SDS-PAGE was performed on a vertical slab gel apparatus (Hoefer, San Francisco, Calif. USA). 12% homogenous gels with 5% stacking gels were used. Electrophoresis was carried out according to Laemmli [9]. MW were estimated using prestained protein standards (14.3–200 kD) (Amersham International and Bethesda Research Laboratories, Md., USA).

Immunoblot

The separated proteins were transferred to nitrocellulose using a Hoefer blotting apparatus, at 300 mA for 4 h [11]. Transfer was performed in a Tris-glycine medium (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3). Nitrocellulose sheets were cut into 5 mm strips. Strips were saturated for 30 min at room temperature in a buffer containing: 50 mM sodium phosphate, 0.5% Tween 20, 0.5% BSA, 0.05% sodium azide pH 7.5. Subsequently strips were incubated overnight at 4°C with patient's serum, diluted 1:10 in the same buffer. Strips were washed 3 times for 20 min with buffer. Bound IgE was detected by ^{125}I rabbit antihuman IgE (Pharmacia International) diluted 1:10 in buffer, incubated overnight at 4°C . Strips were washed 3 times for 20 min. Control strips were processed either with normal human serum pool or buffer, respectively. Autoradiography was performed at -70°C for 48–72 h, using Lanex R FAST intensifying screens and Kodak ORTHO G x-ray films.

Inhibition Studies

RAST. 100 μl of each patient's serum were preincubated with either 150 μl concentrated hair/dander extract (CAN XI D) containing 225 μg total protein or with a 1:2, a 1:4 or a 1:8 dilution. Incubation was performed at 4°C overnight. Samples incubated with PBS only were used as controls. Thereafter RAST discs (e2, e5; Pharmacia Diagnostics) were added and the assay was continued according to standard RAST technique. Results were calculated using the following formula:

$$\text{Inhibition (\%)} = \frac{\text{cpm}_{100} - \text{cpm}_x}{\text{cpm}_{100}} \times 100,$$

where cpm_{100} = uninhibited and cpm_x = inhibited.

Table 1. Patient serologic data

Patient No.	Positive prick test	Positive RAST	Total IgE U/ml
1	dog, cat, horse, cow, pig	e1:4, e5:4	ND
2	dog, cat	e1:4, e5:4	> 1,000
3	dog, cat, cow, grass pollen	e1:4, e5:4, g3:4	ND
4	dog, cat, guinea pig, horse, grass pollen	e3:1.5, e5:4, g3:4	ND
5	dog, cat, horse	e1:2.4, e5:4	600
6	dog, rabbit, cat, guinea pig, horse, mite	e3:3:3, e5:4, d1:2.7	ND
7	dog, horse, cow, grass pollen	e3:1.8, e5:4, g3:4	530
8	dog, cat	e5:4	ND
9	dog, cat, horse, pig	e1:4, e3:3, e5:4	ND
10	dog, cat, horse, cow	e1:4, e3:3.6, e5:4	> 1,000
11	dog, rabbit, cat	e5:4	ND
12	dog, cat	e1:3.6, e2:3, e5:4	ND
13	dog, rabbit, cat, guinea pig, horse, cow, mite	e1:4, e2:4, e5:4, d1:2.2	ND
14	dog, rabbit, cat	e2:4, e5:4	800
15	dog, cat, rabbit, mite, grass pollen	e1:4, e2:4, e5:3, g3:2.4, d1:2.4	> 1,000
16	dog, rabbit, cat, guinea pig, horse	e1:3.9, e2:3, e5:4	> 1,000
17	dog, rabbit, cat, guinea pig, horse, grass and birch pollen	e1:2.9, e2:3, e5:3, g3:3.6, t3:3.6	> 1,000
18	cat, mite	e1:2, d1:4, d2:4	730
19	dog, cat, guinea pig, horse, cow, grass and birch pollen, mite	e3:3.9, e5:3.9, p3:3.1, t3:3.1, d1:2.8	ND
20	dog, birch pollen, mite	e5:3.6, t3:4, d1:2.4	ND
21	dog, cat, horse, mite	e1:2.7, e3:2.2, e5:4, d1:4	500
22	dog, rabbit, cat, horse	e5:4	ND
23	dog, rabbit, cat, guinea pig, horse, cow, grass and birch pollen	e1:2.9, e5:4, g3:4, t3:3.4	400
24	dog, rabbit, cat, guinea pig, grass and birch pollen	e5:4, g3:3.8, t3:4	ND
25	dog, rabbit, cat, guinea pig, horse, grass pollen, mite	e1:4, e3:3, e5:4, g3:4, d1:4	ND
26	dog, cat, horse, mite	e1:4, e5:4, d1:3.2	880

Information regarding skin prick tests and RASTs of various allergens as well as total IgE of representative patients shown in figures 1–6. ND = Not done.

Immunoblot. 1 ml of patient's serum diluted 1:20 was mixed with 1 ml of buffer or extract [hair, skin, hair/dander and dog's albumin (Pel Freeze), respectively] containing 300 µg protein/ml and incubated overnight at 4 °C. Thereafter immunoblotting was performed as described above.

Results

Information regarding skin prick tests and RASTs of various allergens as well as total IgE of representative patients, whose results are shown in figures 1–6, is given in table 1.

RAST Inhibition

The hair/dander extract (CAN XI D) was tested in RAST inhibition experiments with sera from selected patients. Binding to e2 and e5 was inhibited in a range of 64–95 and 32–95%, respectively (fig. 1; patients 12–17 in table 1).

Immunoblot Studies

75 patients were tested by means of immunoblotting on hair/dander extract (CAN XI D) and saliva extract. Selected sera were investigated using extracts made of hair, skin, salivary glands (parotis and submandibularis), serum and liver. SDS electrophoresis was carried out under both reducing and non-reducing conditions; no differences of IgE-binding patterns were observed. SDS-PAGE for immunoblots shown in figures 2–6 were performed under reducing conditions.

Reactivity of Patients' IgE with Dog Hair/Dander Extract (CAN XI D)

In 88% of patients' sera IgE antibodies to proteins of MW of 19 and 23 kD, respectively, were identified. 44% of patient's IgE reacted with a protein of a MW of 69 kD (fig. 2; patients 1–26 in table 1).

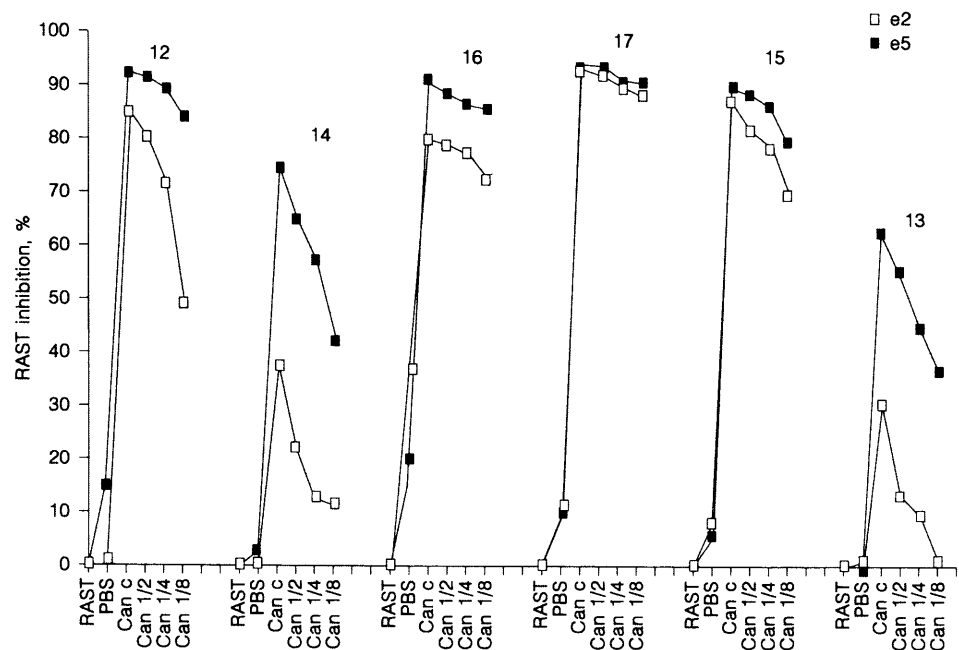


Fig. 1. Rast inhibition experiments. 100 μ l of 6 patients' sera (patients 12-17) were preincubated with either 150 μ l concentrated hair/dander extract (CAN XI D = Can c) containing 225 μ g total protein or with a 1:2 (Can 1/2), a 1:4 (Can 1/4) or a 1:8 (Can 1/8) dilution of this extract. Incubation was performed at 4 °C overnight. Samples incubated with PBS only were used as controls. Thereafter RAST discs (e2, e5; Pharmacia Diagnostics) were added and the assay was continued according to standard RAST technique. Additionally RAST was performed without any admixture. Patient numbers correspond to table 1.

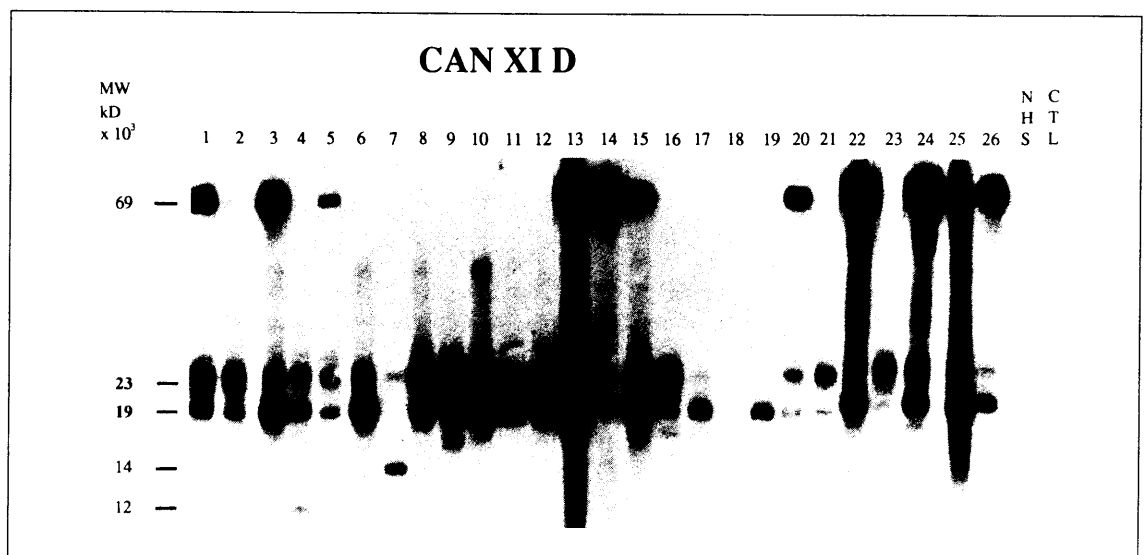


Fig. 2. Binding patterns of IgE antibodies to dog dander/hair allergens. Proteins of a dog hair/dander extract (CAN XI D) were separated by SDS-PAGE and the IgE binding of patients' sera was investigated by means of immunoblotting. NHS = Normal human serum pool; CTL = buffer control. Patient numbers correspond to table 1.

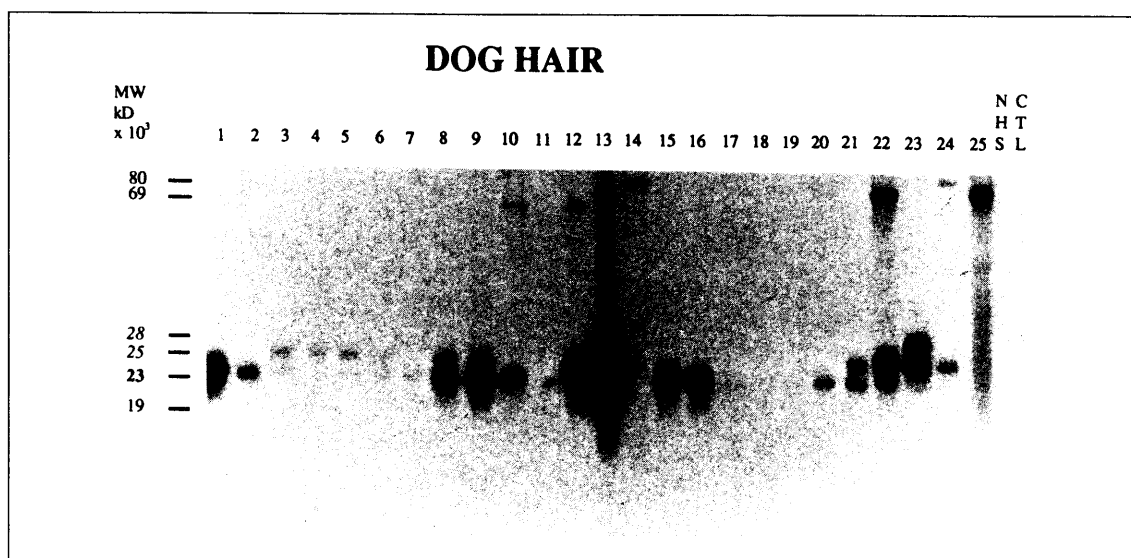


Fig. 3. Binding patterns of IgE antibodies to dog hair allergens. Proteins of a dog hair extract were separated by SDS-PAGE and the IgE binding of patients' sera was investigated by means of immunoblotting. NHS = Normal human serum pool; CTL = buffer control. Patient numbers correspond to table 1.

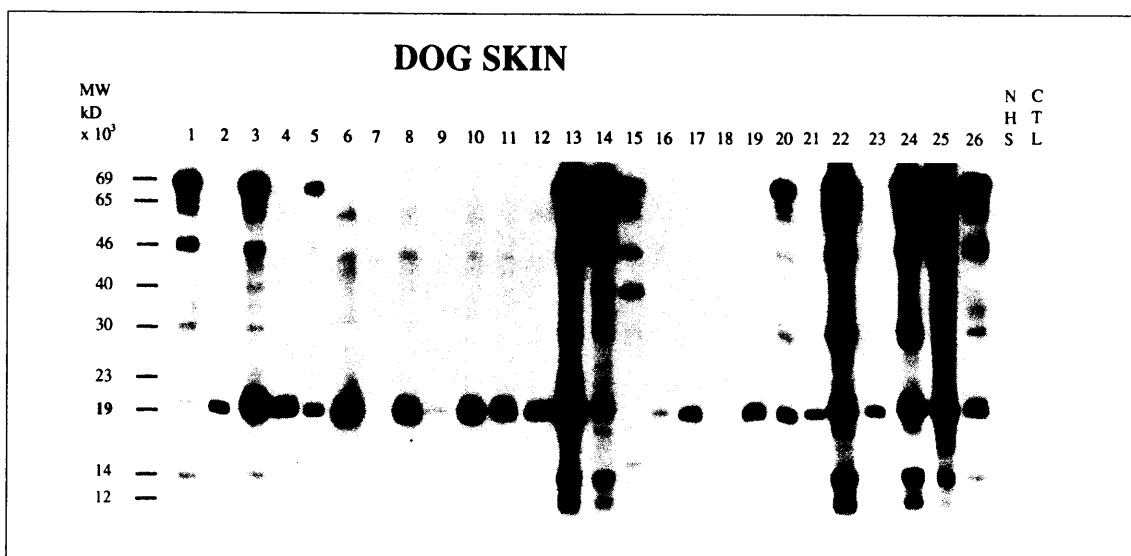


Fig. 4. Binding pattern of IgE antibodies to dog skin allergens. Proteins of a dog skin extract were separated by SDS-PAGE and the IgE binding of patients' sera was investigated by means of immunoblotting. NHS = Normal human serum pool; CTL = buffer control. Patient numbers correspond to table 1.

Reactivity of Patients' IgE with Dog Hair Extract

A 23-kD protein was recognised in 48% of patients' sera. 12% detected the 69-kD protein. 44% of the patients showed IgE binding to a protein of a MW of 25 kD (fig. 3; patients 1–25 in table 1).

Reactivity of Patients' IgE with Dog Skin Extract

A 19-kD protein was detected in 92% of patients' sera. 44% of patients' sera detected the 69-kD protein. Minor allergens with molecular weights of MW of 65, 40, 30, 14, and 12 kD were detected with an incidence of 30% (fig. 4; patients 1–26 in table 1).

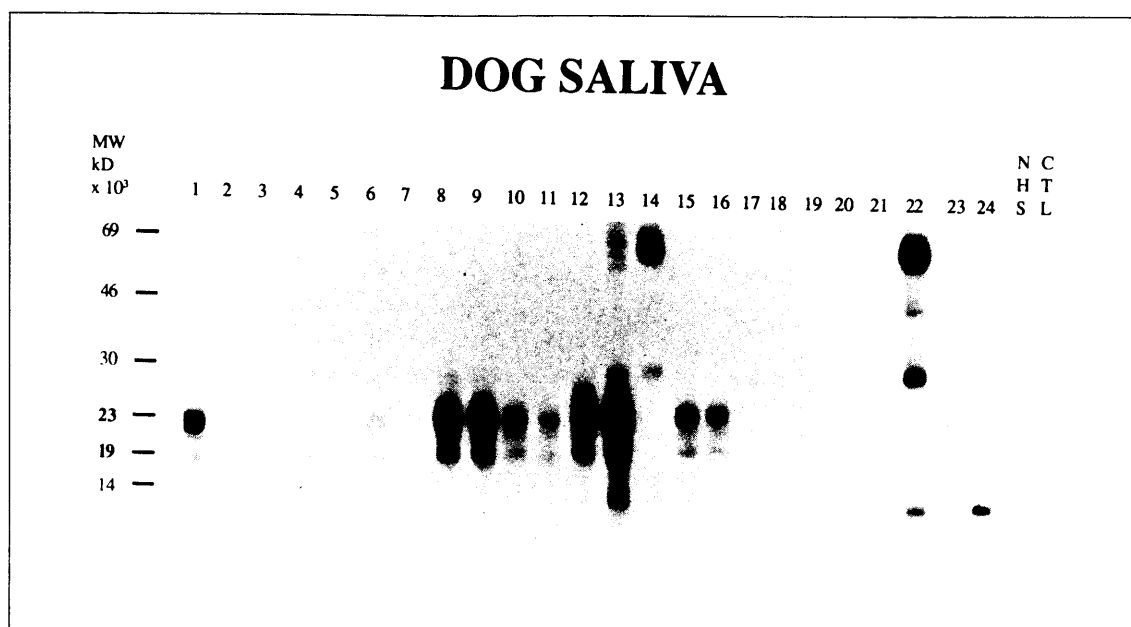


Fig. 5. Binding patterns of IgE antibodies to dog saliva allergens. Proteins of a dog saliva extract were separated by SDS-PAGE and the IgE binding of patient's sera was investigated by means of immunoblotting. NHS = Normal human serum pool; CTL = buffer control. Patient numbers correspond to table 1.

Reactivity of Patients' IgE with Dog Saliva

70% patients have IgE antibodies directed against proteins of a MW of 19 and 23 kD. The 69-kD protein is recognised by 40% of patients' sera. Minor allergens were detected at 30 and 14 kD with incidences of 33 and 42%, respectively (fig. 5; patients 1–24 in table 1)

Reactivity of Patients' IgE with Serum, Liver and Salivary Glands

The 69-kD protein was detected with incidences of 32 and 50% in liver and serum, respectively. Additionally 16% of the patients showed a reactivity with a 30-kD protein in liver extracts. Probed with an extract made of salivary glands 20% of patients' sera tested showed a reactivity with the 69-kD protein, 16% reacted with a 30-kD protein, and 12% detected a 19-kD protein [data not shown].

Inhibition Experiments

To elucidate the relationship of the 19-, 23- and 69-kD IgE-binding proteins found in several extracts inhibition experiments were performed. Preincubation with dog skin extract inhibited IgE binding to the 19-kD protein and to the 69-kD protein on the hair/dander extract (CAN XI D), whereas binding of IgE antibodies to the 23-kD protein was not affected (fig. 6, lane D). Using dog hair extract as

inhibitor, (fig. 6, lane F) inhibition of IgE binding to the 69- and 19-kD protein occurred. Preincubation with dog albumin selectively inhibited IgE binding to the 69-kD band (fig. 6, lane H). Preincubation of patients' sera with dog hair/dander extract (CAN XI D) and subsequent probing on saliva inhibited IgE binding to the 19- and 23-kD proteins. (fig. 6, lane J). Autoinhibition experiments are shown in figure 6, lanes A, B and K, L, respectively.

Discussion

In pets like cats, dogs or guinea pigs hair and dander are the major sources of allergens leading to type I allergies. Several studies attempted to identify the major allergens of dog. Besides albumin De Groot et al. [12] described a protein of 25 kD, tentatively designated as *Can f* I, with an average contribution to total IgE-binding activity of 70% [12], thus qualifying as major allergen. In LIA inhibition experiments Schou et al. [5] demonstrated that 50% of total allergenic activity of dog hair/dander extract is caused by *Can f* I. To assess the quality of the dog hair/dander extract (CAN XI D) used for immunoblot studies, RAST inhibition experiments were performed resulting in

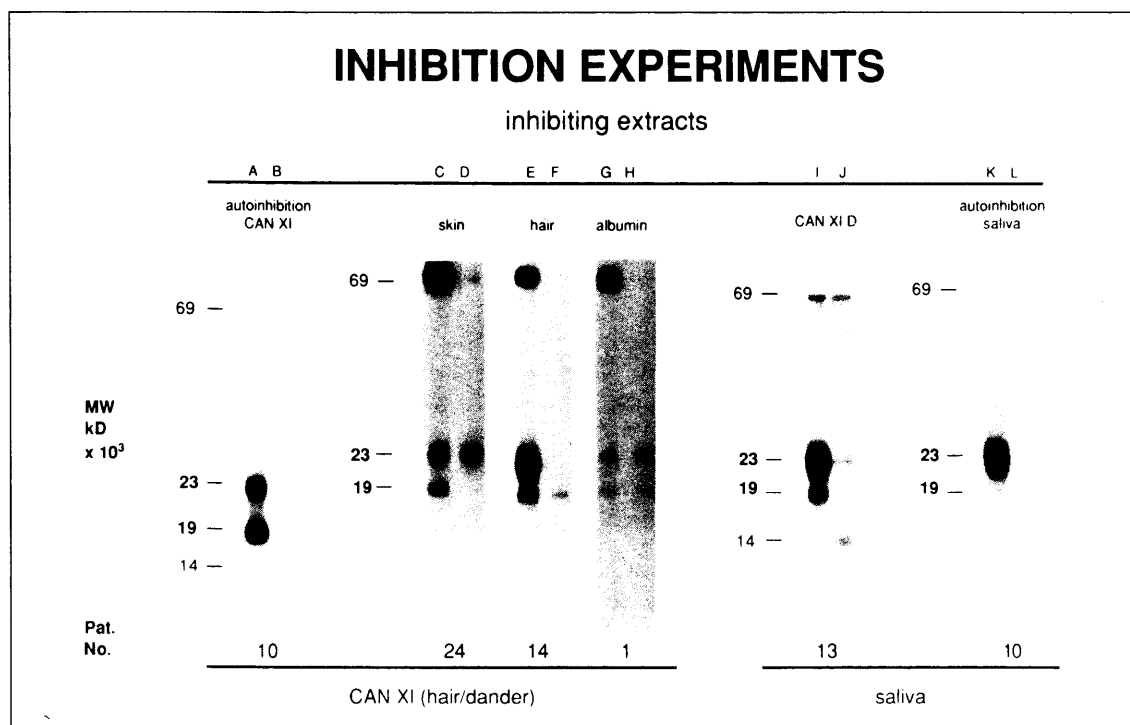


Fig. 6. Immunoblot inhibition experiments. Proteins of hair/dander extract (CAN XI D; lanes A–H) were separated by SDS-PAGE. Patients' sera were preincubated either with buffer (lanes A, C, E, G) or with hair/dander extract (CAN XI D; lane B), dog skin extract (lane D), dog hair extract (lane F) and dog albumin (lane H). IgE binding of patients' sera was investigated by means of immunoblotting. Proteins of dog saliva extract (lanes I–L) were separated by SDS-PAGE. Patients' sera were preincubated either with buffer (lanes I and K) or with hair/dander extract (CAN XI D; lane J) and saliva extract (lane L). Thereafter IgE binding of patients' sera was investigated by means of immunoblotting. Patient numbers correspond to table 1.

inhibitions ranging from 64 to 95 and 32 to 95% for e2 and e5, respectively.

As dog hair/dander extract is a mixture of material from various origins more specific extracts are needed to identify the major allergen sources. Therefore extracts from various sources were used in immunoblotting experiments. As molecular cloning evolved as a standard technique for characterisation of allergens, liver extracts as a candidate epithelial tissue for c-DNA clone bank construction were also included in this study.

96% patients' sera had IgE antibodies reactive with proteins of 19 and 23 kD in a hair/dander extract (CAN XI D) (fig. 2). It became evident that a 23-kD IgE-binding protein is preferentially found in dogs' hair and saliva, whereas a 19-kD allergen is predominantly present in dogs' skin and saliva. Inhibition experiments showed that the 19- and 23-kD allergens found in skin, hair and saliva correspond to the 19- and 23-kD proteins of the hair/dander extract (CAN XI D). These inhibition experiments

also revealed that the proteins represent immunologically independent allergens since preincubation of patients sera with skin extract containing only the 19-kD protein inhibited IgE binding to the 19-kD protein of the hair/dander extract (CAN XI D) leaving IgE reactivity against the 23-kD protein unaffected (fig. 6, lanes C and D). A reverse phenomenon was observed when the 23-kD-containing hair extract was used as inhibitor (fig. 6, lanes E and F). Using hair/dander extract (CAN XI D) as inhibitor binding to the 23- and 19-kD proteins of dog saliva was abandoned (fig. 6, lanes I and J). The 69-kD IgE-binding protein was shown to be dog albumin by means of inhibition experiments (fig. 6, lanes G and H). We think that the 23-kD protein corresponds to the major allergen described by De Groot et al [12] and Schou et al. [5] whereas the 19-kD protein represents a second major allergen of the dog, which has not been described so far.

The fact that the 23-kD protein is identified in hair but not in skin preparations containing the hair-producing fol-

lices may be explained by the possibility that the 23-kD protein of hair is derived from a precursor molecule without an IgE-binding epitope and IgE-binding activity of the 23-kD protein is obtained during the hair maturation process.

The 19- and 23-kD proteins could not be identified in salivary glands [data not shown]. This may be due to the fact that the salivary glands used in our experiments were prepared with some delay after the death of the dogs, so we cannot exclude degradation of the proteins.

The distribution of the 19- and the 23-kD proteins in skin, hair and saliva leads to several speculations. Skin and hair may contaminate dog saliva with the 19- and 23-kD proteins when dogs are licking their furs. On the other hand, like in cats saliva may represent the major source of allergens and skin and hair are contaminated [13]. Further experiments employing c-DNA cloning of allergens from various dog tissues should resolve these questions.

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