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Application of Allergy Diagnosis in Forensic Serology

The general approach to the characterization of bloodstains by "antibody profiling" has been described earlier [*1*]. Antibodies are produced by an individual against foreign organic matter (antigens). As each antibody is designed to react with a specific antigen, a study of the types of antibody present in a person's blood may provide information about that individual's past health record. The use of the Radio-Allergo-Sorbent-Test (RAST®) to measure allergy-associated antibodies in dried blood has also been described earlier [*2,3*]. These IgE antibodies are detected by incubation of bloodstain extracts with purified allergen coupled to small paper disks. It has been shown that at least three different allergen disks could be incubated simultaneously in a bloodstain extract made from less than 50 μl of whole blood [*3*].

Ragweed pollen (genus *Ambrosia*) is known to be the major cause of hypersensitivity in North America. From 16 to 28% of the North American population are believed to be potential allergy sufferers [*4*]. According to Gleich and Jacob [*5*], up to 50% of the total IgE protein in North Americans who suffer from hay fever is associated with ragweed allergens.

In contrast to the United States, where ragweed pollen is recognized as the dominant allergen, grass pollen is believed to be the most common cause of hypersensitivity in the British Isles.

Two blind trials were organized, one in cooperation with operational forensic science laboratories in the United Kingdom, and the other with laboratories in North America. The intention was to assess the incidence of sensitivity to grass pollens and other allergens. Using the RAST, IgE antibodies to specific allergens were measured in the extracts of small bloodstains. The allergens studied in the U.K. survey were chosen after an analysis of IgE profiles of 1196 individuals. In the North American survey, bloodstains were examined for ragweed and other pollen-specific antibodies.

This paper describes the reproducibility of the technique and how bloodstains may be discriminated; it also discusses the information provided by the RAST on the clinical and geographical history of the donor.

Materials

In the North American survey, 195 bloodstains, obtained by finger-prick from 193 different individuals, were submitted to the Aldermaston Central Research Establishment in a "blind" experiment from 14 United States and Canadian laboratories. The stains

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were prepared on cotton cloth or filter paper and sealed in plastic bags. The stains were generally 2 to 3 weeks old at the time of examination. A statement of any history of ragweed allergy or hay fever among the donors was retained by each laboratory. In addition, 51 stains from white U.K. citizens, 27 of whom reported a history of hay fever, were included as controls.

Allergen disks [*A. elatior* (common ragweed), *A. psilostachya* (western ragweed), *A. trifida* (giant ragweed), and *Dactylis glomerata* (cocksfoot grass pollen)] and anti-human ^{125}I-IgE were obtained from Pharmacia GB Ltd. Normal hen serum was purchased from Salisbury Laboratories, Salisbury, Wiltshire, U.K.

For the U.K. survey, blood plasma from 1196 adults with suspected atopic allergies was analyzed at the RAST Allergy Unit, Benenden Chest Hospital, Cranbrook, Kent, U.K. The anonymously coded specific IgE profiles together with total IgE determinations were stored in a Hewlett-Packard minicomputer.

Eight forensic science laboratories in the United Kingdom submitted 82 pairs of bloodstains in a blind experiment where the pairs could be from the same or different donors. The bloodstains (mean age at examination, 7 days) were prepared on a variety of textile and paper substrates. The allergen disks used were *D. glomerata, Dematophagoides pteronyssinus* (house dust-mite), and cat epithelium.

Method

On receipt, the bloodstains were extracted by centrifugation [*6*]. Approximately 1 cm^2 of stain was extracted three times with 50-μl aliquots of phosphate-buffered saline at pH 7.2. The extracts were stored at -10°C until required. One hundred microlitres of a stain extract were incubated simultaneously with either a disk of each of the three ragweed species or the allergen disks *D. glomerata, D. pteronyssinus,* and cat epithelium, respectively. Each allergen disk was identified by a small characteristic cut. Each extract contained about 8 mg total protein (equivalent to 40 μl whole blood) as determined by the Lowry AutoAnalyzer® method [*7*].

After overnight incubation at room temperature, the disks were washed at least three times in about 2.5-ml aliquots of phosphate-buffered saline, and each disk was placed in a separate tube. Fifty microlitres of reconstituted anti-human ^{125}I-IgE (giving 60 000 to 80 000 counts in 100 s) were added and the disks allowed to incubate overnight at room temperature. During this period, the total activity added to each tube was counted for 100 s (Nuclear Enterprises Gamma Counter NE 8311). The counting efficiency was 50%. Following this incubation, the excess anti-human ^{125}I-IgE was removed by aspiration and the disks were washed as above. The disks were placed in clean tubes and their activity counted for 10^3 s. The background radiation was determined by removing the disks and counting the empty tubes. The component of background radiation from the counter was kept constant by allocating one tube holder per disk. Negative controls for the ragweed allergens were prepared by incubating disks with 100 μl normal hen serum instead of a bloodstain extract. The corrected counts for these disk were considered a measure of nonspecific binding of labeled antibody and were routinely subtracted from all other corrected disk counts. Negative control disks, however, were found to be unnecessary for the other allergens.

The antibody activity of a bloodstain was defined as

$$a \times 10^3/bc$$

where

a = activity of the disk (counts per 10^3 s) corrected for background and nonspecific binding;

b = total protein content of the extract (mg/ml); and
c = total activity added (counts per 10^2 s).

In practice, positive reactions were easily identified from the corrected disk counts a, and total protein determinations needed to be made only in such cases. In this way, an acceptable reproducibility for positives was maintained and negatives were screened out efficiently. In our experience, the activity of negative disks rarely exceeded 5 counts/s after correction for background activity and the negative disk control. For the present study, however, protein contents of all extracts were determined.

Results and Discussion

The locations of the North American laboratories from which bloodstains were received and the percentage of ragweed-RAST positives in each case are shown in Fig. 1. The 68 individuals who subsequently reported a pollen hypersensitivity were later tested for their reaction to *D. glomerata*. Summaries of the results are shown in Tables 1 and 2.

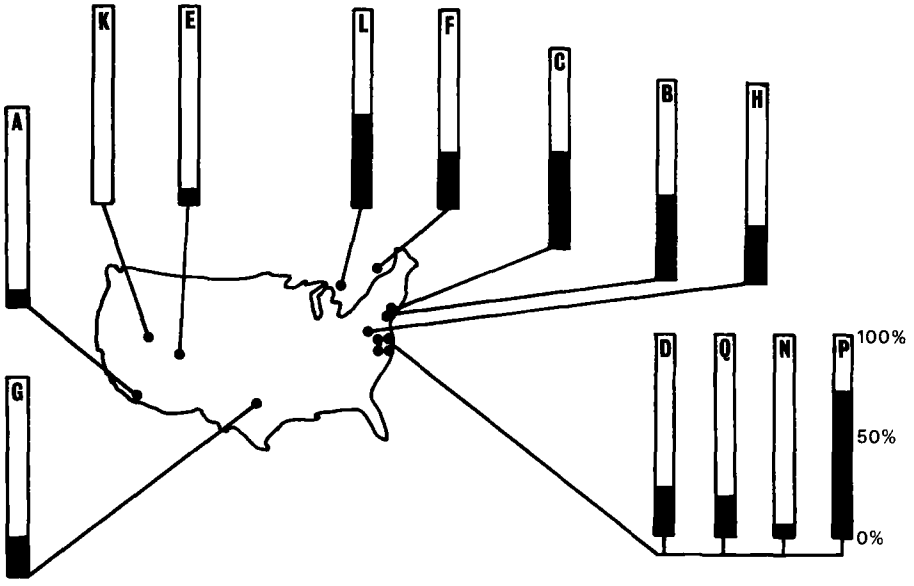

FIG. 1—*Location of the 13 North American mainland laboratories participating in the survey (Laboratory T is located in Japan). Solid bars show the incidence of ragweed-RAST positive diagnoses in each case.*

TABLE 1—*Ragweed-RAST diagnoses for bloodstains from 193 individuals.*

Result	*n*	%
Positive	46	24
Negative	138	71
Inconclusive	9	5

Of the 46 stains that gave a positive reaction to at least one ragweed disk, only two came from donors who reported no allergies. In one of these, the person had received blood transfusions within the recent past and may have passively acquired IgE antibodies.

TABLE 2—*Ragweed and* D. glomerata *RAST diagnoses for the 68 donors reporting a pollen allergy.*

Result	Ragweed, *n*	*D. glomerata, n*
Positive	44	21
Negative	20[a]	47
Inconclusive	4	0

[a] Of these, 9 were positive for *D. glomerata.*

Some of the false negatives, that is, those with a reported ragweed allergy but a negative RAST diagnosis, arose from persons with a history of hypersensitivity in their youth but who had no current hay fever. Many other ragweed-RAST negative cases where the bloodstain donor reported hay fever were found to be positive to grass pollen (*D. glomerata*). Of the 51 control donors (white U.K. citizens), 50 were negative to ragweed.

The three ragweed species gave cross reactions as shown by the correlation of antibody activity, which was significant at the 0.1% confidence level for each pair of disks. The antibody activity with respect to ragweed disks showed no significant correlation with *D. glomerata* antibody activity. Similarly, Lichtenstein et al [*8*] found that ragweed and rye grass allergens had no immunological cross reaction.

The highest antibody activity with ragweed disks was obtained with *A. psilostachya,* and of the 46 individuals who gave at least one clear positive reaction to a ragweed disk, 44 were positive to this particular disk. Table 3 shows the mean antibody activities and standard deviations for all bloodstains. An assumption of a log-Normal population distribution for positives and a Normal, that is, random error, distribution for negatives has been made.

TABLE 3—*Antibody activities of bloodstains tested with ragweed disks.*

Test	$\overline{X} \pm \sigma$
A. elatior[a]	3.86 + 6.51 − 2.43
A. psilostachya[a]	5.42 + 8.68 − 3.33
A. trifida[a]	2.61 + 3.57 − 1.51
Negatives[b] (all disks)	0.75 ± 0.26

[a] Based on a log-Normal distribution.
[b] Based on a Normal distribution.

The adjustment of the total protein content of the bloodstain extracts to approximately 8 mg/100 μl adequately avoided the problem of nonlinearity between the amount of blood protein added and the amount of specific antibody bound to the allergen disk caused by saturation of disk sites [*9*].

The work described here does not represent a truly random survey since no precautions were taken to avoid selection effects. Also, the collection of bloodstains from North America took place during early spring. If blood had been taken during the ragweed season in late summer, antibody levels in the sensitive donors would have been much higher [*5*].

There has been some controversy regarding a possible correlation between month of

birth and hypersensitivity to pollen. This work agrees with those authors [*10*] who suggest that no such correlation exist. Other studies have shown that IgE levels are independent of sex and blood and serum groups ABO, MN, Rh, Gm, and Km [*11*].[2]

The severity of ragweed allergy is known to be highest in the northeast section of the American continent [*12*]. The present results support this finding. The participating laboratories (excluding T, located in Japan) can be grouped into two geographical regions: Group 1 includes laboratories B, C, D, F, H, L, N, P, and Q; and Group 2 includes laboratories A, E, G, and K (see Fig. 1). The mean incidence of ragweed-RAST positives was significantly higher in Group 1 ($P < 0.01$).

Analysis of the plasma IgE profiles from the RAST Allergy Unit revealed that the most common causes of hypersensitivity in the United Kingdom fell into three groups: grass pollens, dusts and mites, and animal epithelia. It was found that the grass pollens as a group cross-reacted, as did the allergens in the dust and mite group. The cat disk was the most efficient of the animal epithelial group. It was concluded that a RAST screen for IgE antibodies to *D. glomerata, D. pteronyssinus,* and cat epithelium would have detected more than 96% of the atopics that were diagnosed by the RAST Allergy Unit using commercially available reagents. This is not too surprising as other common causes of atopic allergy can be associated with the above three allergens. For example, Speer [*13*] found that 86% of food atopies are accompanied by dust and pollen hypersensitivities.

There is considerable overlap of total IgE levels in normal and allergic populations. Normal levels are most frequently between 20 to 30 units/ml [*14,15*]. However, in the present survey nearly 25% of individuals with total IgE levels greater than 160 units/ml did not give positive reactions in the RAST. Therefore, determination of total IgE was considered to be of little value even as a presumptive test for atopic allergy in the present context.

In the U.K. blind trial, the 82 pairs of bloodstains originated from 104 different individuals, of whom 29 were diagnosed as positive to at least one of the three allergens tested. A summary of these results is provided in Table 4. Three of the 16 individuals who were

TABLE 4—*RAST diagnoses for the 104 individuals represented in the U.K. blind trial.*

Test	*n*	Confirming History, *n*
D. glomerata	16	13
D. pteronyssinus	7	6[a]
D. glomerata and *D. pteronyssinum*	5	4[b]
D. glomerata and cat epithelium	1	1
Negative	75	73[c]

[a] One reported a suspected hay fever and another an animal fur allergy.
[b] One reported dust and animal fur allergies.
[c] Number reporting no allergies within the context of this survey.

diagnosed positive to *D. glomerata* reported no symptoms. However, these three cases had antibody activities noticeably lower than the mean of the remaining 13.

Forty-one pairs of bloodstains were one-donor pairs, none of which was wrongly discriminated, and 41 were two-donor pairs. Of these dissimilar pairs, 17 were differentiated by detecting specific IgE antibodies. Nineteen individuals who gave at least one positive response to the allergen disks appeared twice in this trial. Four of these individuals gave

[2] D. J. Werrett, unpublished observations.

positive responses to two allergen disks. Thus a total of 23 duplicate determinations of the antibody activity was obtained. The mean coefficient of variance was 13.3%.

Table 5 shows the mean antibody activities and standard deviations for these bloodstains. Again, an assumption of a log-Normal population distribution for positives and a Normal (random error) distribution for negatives has been made.

TABLE 5—*Antibody activity of bloodstains in the U.K. blind trial.*

Test	$\bar{X} \pm \sigma$
D. glomerata[a]	18.36 + 16.47 − 8.67
D. pteronyssinus[a]	11.64 + 8.50 − 4.91
Cat epithelium	21.1; 22.1[b]
Negatives[c] (all disks)	1.42 ± 0.41

[a] Based on a log-Normal distribution.
[b] The two values of antibody activity for the same donor are given.
[c] Based on a Normal distribution.

As in the ragweed survey, a high correlation was found between the RAST diagnoses and the specific allergies subsequently reported by the bloodstain donors (see Table 4).

If the overall incidence of atopic allergy is taken as 20%, then it can be shown that the discriminating power [*16*] of the RAST is approximately 0.3. In comparison to some serological tests [*16*], the RAST is not highly discriminating when used at random. However, most people with specific atopic allergies (such as hay fever) are aware of their condition and will have raised IgE antibody levels to the allergen in question. In forensic investigations, the decision to use the RAST with a control sample of blood from a suspect or victim will be influenced by the allergy history of that individual. If the suspect/victim reports having specific allergies, then in our experience there is a better than 90% chance of obtaining a positive RAST diagnosis from that person's blood. Clearly, if a case bloodstain also originated from the same person, then there would be a high probability that the two samples would match both qualitatively and quantitatively. Conversely, if the stain did not originate from the suspect, then the two samples would most probably be discriminated.

Chi-squared analysis of the bloodstain data showed that there was no significant association between allergy status and the sex of the donor. The age distribution of negatives was indistinguishable from that of positives. However, it should be noted that food allergies are much more common in children [*17*].

A further advantage of the RAST as a forensic serological technique is that it is effectively nondestructive; only specific antibodies are removed by the disk, and the remaining constituents of the bloodstain extract are unaffected. If this residual extract is to be used for the electrophoretic separation of enzyme polymorphs (such as phosphoglucomutase), then it is suggested that the initial incubation of disks with blood be shortened to 3 h to reduce the risk of losing enzyme activity. A separate experiment showed that equilibrium is almost complete in this time. However, equilibrium of a disk coated with specific antibody and the anti-human ^{125}I-IgE requires 16 to 24 h. In both incubation stages, considerably more antibody is bound to the disks at room temperature than at 4°C.

The use of the Rast in forensic serology is presently confined to blood. Specific IgE antibodies are often detectable in nasal secretions [*18*], but attempts in this laboratory to determine such antibodies in up to 0.5 ml of liquid semen or saliva have not been successful.

Because of the geographical specificity of some allergens, it is recommended that be-

fore the RAST is employed in forensic investigations, a survey should be carried out to determine the frequencies of local allergies. It is anticipated that an IgE antibody profile may, in some circumstances, contain information on the geographical origins of the individual in addition to its other descriptive and discriminating aspects.

In conclusion, when a bloodstain is left at a scene of crime, the presence in the stain of specific antibodies may prove useful in directing investigations towards certain individuals.

Summary and Conclusions

A survey of ragweed allergy in North Americans has been carried out. The Radio-Allergo-Sorbent Test (RAST) was used to measure IgE antibodies to ragweed pollen in the extracts of small bloodstains. Forty-six (24%) of 193 individuals gave a positive reaction to ragweed disks; only two of these individuals reported no hypersensitivity. Stains from allergic donors were also tested for their reactivity to *D. glomerata* (cocksfoot). A parallel study of ragweed antibodies was also carried out using stains from white U.K. citizens.

In addition, 82 pairs of bloodstains from 104 British donors were submitted to this laboratory in a blind trial designed to discriminate bloodstains on the basis of the allergy status of the donor. The choice of allergen disks was decided by an initial survey of patients attending an allergy clinic. Twenty-eight percent of the bloodstain donors were diagnosed as positive to at least one of these allergens. More than 40% of the stain pairs from different individuals were differentiated, and no errors of discrimination were recorded.

In both surveys, a high correlation was found between the RAST laboratory diagnosis and the donor's subjective assessment of his/her own allergies.

The finding of specific antibodies in a bloodstain provides three levels of information of use to a forensic scientist. First, it enables the stain to be differentiated from others which contain no specific antibody. Second, it provides clinical information about the person who left the stain, that is, he may suffer from a hypersensitivity. Third, antibodies to some antigens are largely confined to certain populations.

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