

The allergens of dog. I. Identification using crossed radio-immunoelectrophoresis

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Summary

The antigens present in an extract of dog hair and dander were examined by crossed immunoelectrophoresis (CIE) and the IgE-binding allergens by crossed radio-immunoelectrophoresis (CRIE), respectively, using sera from 60 British and Finnish animal-allergic subjects. The extract was comprised of a minimum of 28 antigens, 11 of which were common to dog serum. IgE antibody in the sera of the dog-sensitive patients bound to 21 of the 28 antigens at varying frequencies and intensities. Binding of any intensity occurred most frequently to two serum proteins: antigen 23 (IgG) binding IgE in 88% of cases, and antigen 3 (dog serum albumin, DSA) in 77% of cases. Dander antigen 8 bound in 63% and antigen 1 in 42% of the sera. Strong IgE binding, however, was most commonly associated with dander antigen 8 followed by antigens 1 and 23 (IgG) then 3 (DSA). The ranking of the antigens as allergens was similar for the two populations except that DSA was more important for the British than for the Finnish subjects.

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Introduction

Inhalant allergy caused by airborne particles such as pollens, animal danders, dusts and moulds can affect up to 30% of the population. Grass pollens and the house-dust mite *Dermatophagoides pteronyssinus* are the major culprits followed closely by cat fur/dander, and for this reason these three allergens are commonly used together as a routine test for atopy [1]. Although it has been known for many years that dog aero-allergens can give rise to asthma and other allergic disorders the magnitude of this problem has only relatively recently been assessed; for example dog allergy was implicated in 56% of asthmatic subjects in a Finnish population [2].

The importance of dog hair and dander is acknowledged by the WHO [3] who have recently established an international standard for dog hair/dander extract [4], one of five such international standards for allergens

extracts now in existence (the others being for timothy grass pollen [5], *D. pteronyssinus* [6], short ragweed pollen [7] and birch [8]). However, unlike the other allergen extracts for which standards exist, relatively little is known of the allergenic composition of dog hair/dander extract and although certain allergens have been described as major, apart from serum albumin which is available commercially, none has so far been isolated and none has been assigned the proposed international nomenclature [9].

As with all allergens extracts examined, dog hair/dander extract comprises a complex mixture of proteins of different molecular weights [10]. Theoretically all of these, if encountered in appropriate quantities, have the potential of acting as allergens.

Confirmation of identity of a single major allergen or of several major allergens has not proved to be straightforward. Blands *et al.* [11] in Denmark identified both dog serum albumin (DSA) and dander antigen 13 as major allergens [12], and α -antitrypsin, γ -globulin and dander antigen 11 as important allergens using a dog dander extract derived from six distinct breeds. Uhlin *et al.* [13] in Sweden found 24 allergens, including DSA and a dander antigen D4, in a poodle and alsatian dandruff extract. Both groups used crossed immunoelectrophoresis (CIE) and crossed radio-immunoelectrophoresis (CRIE) for

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separation of antigens and identification of allergens, with Uhlin *et al.* incorporating an intermediate gel of anti-dog serum to differentiate between serum- and dander-derived antigens. Löwenstein, also using CIE and CRIE in more general studies of mammalian allergens [14,15], found numerous allergens in the dog, but failed to identify any of these as major, whereas Yman *et al.* [16,17] using polyacrylamide gel electrophoresis and blotting techniques considered DSA to be an important allergen. The aim of this work was to elucidate further the antigens and allergens in a dog hair/dander extract, using CIE with an intermediate gel and 60 dog-allergic sera from the U.K. (London) and Finland (Helsinki).

Materials and methods

Production of a dog hair/dander extract

Dog hair/dander extract was produced according to the scheme in Fig. 1. Briefly, unwashed hair clipped from dogs of a variety of breeds was obtained from London dog-grooming parlours. Amounts of 500 g of hair were immersed overnight at 4°C in 10 l of 0.125 M ammonium bicarbonate solution, pH 8.6, containing 0.02% (w/v) of sodium azide and stirred intermittently. The extract was seived, centrifuged at 7500 r.p.m. in a Sorvall centrifuge (1 hr at 4°C) and the supernatant filtered through a bank of Millipore filters of pore size 1.2, 0.45 and 0.2 µm. It was concentrated to 1/10 of the volume in an Amicon stirred cell (model 8400; Amicon Ltd, U.K. containing a UM 10 Diaflo ultrafiltration membrane (molecular weight cut off 10 kD)) at 4°C, dialysed against 0.005 M ammonium bicarbonate solution (two changes each 24 hr) followed by distilled water (two changes each 24 hr) at 4°C and further filtered through a 0.2 µm membrane and freeze-dried on an Edwards Modulyo Freeze-Dryer overnight. The dried material was weighed and stored at -20°C until use.

The protein content of the extract was estimated by amino acid analysis. The samples were prepared exactly as described by Calam *et al.* [18] and analysed by reversed-phase HPLC on a Beckman Ultrasphere ODS column as described by Noyes [19].

The content of dog serum albumin in the dog hair/dander extract was estimated using rocket immunoelectrophoresis according to the method of Weeke [20] with a standard of different concentrations of dog albumin (Sigma Chemical Co. Ltd, Poole, U.K.) and monospecific rabbit anti-dog albumin serum (Nordic Immunological Laboratories, Maidenhead, U.K.).

Rabbit antisera

Rabbit anti-dog hair/dander serum was raised in rabbits according to the immunization schedule of Harboe and

Ingild [21] using three rabbits and a course of injections and bleeding over 6–12 months. A 45% ammonium sulphate precipitation was performed on the pooled sera to prepare an antibody-rich fraction of hyperimmune rabbit serum.

Rabbit anti-dog serum was purchased from ICN Immuno Biologicals (formerly Miles-Yeda), High Wycombe U.K., for use in the intermediate gel of the CIE.

Human sera

The 60 human sera were obtained as follows: six (Nos 1–6) were RAST positive sera previously collected from animal allergic subjects and stored at NIBSC, two (nos 46 and 60) were from NIBSC staff and a visitor to NIBSC with dog allergy, 34 were from dog-allergic patients attending London teaching hospitals: Clinical Research Centre (CRC) (nos 7–29), St Bartholomews (No. 45), and Guy's Hospital (Nos 43–44 and 47–59) kindly donated by Drs Mitchell, Davies and Lessof; and 18 sera (Nos 25–42) were from dog-allergic patients attending the Allergy Clinic in Helsinki Hospital, Finland, a gift from Dr S. Mäkinen-Kiljunen. The British sera were selected on the basis of a history of animal allergy, a strong positive skin test to dog extract and/or a strong positive RAST, and the sera from Finland were from clinically sensitive patients with positive skin tests. The London group of patients tended to have more allergies, in addition to dog, as determined by skin test than did the Finnish group, and this was largely accounted for by the higher percentage of the London group with circulating IgE to grass pollens and *D. pteronyssinus* house-dust mite. The proportion with cat allergy (RAST or skin test positive) was the same in the two populations, representing approximately 75% of the subjects. Clinical details were available for all but eight of the subjects; 60% of them were known to have asthma, 27% hay fever, 37% eczema, 15% urticaria and 43% more than one of these conditions.

The total IgE of the sera was determined in the clinics donating the samples by the method of PRIST® (Pharmacia, Uppsala, Sweden) following the manufacturers instructions. Dog hair and dander specific IgE was measured using an optimized system of RAST [22] as follows. Dog hair and dander paper discs (20 µg dry weight of extract/disc) were incubated overnight at room temperature with 100 µl of a 1/5 dilution in 5% human serum albumin of each of the human sera. They were washed, incubated with 50 µl of ¹²⁵I-labelled anti-human IgE (Pharmacia, Uppsala, Sweden) overnight at room temperature, washed again, the bound counts per minute (c.p.m.) were read in a gamma counter (LKB, Multi-gamma), expressed as percentages of the c.p.m. added to the discs and converted to a RAST class equivalent using

a reference serum pool and Phadebas Reference Kits (Pharmacia). RAST class 1 was found to be equivalent to 1.5–2.1% of total counts bound, RAST class 2 to 2.2–8.5%, RAST class 3 to 8.6–24.6%, RAST class 4 to 24.7–35.0% and RAST class 5 to >35%.

CIE/CRIE

CIE/CRIE were performed essentially according to the method of Løwenstein [12] using rabbit anti-dog hair/dander serum in the anodic and cathodic portion of the gel and an intermediate gel containing rabbit anti-dog serum [13]. Autoradiographs were produced by exposure of X-ray films (Kodak X-omat S) in cassettes containing single intensifier screens (Protex, Cuthbert Andrews, U.K.) at -70°C for 2, 4 and 7 days [23] and the accompanying CIE slides were stained in Coomassie brilliant blue R250. Controls for the intensity of radioactive binding were performed using a gel technique. Briefly, a 1% solution of agarose containing goat anti-rabbit IgG at $10\ \mu\text{l cm}^{-2}$ was poured onto a $7 \times 5\ \text{cm}$ glass plate. Nine dilutions of the ^{125}I -labelled anti-human IgE were made in $50\ \mu\text{l}$ of PBS such that nine concentrations of $10\text{--}50\ \mu\text{l}$ of neat anti-IgE in $5\ \mu\text{l}$ steps were obtained. A mixture of equal volumes ($20\ \mu\text{l}$) of each of these was made with rabbit serum and $7\ \mu\text{l}$ of each mixture was pipetted into nine 3-mm wells punched in the prepared agarose plate. The plate was left overnight at room temperature for immunoprecipitation to occur, washed and pressed with the CIE plates and autoradiographed as for the CRIEs. Values of 1 (weak) to 9 (strong) were given to the intensity of the darkening of the X-ray film.

A reference pattern for CIE was drawn up from the stained plates and the precipitation arcs numbered from the anode to the cathode. The degree of darkening (1–9) of the CRIE X-ray film was assessed for each arc with each patient's serum. To simplify the score for each of the numbered antigens the darkening of the X-ray film was reallocated into three categories: weak (1–3), intermediate (4–6) and strong (7–9).

Results

Preparation of the extract

The extraction of 500 g of dog hair resulted in 400 mg dry weight of freeze-dried extract, 20% (80 mg) of which was estimated to be protein by amino acid analysis. Albumin, estimated by rocket immunoelectrophoresis using Sigma DSA as the standard, represented 10.7% of the protein.

CIE

The pattern of antigen–antibody precipitation arcs identified by CIE using an intermediate gel containing anti-dog

serum is illustrated in Fig. 2a and diagrammatically in Fig. 2b, where the arcs are numbered from the anode to the cathode as antigens 1–27. An additional antigen was seen at the anodic end of the gel in some slides and was given the number 0, making 28 discernable antigens in all. CIE using an intermediate gel containing monospecific antisera to DSA and IgG clearly identified these as antigens 3 and 23, respectively (not shown here). Eleven antigens (3, 5, 12, 14, 15, 17, 19, 20, 21, 23 and 26) were precipitated by the rabbit anti-dog serum.

Total and specific IgE

The total IgE and dog-specific IgE (RAST score) of the human sera are shown in Table 1. The average total IgE for the sera derived from London hospitals was far higher than that for those from Finland (3508 v 1031). Approximately 75% of the sera had a dog-specific RAST score of 3 or more and over 33% were RAST class 4 or more. The Finnish sera had a greater percentage (89%) of dog RAST class 3 or more than the London sera (65%).

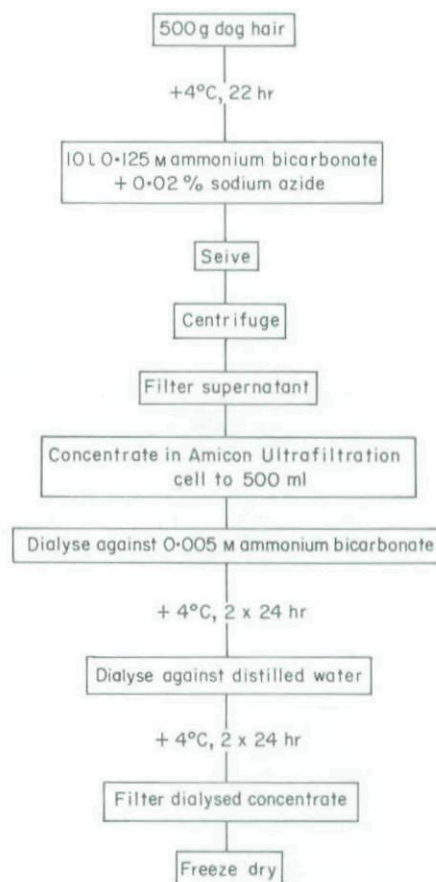


Fig. 1. Schedule for production of dog hair/dander extract.

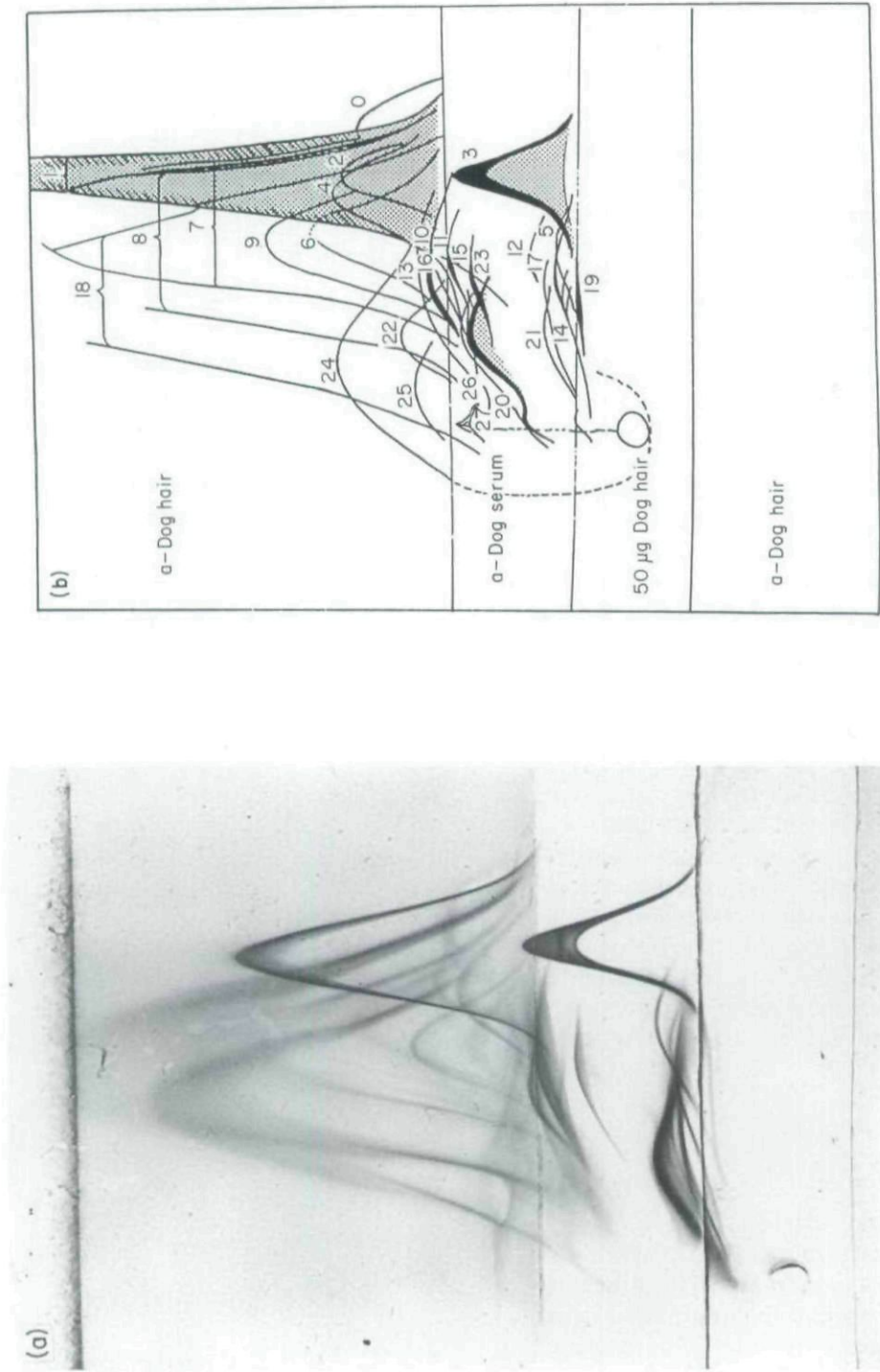


Fig. 2. (a) CIE of dog hair/dander extract. Conditions as in text. (b) Line diagram of (a) showing antigens numbered from anode to cathode.

Table 1. Details of IgE measurements in human sera used in CRIE

Patient	Total IgE (IU)	Dog-specific IgE*	
		TC (%)	RAST class
1	—	37	5
2	—	22	3
3	—	34	4
4	—	34	4
5	—	31	4
6	—	16	3
7	2530	15	3
8	1085	2	1
9	2910	4	2
10	4725	6	2
11	2750	15	3
12	1190	33	4
13	—	29	4
14	4250	39	5
15	449	7	2
16	164	2	1
17	—	35	4
18	8200	35	4
19	6400	37	5
20	—	15	3
21	—	1	1
22	5750	21	3
23	—	12	3
24	5200	7	2
25	50	20	3
26	780	36	5
27	840	37	5
28	165	24	3
29	460	37	5
30	153	32	4
31	—	6	2
32	66	7	2
33	—	29	4
34	9000	26	4
35	97	22	3
36	170	15	3
37	—	25	4
38	—	27	4
39	210	14	3
40	390	16	3
41	—	29	4
42	—	9	3
43	—	17	3
44	—	34	4
45	—	17	3
46	—	5	2
47	—	22	3
48	—	6	2
49	—	7	2
50	—	17	3
51	—	8	2
52	—	3	2
53	—	34	4
54	—	5	2
55	—	32	4
56	—	42	5
57	—	32	4
58	—	31	4
59	—	21	3
60	—	—	—

* RAST score assigned as percentage of total counts bound (TC%) and as RAST class 1–5 (as defined in the Materials and methods).

CRIE

Three typical IgE binding patterns in the human sera to the separated elements of the dog hair/dander extract in CRIE were evident; some sera bound only a single protein such as DSA, others bound a large number of both dander and serum antigens and closely resembled the binding of the serum pool, whereas a third arrangement was for the sera to bind strongly to two or three dander antigens (Fig. 3). All the antigens apart from seven (antigens 6, 17, 20, 22, 25, 26 and 27) were bound by at least one of the 60 sera. Thus 21 of the 28 antigens (eight serum- and 13 dander-specific) were allergens. Antigen 23 (IgG) was bound by IgE by 53 (88%; London 89%, Finland 87%), antigen 3 (DSA) by 46 (77%), antigen 8 by 38 (63%; London 50%, Finland 82%) and antigen 1 by 25 (42%) of the 60 human sera. However, despite the high frequency of IgE binding to serum antigens, really strong binding was found most frequently to dander antigen 8 (32%), whereas that to another dander antigen, antigen 1, or serum IgG (antigen 23) was only 18% and to serum albumin, antigen 3, was 15%. A histogram showing the relative frequency and intensity of IgE binding by the antigens is presented in Fig 4. The frequency of strong and intermediate intensity binding ranks the importance of the antigens of the dog hair/dander extract as allergens as follows: antigen 8 > 23 > 1 > 7 > 3 > 18.

Discussion

This study resulted in two main findings. One was that, amongst the numerous antigens found to be allergens, none qualified as a major allergen. The second finding was that no gross differences were observed in the IgE binding of the sera from the two geographical locations. Firstly, a major allergen, defined as one which strongly binds IgE in 50% or more of the sera [12], was not found. Both DSA and dog IgG bound IgE at a high frequency (88 and 77%, respectively) but not strongly (only 15 and 18%, respectively) in the majority of sera. Dander antigen 8 was also bound by the majority (63%) of the sera but was only bound strongly by 32% of them. Antigen 8 could be equivalent to the major allergen, antigen D4, of Uhlin *et al.* [13] based on its similar electrophoretic mobility, its dander origin and its importance as an allergen. It has recently been found (unpublished data) to be identical to antigen 13 of Blands *et al.* [11] by use of monospecific rabbit antiserum to antigen 13 from Dr Carsten Schou, ALK, Denmark. However, our finding of no major allergens according to the above definition contrasts with that of other workers [11, 13, 16, 17] but agrees with that of two studies of mammalian allergens by Löwenstein [14, 15]. That DSA is not an important allergen is supported by a study in Finland by Vanto *et al.* [24] where

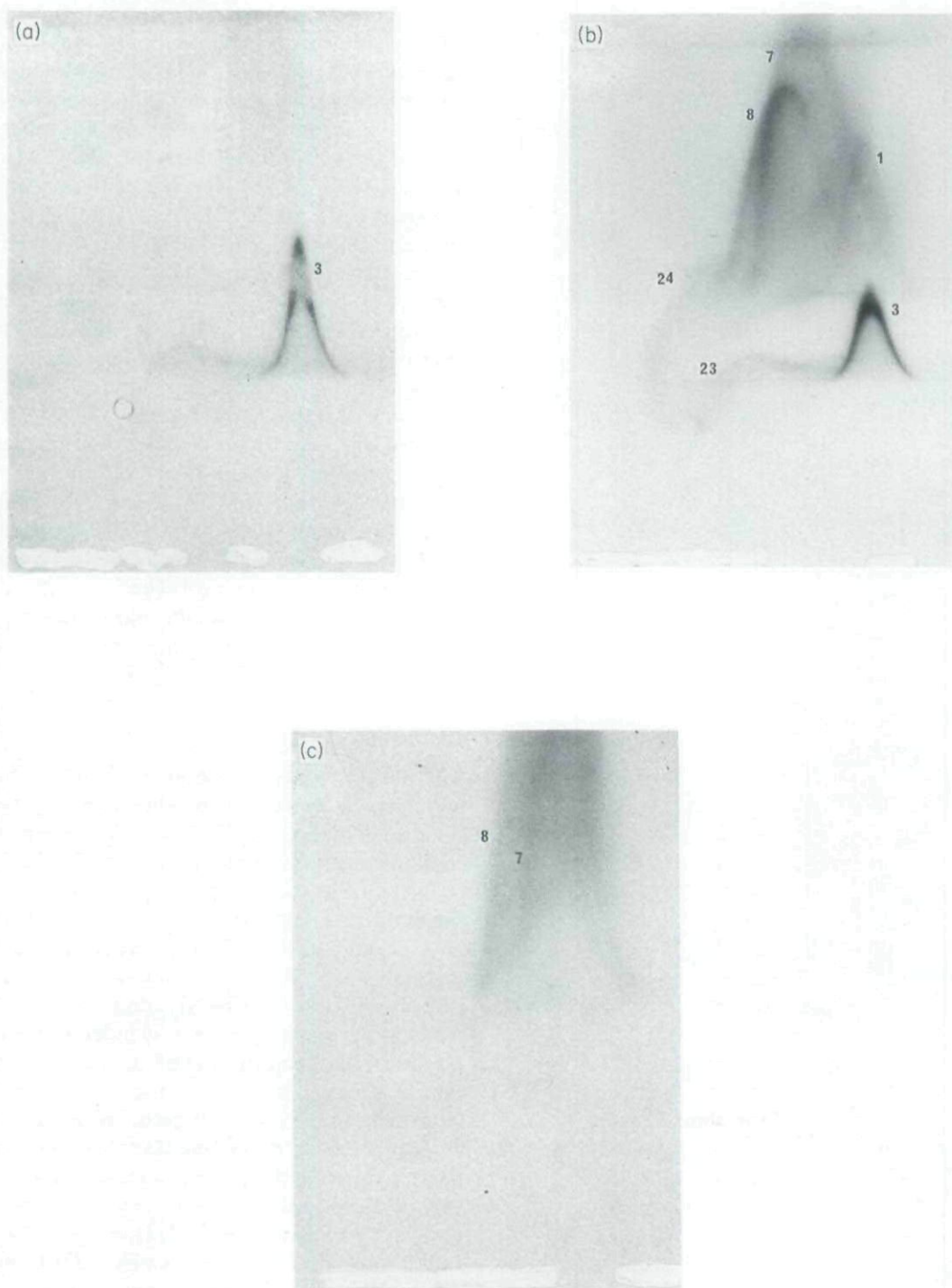


Fig. 3. Typical radiostaining patterns in CRIE following incubation with human sera. (a) IgE binding of serum 56 predominantly to dog serum albumin (antigen 3). (b) IgE binding of serum pool (NIBSC 79/530) showing binding to antigens 1, 3, 7, 8, 23 and 24. (c) IgE binding of serum 55 showing binding to antigens 7 and 8.

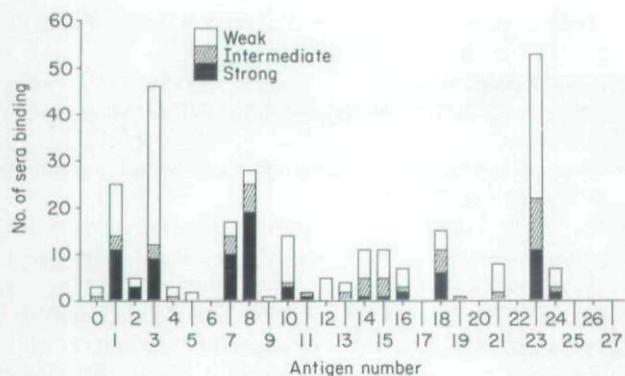


Fig. 4. Frequency and intensity of IgE binding by the 60 sera to antigens present in dog hair/dander extract by CRIE.

only 11% of 80 dog skin test-positive subjects gave positive reactions to DSA and by a small study at Guy's Hospital where only two out of 14 of the dog-allergic subjects gave a positive skin-prick test to a 1 mg/ml solution of DSA [25]. Furthermore, a RAST study of this group of 60 subjects showed only 12% of the sera to be strongly positive to DSA, and inhibition of dog hair/dander RAST was 50% at a concentration of 1 mg/ml DSA [10]. The explanation for the conflicting results regarding the presence of major allergens in dog hair/dander extracts probably lies in the variations of the methods used including the source of extracts, the rabbit antisera, the threshold of positivity and the selection of human sera.

The second finding concerned the relative importance of allergens in the sera from Britain and Finland. Interestingly, the two groups proved almost identical, antigen 23 (IgG) was bound commonly, but weakly in both groups, and antigen 3 (DSA) was also similarly bound although the combined strong and intermediate binding was twice as high in the London group (24%) as in the Finnish group (11%). Antigen 8, the dander allergen most closely approaching a major allergen, was bound by a lower percentage of the London sera than the Finnish when all levels of binding were considered, but this difference was accounted for almost entirely by the high proportion of Finnish sera weakly binding the allergen. When strong and intermediate binding was examined the two groups were again very similar (London 35%, Finland 44%). It can thus be concluded that the same antigens act as allergens in subjects from Britain and Finland.

The sera used in this study, especially those from NIBSC London, were selected on the basis of strong dog positivity either by RAST or skin test and do not represent consecutive patients from a single allergy clinic in one geographical area. Thus it may not be valid to draw

conclusions regarding which are the major or minor allergens of the dog, and the findings here only relate to this group of sera selected by these criteria. The conclusions can be drawn, however, that, using the methods described here, dog hair/dander contains 28 antigens, the majority of these are capable of binding IgE from dog allergic patients and that antigens 23 (IgG), 3 (DSA) and 8 appear to be the most important allergens.

CRIE has been criticized in the past because of its reliance on the presence and concentration of the rabbit antibodies [26] that determine the presence and, to some extent, the intensity of radio-binding of IgE in CRIE. However, this method was used in the present study since it provided a starting point for the identification of allergens in dog extracts. Other methods, such as SDS-PAGE and immunoblotting can be criticized on the grounds that the proteins may not be in their natural, conformational state following treatment prior to electrophoresis [27]. Further studies using these methods, however, are currently being employed to confirm and supplement this data. Having identified the allergens it is then intended to isolate and purify the most important of them.

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