

Histamine Release from Peripheral Blood Leukocytes with Purified Bee Venom Allergens: Effect of Hyperimmune Beekeeper Plasma

P.M. Clinton, D.M. Kemeny, L.J.F. Youlten, M.H. Lessof

Department of Allergy and Allied Respiratory Disorders, United Medical and Dental Schools of Guy's and St. Thomas' Hospitals, London, UK

Abstract. The response of 15 strongly bee-venom-allergic patients to highly purified venom allergens was compared using skin prick test titration, peripheral blood leukocyte (PBL) histamine release and radioallergo-sorbent test with three highly purified bee venom allergens: phospholipase (PLA₂), hyaluronidase (HYAL) and acid phosphatase (ACID P). Sensitivity to the three allergens ranked in the same order for all three tests and in each case PLA₂ was found to be the most potent allergen. In the presence of hyperimmune beekeeper plasma, maximum histamine release was reduced significantly for all three allergens ($p < 0.001$). Furthermore, hyperimmune beekeeper plasma increased the amount of allergen required for a comparable release of histamine (mean shift in dilution curve PLA₂ 917-fold; HYAL, 492-fold; ACID P, 61-fold). The release of histamine from whole blood was also compared with PBL + 10% normal human serum (NHS). For all three allergens maximum release was much lower from whole blood compared with washed cells + 10% NHS ($p < 0.001$).

These data confirm PLA₂ as the major bee venom allergen by all three tests. Hyperimmune beekeeper plasma reduces maximum histamine release and increases its threshold. Histamine release in response to ACID P appears harder to block with hyperimmune beekeeper plasma than that provoked by PLA₂ or HYAL ($p < 0.01$). Whole blood releases less histamine and requires more allergen than washed cells, indicating that sensitivity of PBL *in vivo* is unlikely to be as great as washed PBL *in vitro*.

Introduction

Immunotherapy with allergen extracts has been widely used to treat patients with bee sting allergy. Such treatment results in an initial rise in specific IgE subsequently followed by a fall which is paralleled by a rise in specific IgG [1-7]. Both the level of allergic mediators present in secretions elicited by allergen challenge [8] and the sensitivity of basophil leukocytes have been found to decrease over the course of treatment [1-3, 9-11] although not in all cases [12].

The mechanism by which immunotherapy produces its protective effect has been the subject of a number of studies. Antigen-specific IgG antibody produced during treatment can block the release of histamine from sensitized human leukocytes *in vitro* [13, 14] and can inhibit the binding of IgE antibodies to antigen in radioallergosorbent test (RAST) [15]. Passive immunization using either serum from patients of immunotherapy [16] or gamma globulin from

hyperimmune beekeepers [17] has been shown to prevent or substantially reduce the allergic reaction to subsequent challenge *in vivo*.

We have used highly purified bee venom proteins to determine whether hyperimmune beekeeper plasma blocked histamine release to all bee venom allergens equally and, secondly, to determine if the response of whole blood to bee venom is the same as washed peripheral blood leukocytes (PBL).

Materials and Methods

Selection of Patients

Fifteen adult subjects were chosen for this study on the basis of clinical history of systemic and immediate reactions to not more than five simultaneous stings. A positive skin test was recorded when the wheal diameter was more than 3 mm larger than that produced by the normal serum albumin diluent control. All subjects tested had high levels of specific IgE to bee venom as measured by the RAST (table 1) and in this respect were atypical of bee-venom-

Table 1. Comparison of the response of skin, PBL and the concentration of IgE antibodies in the study group

Subject	Skin test	RAST	Major allergen by				
			HR			SPT	IgE ELISA
			+NHS	+BP	WB		
1	10	4+	P	P	P	P	P
2	10	4+	A	A	ND	H	P
3	10	2+	A/P	A	A	A/P/H	A/P
4	100	3+	P	P	P	P	P
5	1	2+	P	P	P	H/P	A
6	10	4+	P	P/A	P	P	A/P
7	10	4+	H/P/A	P	H	H/P	A/P
8	1	3+	P	P	H	H	A/P
9	100	3+	P	P	P	P	P
10	10	2+	P	H	A	ND	P
11	10	4+	P/A	P/A	P/A	P	P
12	1	4+	P	P	P	P	A
13	10	4+	P	A	P	P	P
14	1	ND	ND	ND	ND	ND	ND
15	1	2+	P/A	P/A	P/A	P	P

Histamine release (HR) was carried out in the presence of NHS, blocking plasma (BP), and from whole blood (WB). The data given for HR, IgE ELISA and skin prick test (SPT) are the antigen which produced the greatest response by the particular test method. ND = Not done; P = PLA₂; H = HYAL; A = ACID P.

allergic patients as a whole. Our reason for selecting such highly sensitive individuals was that we felt they were more likely to have persisting sensitivity of their basophils when recruited to this study. All had been given immunotherapy, although 5 had suffered adverse reactions and either failed to complete the immunotherapy course or had a history of recurring adverse reactions to maintenance doses of venom (table 1). Seven subjects were male, eight were female and the majority were nonatopic.

Venom Antigens

In bee venom the most important antigens are phospholipase A₂ (PLA₂) [18], hyaluronidase (HYAL) [19, 20] and acid phosphatase (ACID P) [21–23]. Mellitin is a minor allergen [24]. The first three are proteins with molecular weights of approximately 20,000, 40,000 and 136,000 daltons, respectively, whilst mellitin is a peptide of 2,840 daltons.

The purified bee venom antigens were all prepared from venom purchased from Nectacoorp (Sofia, Bulgaria), which was a kind gift of Prof. C.A. Vernon, Chemistry Department, University College, London, UK. PLA₂, HYAL and ACID P were purified as previously described [20, 25]. These venom proteins were highly purified with less than 0.01% contamination of PLA₂ by HYAL and ACID P, and less than 0.1% contamination of ACID P by HYAL and PLA₂ or of HYAL by PLA₂ and ACID P. None of the proteins gave rise to greater than 4% histamine release over the dilution

range tested, when incubated with washed leukocytes from a highly atopic but bee-venom-insensitive control subject.

Blocking Antibody

Plasma was taken from a hyperimmune, nonatopic beekeeper which, on quantitation [26], was found to contain specific IgG at concentrations of 24 µg/ml for anti-PLA₂, 7.8 µg/ml for anti-HYAL, 7 µg/ml for anti-ACID P and undetectable levels of venom-specific IgE. Because there was over 3 times as much anti-PLA₂ in the blocking plasma as anti-HYAL or anti-ACID P, this was corrected by diluting 1:2 in normal serum when studying PLA₂-induced histamine release.

Serum from a nonatopic donor (matched for age, sex and blood group -0) was used as a negative control. Although serum was not an entirely appropriate control for plasma, several experiments indicated that this made no difference to the histamine release or efficacy of blocking. Dose-response curves for histamine release from washed leukocytes taken from a venom-insensitive, mite-allergic control subject in response to *Dermatophagoides pteronyssinus* in the presence of normal serum and plasma, and beekeeper serum and plasma were all superimposable (results not shown). Likewise, the dose-response curves for histamine release from subject 14 were also superimposable when washed leukocytes were incubated in the presence of normal plasma or serum (results not shown).

Skin Prick Tests

Patients were tested with honey bee venom, PLA₂, HYAL, ACID P and mellitin over a range of concentrations from 0.01 µg/ml in 10-fold increments to 100 µg/ml. The diluent and negative control was sterile saline. Histamine was used as a positive control. A positive result was taken as a wheal size larger than 3 mm compared with the negative control.

Histamine Release Assay

Washed leukocytes were prepared from 15 ml of blood as described previously [27]. The washed cells were resuspended in 7.2 ml Tris ACM (25 mM Tris(hydroxymethyl)aminoethane, 120 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 0.03% human serum albumin) + 300 U (0.3 ml) heparin (added to prevent plasma clots), giving an average cell concentration of 5.5×10^6 cells/ml (range $3-8 \times 10^6$ cells/ml). Whole-blood preparations were obtained by diluting 5 ml of blood in 1.25 ml Tris ACM and 125 U heparin, giving an average cell concentration of 4.4×10^6 cells/ml (range $2-10 \times 10^6$ cells/ml). PLA₂, HYAL and ACID P were used over a range of six dilutions from 0.04 ng/ml to 4 µg/ml (final concentration) in 10-fold increments when testing hyperimmune beekeepers' plasma. In these experiments, 40 µl of each antigen dilution in duplicate (including 40 µl of Tris ACM to determine spontaneous histamine release) were added to 0.5 ml polypropylene microcentrifuge tubes. To each 40-µl aliquot of antigen or buffer was added 10 µl of beekeeper plasma or control serum. These were incubated for 30 min at room temperature in a similar protocol to that described elsewhere [13] before 50-µl aliquots of washed leukocytes were added, and a further incubation carried out for 60 min at 37 °C. The tubes were then cooled to 4 °C to prevent further release before centrifugation at 800 g for 20 min at 4 °C. Aliquots of 50 µl were removed from the supernatants of each tube and stored at -20 °C until assayed for histamine content as described previously [28].

Total histamine levels were determined by boiling 50 µl washed

Fig. 1. Time course of histamine release in the presence or absence of blocking plasma. The length of preincubation of PLA₂ with hyperimmune beekeeper plasma (○) had no effect on the blocking indicating that this occurred almost instantaneously. In the presence of normal human serum (●) there was some drop in histamine release with prolonged incubation possibly due to loss of antigen during storage.

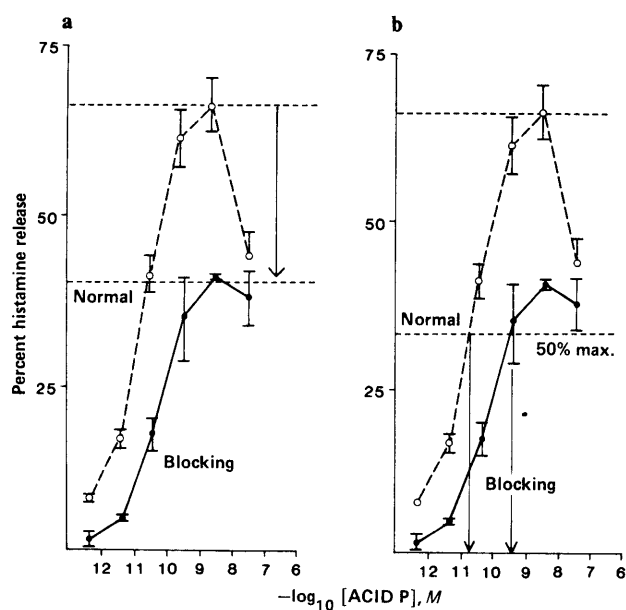
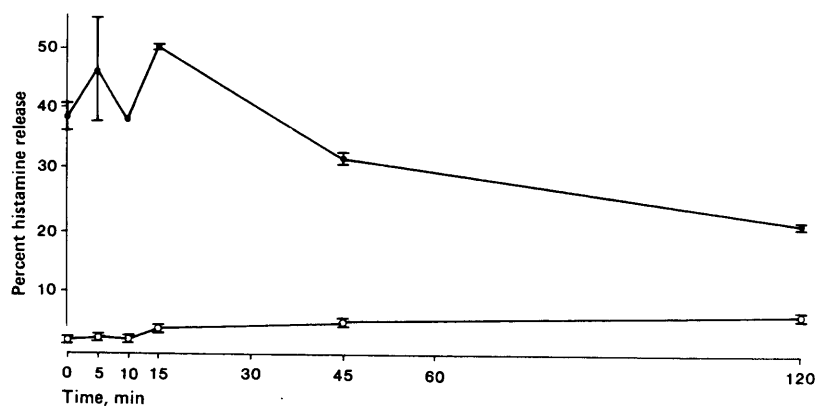


Fig. 2. Typical histamine dose-response curves in the presence of normal (○) and blocking (●) plasma showing reduction of maximum histamine release and a shift to the right in the dose-response curve. **a** Percent inhibition of maximal histamine release. **b** Threshold of histamine release.

Table 2. Increase in the amount of antigen required for histamine release in the presence of hyperimmune beekeeper plasma

Patient	PLA	HYAL	ACID P
1	150	100	3
2	250	50	20
3	800	25	20
4	2,000	100	ND
5	50	1,000	70
6	500	90	7
7	500	1,500	100
8	2,000	2,000	500
9	2,000	1,000	15
10	500	0	10
11	2,000	5	20
12	2,000	6.7	20
13	700	20	50
14	800	1,000	10
15	100	ND	6
Mean	957	492	61

The shift in the histamine release dose-response curve in the presence of hyperimmune beekeeper plasma was less for ACID P than for PLA₂ or HYAL ($p < 0.01$, Student's *t* test). ND = Not done.

leukocytes in 50 μ l Tris ACM and 50 μ l whole blood in 100 μ l Tris ACM for 10 min before centrifugation as above and subsequent collection of the supernatants for histamine assay.

A positive result was taken as a release of more than 10% of total cell histamine (after the value for spontaneous histamine release had been subtracted). Sensitivities to the three antigens were deter-

mined by comparison of the lowest molar concentration of antigen that gave 50% of the maximum percent histamine release for that antigen. A third experiment was carried out to ascertain if the length of preincubation of antigen with beekeeper antibody made a significant difference to the final extent of blocking of histamine release. Two dilutions of the venom antigens (1 ng/ml and 5 μ g/ml)

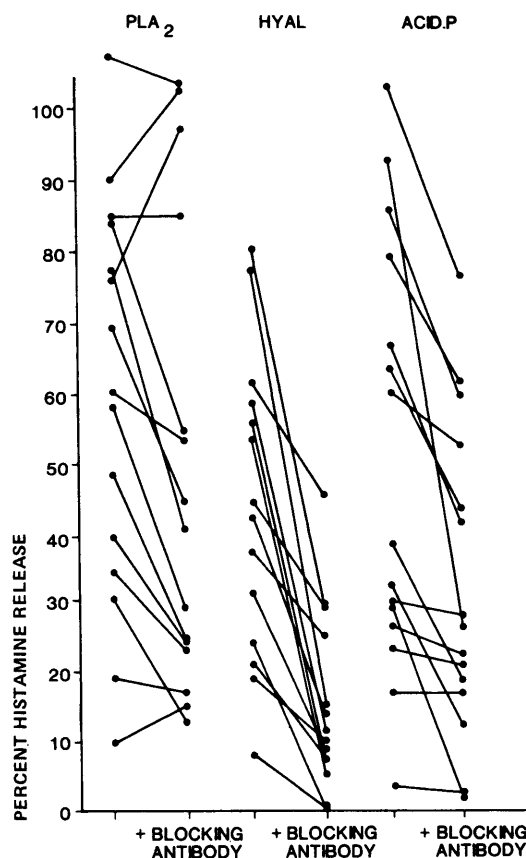


Fig. 3. Maximum histamine release in the presence and absence of hyperimmune beekeeper plasma with PLA₂, HYAL and ACID P. In most cases, hyperimmune beekeeper plasma reduced maximum histamine release.

were incubated with beekeeper plasma and normal serum in duplicate as described above, for 0, 5, 10, 15, 45 and 120 min before washed leukocytes from subject 14 were added and histamine release determined in the usual way. No difference was observed (fig. 1) in the amount of histamine released and the length of preincubation with the hyperimmune beekeeper plasma although there was some fall in the release when the allergen was preincubated with normal serum for 120 min which might be due to loss of antigen on storage.

Quantitation of Blocking

The amount of blocking was determined by comparing firstly the value for maximum histamine and secondly the threshold of histamine release, which is defined as the amount of allergen required for 50% maximum release as shown in figure 2. In some cases there was insufficient release after addition of the blocking plasma and comparisons had to be made at 20 or 30% of maximum.

Assays for IgE and IgG Antibodies

IgE and IgG antibodies were measured using a sandwich enzyme-linked immunosorbent assay (ELISA) as described previously [29, 30]. The results are expressed as nanograms or micro-

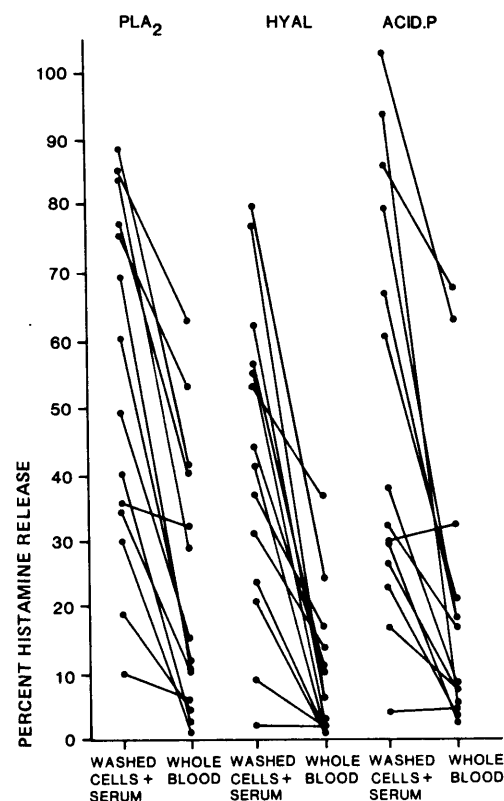


Fig. 4. Comparison of maximum histamine release with whole blood and washed cells + normal human serum showing that maximum release was lower in whole blood.

grams per milliliter using reference sera calibrated by ¹²⁵I-radiolabeled antigen saturation [26]. IgE antibodies to whole bee venom were measured by RAST as described elsewhere [26, 31] and results expressed as a score using the Phadebas reference kit [Pharmacia (GB) PLC].

Results

Comparison of Histamine Release, Skin Test and RAST

The majority of patients were most sensitive to PLA₂ as determined by histamine release, skin test and RAST (table 1). These three tests agreed very well when determining a positive or negative result, with a concordance of 81% for histamine release and skin test, 93% for histamine release and RAST, and 79% for skin test and RAST. The majority of patients were also most sensitive to PLA₂ as determined by hista-

mine release in the presence and absence of both exogenous blocking antibody and endogenous IgG (whole-blood preparation; table 1).

Change in Maximal Histamine Release in the Presence of Hyperimmune Beekeeper Plasma

The percentage of total cell histamine released at the optimum allergen concentration is shown in figure 3. For all three allergens there was a marked reduction in maximum histamine release ($p < 0.01$, paired t test). The threshold histamine release was shifted to the right for all three allergens and was less for ACID P compared with PLA₂ and HYAL (table 2).

Comparison of Washed PBL and Whole-Blood Histamine Release

The maximum amount of histamine released was much lower from whole blood than for washed cells + 10% normal human serum (fig. 4). The resulting curve was often too low for any real estimate of the threshold dose to be established.

Discussion

In this study three questions have been addressed. First, is the sensitivity to highly purified allergens the same for skin mast cells, PBL and specific IgE antibodies. Second, what effect does hyperimmune beekeeper plasma have on PBL and, third, how does histamine release from washed PBL compare with whole blood.

For skin test titration, PBL histamine release and RAST PLA₂ proved to be the most potent bee venom allergen and this agrees with previous studies with RAST [31] and histamine release [32]. The response of PBL to allergen was substantially reduced in the presence of hyperimmune beekeeper plasma. Previous studies of the effects of immune serum on histamine release by Lichtenstein et al. [33] showed that IgG is largely responsible for this and not nonspecific factors [34]. In contrast to Lichtenstein et al. [33], we found that maximum histamine release as well as the threshold of release were reduced. In our study, we used highly purified bee venom allergens and this may explain the difference between our data. Of the three allergens the shift in the histamine release curve was less for ACID P than PLA₂ or HYAL but the reduction in maximum release was similar. It is difficult to draw firm conclusions about the relative ease with

which allergens can be blocked but the concept that some larger allergens such as ACID P might be harder to block remains a possibility. Whole-blood histamine release was much lower than that from washed PBL, whether due to specific or nonspecific factors we cannot say.

Acknowledgements

Thanks are due to Mrs. Sheelah Lockwood for typing this manuscript. P.M.C. is a Rodney and Hilda Poulten fellow. This work was supported by the Asthma Research Council.

References

- 1 Levy, D.A.; Lichtenstein, L.M.; Goldstein, E.O.; Ishizaka, K.: Immunologic and cellular changes accompanying the therapy of pollen allergy. *J. clin. Invest* 50: 360-369 (1971).
- 2 Evans, R.; Pence, H.; Kaplan, H.; Rocklin, R.E.: The effect of immunotherapy on humoral and cellular responses in ragweed hayfever. *J. clin. Invest.* 57: 1378-1385 (1976).
- 3 Norman, P.S.; Lichtenstein, L.M.; Kagey-Sobotka, A.; Marsh, D.G.: Controlled evaluation of allergoid in the immunotherapy of ragweed hay fever. *J. Allergy clin. Immunol.* 70: 248-260 (1982).
- 4 Clayton, W.F.; Reisman, R.E.; Georgitis, J.W.; Wypych, J.I.; Arbesman, C.E.: Effect of prolonged venom immunotherapy on serum venom-specific IgE and IgG. *Clin. Allergy* 13: 301-307 (1983).
- 5 Kemeny, D.M.; MacKenzie-Mills, M.; Harries, M.G.; Youlten, L.J.F.; Lessof, M.H.: Antibodies to purified bee venom proteins and peptides. II. A detailed study of changes in IgE and IgG antibodies to individual bee venom antigens. *J. Allergy clin. Immunol.* 72: 376-385 (1983).
- 6 Djurup, R.; Malling, H.J.; Sondergaard, I.; Weeke, B.: The IgE and IgG subclass antibody response in patients allergic to yellow jacket venom undergoing different regimens of venom immunotherapy. *J. Allergy clin. Immunol.* 70: 46-55 (1985).
- 7 Hunt, K.J.; Askenase, P.W.: Enzyme-linked immunosorbent assay (ELISA) of changes in specific IgG antibodies to five venoms during venom immunotherapy. *Clin. Allergy* 15: 17-27 (1985).
- 8 Creticos, P.S.; Adkinson, N.F., Jr.; Kagey-Sobotka, A.; Proud, D.; Meier, H.L.; Naclerio, R.M.; Lichtenstein, L.M.; Norman, N.S.: Nasal challenge with ragweed pollen in hay fever patients: effect of immunotherapy. *J. clin. Invest.* 76: 2247-2253 (1985).
- 9 Stahl Skov, P.; Norn, S.; Weeke, B.: Basophil histamine release in patients with hay fever: results compared with specific IgE and total IgE during immunotherapy. *Clin. exp. Immunol.* 27: 432-439 (1977).
- 10 Malling, H.-J.; Stahl Skov, P.; Permin, H.; Norn, S.; Weeke, B.: Basophil histamine release and humoral changes during immunotherapy. *Allergy* 37: 187-190 (1982).
- 11 Kimura, I.; Tanizaki, Y.; Goda, Y.; Komagoe, J.; Kitani, H.: Decrease in reactivity of basophils by immunotherapy with housedust extract. *Clin. Allergy* 15: 1-7 (1985).

- 12 Ohman, J.L., Jr.; Findlay, S.R.; Leitermann, K.M.: Immunotherapy in cat-induced asthma: double-blind trial with evaluation of in-vivo and in-vitro responses. *J. Allergy clin. Immunol.* 74: 230-239 (1984).
- 13 Lichtenstein, L.M.; Osler, A.G.: Studies on the mechanisms of hypersensitivity phenomena. XII. An in vitro study of the reaction between ragweed pollen antigen, allergic human serum and ragweed-sensitive human leukocytes. *J. Immunol.* 96: 169-179 (1966).
- 14 May, C.D.; Lyman, M.S.; Alberto, R.; Aduna, N.: On the measurement of blocking antibody: blocking of antigenic histamine release compared to maximal antigen binding by antibodies in serum. *J. Allergy clin. Immunol.* 49: 54-58 (1972).
- 15 Zimmerman, E.M.; Yunginger, J.W.; Gleich, G.J.: Interference in ragweed pollen and honeybee venom radioallergosorbent tests. *J. Allergy clin. Immunol.* 66: 386-393 (1980).
- 16 Cooke, R.A.; Barnard, J.H.; Hebal, S.; Stull, A.: Serological evidence of immunity with coexisting sensitization in a type of human allergy (hay fever). *J. exp. Med.* 62: 733-750 (1935).
- 17 Lessof, M.H.; Sobotka, A.K.; Lichtenstein, L.M.: Effects of passive antibody in bee venom anaphylaxis. *Johns Hopkins med. J.* 142: 1-7 (1978).
- 18 Sobotka, A.K.; Franklin, R.M.; Adkinson, N.F.; Valentine, M.D.; Baer, H.; Lichtenstein, L.M.: Allergy to insect stings. II. Phospholipase A₂: the major allergen in honey bee venom. *J. Allergy clin. Immunol.* 57: 29-40 (1976).
- 19 King, T.P.; Sobotka, A.K.; Kochoumian, L.; Lichtenstein, L.M.: Allergens of honey bee venom. *Archs Biochem. Biophys.* 172: 661-671 (1976).
- 20 Kemeny, D.M.; Dalton, N.; Lawrence, A.J.; Pearce, F.L.; Vernon, C.A.: The purification and characterisation of hyaluronidase from the venom of the honey bee, *Apis mellifera*. *Eur. J. Biochem.* 139: 217-223 (1984).
- 21 Karpas, A.B.; Baer, H.; Hooton, M.L.; Evans, R.: A high molecular weight allergenic fraction of honey bee venom. *J. Allergy clin. Immunol.* 60: 155-162 (1977).
- 22 Hoffman, D.R.; Shipman, W.H.: Allergens in bee venom I. Separation and identification of the major allergens. *J. Allergy clin. Immunol.* 58: 551-562 (1976).
- 23 Hoffman, D.R.: Allergens in bee venom. III. Identification of allergen B of bee venom as an acid phosphatase. *J. Allergy clin. Immunol.* 59: 364-366 (1977).
- 24 Paull, B.R.; Yunginger, J.W.; Gleich, G.J.: Mellitin: an allergen of honey bee venom. *J. Allergy clin. Immunol.* 59: 334-338 (1977).
- 25 Barboni, M.; Kemeny, D.M.; Campos, S.; Vernon, C.A.: The purification of acid phosphatase from bee venom. *Toxicon* 25: 1097-1103 (1987).
- 26 Kemeny, D.M.; Lessof, M.H.: The immune response to bee venom. II. Quantitation of the absolute amounts of IgE and IgG antibodies by saturation analysis. *Int. Archs Allergy appl. Immunol.* 83: 113-120 (1987).
- 27 Clinton, P.M.; Kemeny, D.M.; Amlot, P.; Urbanek, R.; Lessof, M.H.: Histamine release from peripheral blood leukocytes in egg-allergic patients. *Clin. Allergy* 16: 345-354 (1986).
- 28 Clinton, P.M.; Kemeny, D.M.; Murdoch, R.M.: The development of a micro-histamine release assay. *Clin. Allergy* 17: 181-189 (1987).
- 29 Kemeny, D.M.; Urbanek, R.; Samuel, D.; Richards, D.: Increased sensitivity and specificity of a sandwich ELISA for measurement of IgE antibodies. *J. immunol. Methods* 78: 217-226 (1985).
- 30 Urbanek, R.; Kemeny, D.M.; Samuel, D.: Use of enzyme-linked immunosorbent assay for measurement of allergen-specific antibodies. *J. immunol. Methods* 79: 123-131 (1985).
- 31 Kemeny, D.M.; Harries, M.G.; Youtlen, L.J.F.; Mackenzie-Mills, M.; Lessof, M.H.: Antibodies to purified bee venom proteins and peptides. I. Development of a highly specific RAST for bee venom antigens and its application to bee sting allergy. *J. Allergy clin. Immunol.* 71: 505 (1983).
- 32 Wahn, U.; Thiemeier, N.; Gens, G.; Farck, G.; Kemeny, D.M.: The allergenic activity of purified bee venom proteins and peptides (Abstract). *J. Allergy clin. Immunol.* 73: 189 (1982).
- 33 Lichtenstein, L.M.; Holtzman, N.A.; Burnett, L.S.: A quantitative in-vitro study of the chromatographic distribution and immunoglobulin characteristics of human blocking antibody. *J. Immunol.* 101: 317-324 (1968).
- 34 Miadonna, A.; Tedeschi, A.; Zanussi, C.: Plasma of normal, but not atopic, persons reducing basophil degranulation induced by anti-IgE. *Clin. Allergy* 14: 29-33 (1984).

Received: August 11, 1988

Correspondence to: Dr. D.M. Kemeny
 Department of Allergy and Allied Respiratory Disorders
 UMDS
 Guy's Campus
 London SE1 9RT (UK)