

Original Research Papers

Complement activating agents in allergenic extracts

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Abstract. *Objective:* Extracts of environmental allergens, moulds and plant pollens are known to consume haemolytic complement (huC) in human serum in vitro through the antibody-independent engagement of the first component C1 of the classical pathway. The present work was undertaken to establish the nature and characteristics of the complement activating agents in allergenic extracts and to probe their relationship with the IgE-binding allergens.

Materials and Methods: A large series of different >10 kDa allergenic products was investigated for their capacity to consume haemolytic complement in the sera of allergic patients with specific anti-allergen IgE antibodies, as well as in normal control sera. UV-spectroscopy was used for categorizing the non-protein components in the extracts.

Results: The experiments confirmed that huC is consumed in an antibody-independent fashion and in a qualitatively similar, but quantitatively distinct manner. The ratio of the complement activating potencies among the different allergenic preparations thereby remained constant and independent of the serum source, while an overt relationship with specific IgE-antibodies could not be established. UV-spectroscopy of the allergenic preparations revealed the presence of chemical decomposition products in nearly every extract and roughly in proportion to the complement activating potencies.

Conclusion: The data support the idea that huC-activation by traditional allergenic extracts is mainly due to by-stander degradation products of the melanoidin (Maillard) or tannin type, which may or may not occur in physical association with the IgE-binding protein allergens.

Key words: Complement – Allergens – Tannins – Melanoidins – Maillard

Introduction

Allergenic extracts of house dust, animal epithelia, some

mite species, moulds and several plant pollens widely used in the diagnosis and treatment of human allergies are known to consume haemolytic complement (huC) in human serum in vitro [1–3]. The mechanism of complement consumption by these extracts in dilute serum involves activation of the first component C1 of the classical pathway [4], though fragmentation of native C3 through the C3-amplification loop may under special conditions be observed as well [5]. The engagement of C1 by constituents of allergenic extracts is antibody-independent, but it remains unclear whether it concerns a direct interaction, or perhaps an indirect mechanism mediated by so far unidentified serum factors [6, 7]. The mechanism resembles the action on complement of certain allergenic polyphenols and flavonoids [8–10]. From a recent study of complement consumption by *Parietaria* pollen allergens and their enzymic fragments it was indeed concluded that the activating sites involve structural elements of flavonoid-peptide conjugation [10].

Although complement consumption in vitro by extracts of environmental dust and animal materials has often been confirmed [5, 11–14], the role of complement in allergic reactions in vivo remains equivocal. Nevertheless, recent studies with ragweed pollen extracts reinforce earlier results with purified house dust allergens, namely that the sera of atopic patients in statistical terms tend to be more susceptible to complement inactivation than observed with the sera of non-allergic controls [2, 5]. This phenomenon has tentatively been attributed to a possible innate hyperreactivity of complement or of mediating serum factors related to the 'atopic' condition, but not to antibody-dependent allergen sensitization [2, 3, 6, 7].

In an attempt to assess the chemical nature and structural prerequisites of the complement activating components in allergenic extracts, we have now compared the effect of a variety of traditional allergen preparations on human haemolytic complement in vitro. The accumulated evidence supports the idea that complement consumption by allergenic extracts is mainly due to by-stander degradation products of the melanoidin or tannin type, which may or may not occur in physical association with IgE-binding protein allergens.

Materials and methods

Allergenic extracts

Aqueous allergenic extracts were produced in our laboratories according to standard manufacturing procedures. The high-molecular allergenic components (HMW) were obtained in the lyophilized form after dialysis against distilled water or ultrafiltration through 10 kDa cut-off membranes. Where mentioned, these preparations were further purified and the subfractions coded strictly following published protocols [15]. Commercially obtained cured Virginia tobacco leaf was extracted by the method of Becker et al. [16] and the 'tobacco glycoprotein' was obtained after a series of salt fractionation and chromatographic isolation steps corresponding to the protocol for house dust fraction E [15]. Green coffee beans were purchased from a specialized store in Madrid, extracted according to Freedman et al. [17] and the >10 kDa components secured by lyophilization. A sample of Chinese green tea obtained from the same store was extracted with boiling water, filtered and lyophilised; it was the only product used in the non-dialysed form.

Dry pollen powders as source material for allergenic pollen proteins were obtained from Biopol (Spokane, WA, USA) and from Greer Laboratories (Lenoir, NC, USA) and were defatted by percolation with ethylether. Moulds for the preparation of allergenic extracts were cultured in our facilities on liquid dialysable nutrient media, using for inoculation certified specimen provided by the Central Bureau for Mould Cultures (CBS, Baarn, The Netherlands). After the exponential growth period of about 3 weeks, the culture media were separated from the mycelia by filtration. The >10 kDa allergenic preparations were subsequently isolated from the culture filtrates by dialysis and lyophilization.

Freeze-dried allergenic preparations were reconstituted in veronal-saline buffer VSB⁺⁺ for haemolytic complement studies (see below), or in 0.01 M phosphate-saline buffer pH 7.4 for recording the UV-absorption spectra in a Philips UV-VIS spectrophotometer in the range of 200–600 nm.

Complement assays

Complement was measured in a haemolytic assay in microtiter modification of the previously described method [2,4,18]. Fresh sheep red blood cells and rabbit amboceptor were obtained from BioMérieux, Madrid. The working dilution for amboceptor was 1:800. Sensitized cells EA were freshly prepared by adding a suspension of washed erythrocytes E in VSB⁺⁺ at 1.5×10^9 cells/ml to an equal volume of the working dilution of amboceptor A. Dose-response curves were obtained by preincubating serial dilutions of reconstituted freeze-dried >10 kDa allergen preparation (76 µl) with 38 µl patient's serum pre-diluted 1:30 in Ca⁺⁺/Mg⁺⁺ - containing veronal-saline buffer (VSB⁺⁺) in V-shaped microtiter wells for 30 min at 37 °C. Residual haemolytic C was evaluated in a second step by adding 38 µl EA (7.5×10^8 cells/ml) to the wells containing the pre-incubated allergen-serum mixtures, and shaking the plates for 1 h at 37 °C. Controls included an EA sample lysed in distilled water and a sample lysed by dilute serum in the absence of allergen. After sedimentation, 100 µl aliquots were pipetted into U-shaped microtiter wells containing 200 µl of VSB⁺⁺ and the colour was read at 405 nm. For comparative purposes, results from dose-response curves were expressed in µg of allergenic preparation required for 50% consumption of available haemolytic complement in the standard assay volume (µg/152 µl). Since individual human sera may differ appreciably in complement reactivity to a single allergen preparation [2,5,7], all experiments were run side-by-side with a highly purified allergen and powerful complement activator (# 72E) isolated from house dust as the standard reference [2,4,6,7,15]. Because the quantitative relationship between the huC-consuming potentials of distinct allergens remains constant and independent of the serum (see Results section), potency calculations for the different allergen preparations refer to the 72E standard as unity, under identical conditions of assay.

Serum samples and specific IgE - antibodies

Blood serum samples of allergic patients were obtained from clinical allergy departments of various hospitals in Spain and The Netherlands, and were analysed for specific IgE antibodies with commercial test kits (CAP-system, Pharmacia) or an in-house enzyme immunoassay (EIA), using cellulose discs coupled with cyanogen bromide to the appropriate >10 kDa allergenic proteins according to Ceska et al. [19]. Results of the in-house immunoassay were evaluated and expressed in percent binding of added enzyme-labelled mouse monoclonal anti human IgE (Ingenasa, Madrid, Spain) as described before [20]. Serum samples were stored in small aliquots at -20 °C for no longer than 4 weeks before use.

Results

The nondialysable and unfractionated >10 kDa components from aqueous allergenic extracts were examined for their capacity to consume haemolytic complement in the sera of specifically sensitized patients with positive skin prick tests and high IgE antibody levels to the allergen under study. However, in conformity with earlier results it was noted that a highly purified allergen preparation from house dust invariably proved to be the most potent activator, independent of detectable IgE or IgG antibodies and no matter what the serum source [2,7]. Furthermore, the huC-consuming potencies of separate allergens remained in a constant ratio to each other and to the house dust standard, though the absolute huC-sensitivity of individual sera differed [2]. This phenomenon is illustrated with the >10 kDa components in extracts of cat dander and *D. pteronyssinus* in the scatter diagrams of Figures 1a, b.

For those allergenic extracts for which a sufficient number of specifically sensitized patients and sera were available, statistical data are collectively listed in Table 1. These analyses at the same time demonstrated a lack of correlation with specific IgE-antibody values as determined in direct binding assays at a single serum dilution. For example, in the 18 serum samples of cat-sensitive patients used for the complement studies (Fig 1b and Table 1), correlation coefficients between C-activation and cat-specific IgE were found of $r=0.21$ (by regression analysis, $p=0.4$) and $r=0.23$ (Spearman rank order, $p=0.3$). In the various sets of serum collections, the respective IgE-binding values were nevertheless interrelated in terms of ranking order (see footnotes to Table 1).

For all the >10 kDa allergenic preparations under study, the huC-consuming potential was established in relation to a standard house dust preparation. In this manner, the constant relationship of the huC-activating potencies of these various preparations allows an inter-allergen comparison relative to the house dust standard defined as unity. The values thus obtained were averaged over a minimum of 3 IgE-positive sera. In those cases for which sera with detectable allergen-specific IgE were not available, e.g. with the tobacco allergens, serum samples of mite-allergic patients and/or normal controls were used. The relative huC-activating potencies of a series of commonly used allergenic extracts are collectively shown in the bar graph of Figure 2a, namely the dust, mould, epithelial, pollen and some food extracts.

The results shown in Figure 2a demonstrate that the phenomenon of in vitro huC-activation is a property

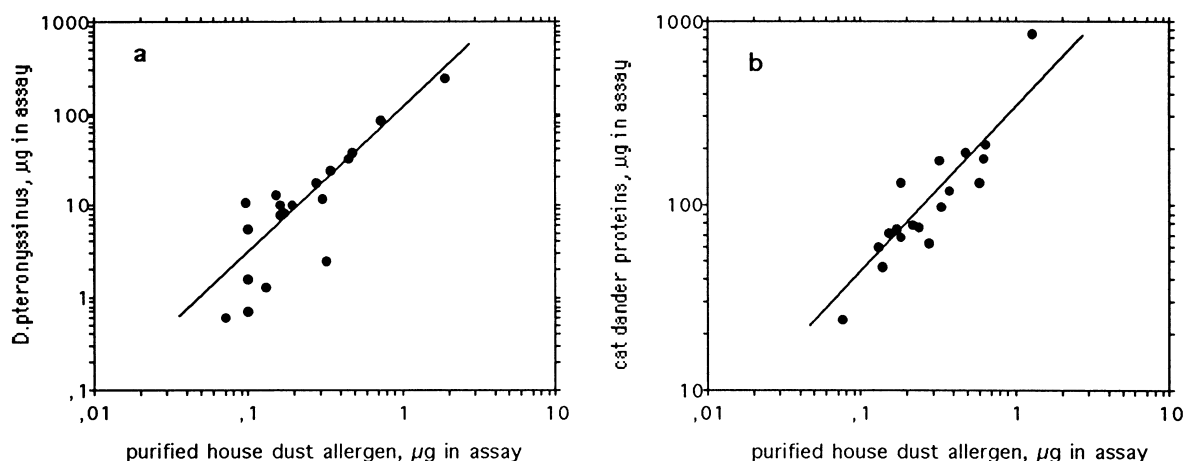


Fig. 1. (a) Scatter diagram showing the quantitative data (μg in pre-incubation volume of 152 μl), for 50% haemolytic complement consumption under standard assay conditions by purified house dust (# 72E) and the >10 kDa components in extracts of full-grown cultures of *D. pteronyssinus*, using the sera of 19 individual patients with detectable IgE antibodies and positive skin tests to house dust and mite (*D. pteronyssinus*) allergenic extracts. Statistical data in Table 1. (b) Scatter diagram showing the quantitative data (μg in pre-incubation volume of 152 μl) for 50% haemolytic complement consumption under standard assay conditions by purified house dust (# 72E) and the >10 kDa components in cat dander extracts, using the sera of 18 individual patients with positive IgE antibodies and skin tests to house dust and cat dander allergenic extracts. Statistical data in Table 1.

qualitatively shared by all the preparations investigated in a quantitatively consistent ratio. The complement activating potency of the dander extracts of cats, dogs or man, as well as of house dust, feathers, tobacco, *D. pteronyssinus* and *Parietaria* remained unaffected by heating for at least 15 min at 100°C. In contrast, the proteolytic and amidolytic activities of some predominant IgE-binding proteases present in various allergenic extracts were completely abolished by heating. In some cases, the binding to specific IgE antibodies diminished as well, though less dramatically. Hence, the thermostable complement activating components are likely to be physically and functionally

distinct from identified allergens such as the proteases Der p 1 and Der p 3.

In an attempt to define the structural elements responsible for huC-activation, attention was focused on the presence of pigmented material as a conspicuous common factor among allergenic extracts. The manifest tan colour of concentrated aqueous extracts of house dust, mite cultures, whole-body mites, insects, decomposed chicken feathers, animal epithelia, or vegetable fibers is due to degradation products generated during extracellular 'enzymic' and 'non-enzymic browning' chemical processes [15]. Such polymeric components belong to the class of the macromolecular Maillard-

Table 1. Statistical data pertinent to the activation in vitro of haemolytic complement by >10 kDa components in allergenic extracts. Correlation coefficients and p-values from regression analysis. All correlations refer to house dust #72E.

Allergen	No of sera	Mean	SD	Range	r	p
μg in assay						
<i>D. pteronyssinus</i>	19	28	57	1–250	0.98	<0.001
House dust	19	0.3	0.4	0.07–1.9		
Human dander*	21	25.2	21.5	5.6–88	0.87	<0.001
House dust	21	7.0	8.3	0.6–35		
Cat dander**	18	148	183	24–850	0.91	<0.001
House dust	18	3.6	2.9	0.8–13		
Guinea pig dander***	7	126.0	87.9	21–290	0.97	<0.001
House dust	7	3.8	2.8	0.6–7.8		
Tobacco****	21	31	35	5.2–140	0.86	<0.001
House dust	21	0.7	0.7	0.15–2.6		

* These sera formed part of a larger group of 58 samples from individual patients with adult atopic dermatitis, where a rank correlation for the direct EIA-IgE values (at a single serum dilution) coefficient (Spearman) was found of 0.752, $p < 0.001$, with a mean IgE-ratio house dust (#72E) : human dander = 1 : 0.71.

** These sera formed part of a larger group of 47 samples from individual adult patients with bronchial asthma/rhinitis and cat allergy, where the rank correlation coefficient for the direct EIA-IgE values at a single serum dilution was 0.854, $p < 0.001$, mean IgE-ratio house dust (#72E) : cat dander = 1 : 1.14.

*** No specific EIA-comparisons were made because only 3 sera had detectable IgE antibodies, though all patients presented positive skin reactions.

**** Specific IgE antibodies not detectable.

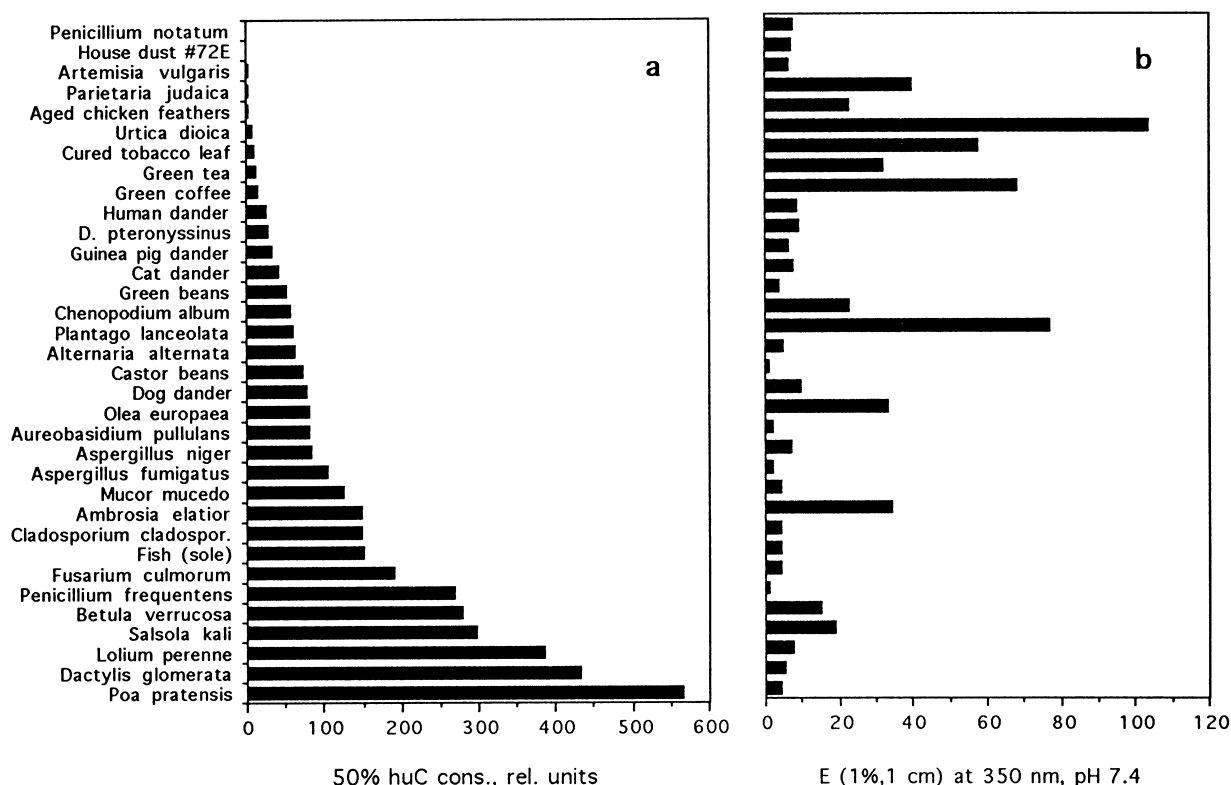


Fig. 2. (a) Bar graph collectively showing the huC-activating potential of the series of >10 kDa allergenic products employed during this investigation. The potencies of the individual preparations refer to the standard house dust preparation # 72E run simultaneously with the same sera and taken as unity. The lowest values represent the highest complement activating potencies. (b) Bar graph showing the extinction coefficients at 350 nm E (1%, 1 cm) of the investigated allergenic >10 kDa preparations in 0.01 M phosphate-saline buffer pH 7.4.

type melanoidins or humic acids, or may in the case of the dander extracts represent soluble derivatives or precursors of the (eu-)melanins. These highly complex and chemically ill-defined compounds are revealed by their absorption spectra, which usually feature a marked elevation of 'protein' absorption at 280 nm, extending well into the visible region without any further discrete maxima [15,21]. The examples shown in Figure 3a clearly demonstrate an abundance of such degradation products in several of the investigated extracts. Indeed, huC-consuming protein derivatives displaying these characteristics have been prepared artificially [22].

In aqueous extracts of pure pollen granules, especially in those of the weeds and trees, polyhydroxylic flavonoid pigments are known to persist even after the usual processes of defatting and extensive dialysis through 10 kDa cut-off membranes [10,15]. Despite the removal of free flavonoid aglycones and water-soluble flavonoid-glycosides by these procedures, the >10 kDa purified proteins in most pollen extracts continue to expose a high proportion of firmly complexed flavonoids and/or catechins. Such pigment components typically show absorption maxima in the 320 - 360 nm range [23], as illustrated by the UV-spectra in Figure 3b.

The extinction coefficients at 350 nm and pH 7.4 of the allergenic preparations under investigation are collectively given in the bar graph of Figure 2b, which indicates that even with these highly complex extracts a tendency is perceived for some of the more potent complement activating preparations to be associated with increased non-protein

UV-absorption at 350 nm, attributable to Maillard-type melanoidins or to condensed tannins comprising flavonoid core structures [10,15,21,23].

Discussion

The results confirm that complement activating agents are widespread among most of the common allergenic extracts. In dilute serum the main target is the C1-complex of the classical pathway, although in special cases modulators may be present which preferentially engage complement through the alternative or lectin pathways, e.g., heteropolysaccharides [24]. The action on C1 may either be direct, as presumed for the occupational allergen plicatic acid [9], or indirect through coagulation factor XII and plasma prekallikrein as inferred for house dust [4,6,7], ragweed pollen [5,16] and tobacco glycoproteins [8,16]. The mechanism of complement consumption differs from that induced by staphylococcal protein A or heat-aggregated human IgG [4,6,7].

Although extracts of the Gramineae pollen are virtually inactive on complement [1,2,7], the deeply pigmented extracts of the pollen of weeds and trees may be strong complement activators [5,10], as confirmed in the present work (Fig. 2a). Furthermore, the ratio of the complement activating potencies of the various different allergenic extracts remained constant and independent of the serum source (Fig. 1., Table 1), although in absolute terms

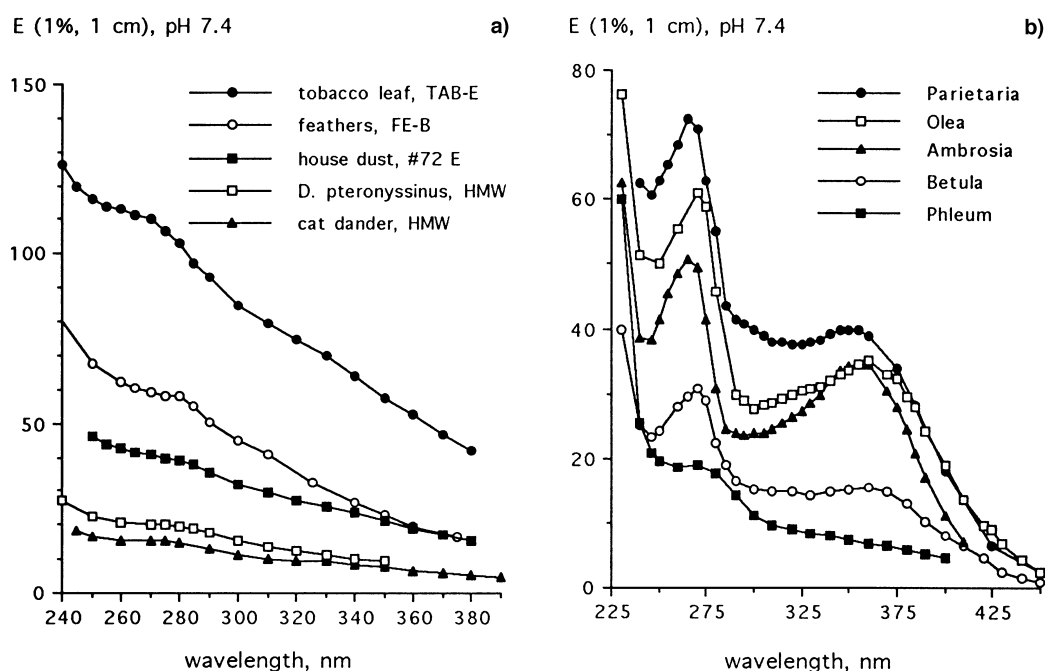


Fig. 3. a) UV-absorption spectra in phosphate-buffered saline at pH 7.4 of some representative >10 kDa dust and animal dander preparations featuring typical melanoidin- or melanin- characteristics. b) UV-absorption spectra in phosphate-buffered saline at pH 7.4 of some representative >10 kDa allergenic preparations from the pollen of grasses, weeds and trees, featuring the typical characteristics of (protein-conjugated) flavonols or condensed tannins.

complement sensitivity to any given allergen varied among individual sera, independent of the presence or absence of allergen-specific IgE or IgG antibodies [2,5,10,14].

Together with previously published data, these findings affirm that the engagement of complement by allergenic extracts in vitro represents a non-allergen-specific phenomenon. Indeed, in a recent study on ragweed pollen allergy no relation between complement consumption and specific IgE-binding was found [25]. Similarly, no correlation of complement consumption with allergen-specific IgE was observed with the extract components of cat dander. The extracts of human dander, aged chicken feathers, cured tobacco leaves and green coffee beans, which frequently elicit local skin reactions in atopic subjects [3,7,15–17,26], displayed impressive complement activating potency (Fig. 2a, Table 1), whereas the corresponding putative allergens only rarely induce detectable specific IgE antibodies [15,27]. The most logical presumption at this point therefore is that complement consumption by allergenic extracts is largely due to non-allergenic (i.e. non-IgE-binding) bystander constituent components.

With regard to the nature of the complement activating agents it seems clear from the spectroscopic evidence that these belong to at least two broad classes of chemically distinct decomposition products, i.e. the (Maillard-type) melanoidins and the (condensed) tannins (Fig. 3a, b). The composite graph of Figure 2b tends to confirm that the complement consuming potency of the allergenic preparations is related to macromolecular melanoidins and/or flavonoid polycondensates in the extracts [21,23,28]. Both classes of compounds are indeed known activators of the complement [3,22,28,29] and the contact phase (coagulation Factor XII) cascades [16]. Preliminary studies show that

artificially prepared model compounds may imitate the action on complement of the natural agents encountered in allergenic extracts [22,28].

Despite the chemical diversity and heterogeneity of the degradation products detected among allergenic extracts, their complement activating action suggests that some common quality may be involved. Tentatively, a possible role in complement activation may be considered for the antioxidant and metal-chelating properties shared by these negatively charged macromolecules. Oxidation of allergenic extracts has in fact been reported to cause a dramatic drop in complement consuming power, as well as in IgE-binding and skin-reactivity [30].

Although it seems clear that specific IgE antibody binding and complement consumption represent two distinct mechanisms triggered by different classes of components in allergenic extracts [24], it may at times be difficult to distinguish between them. For example, it has recently been shown that the dominant IgE-binding protein allergens from the pollen of *Parietaria judaica* are at the same time powerful complement activators due to the fact that the allergens have become 'tanned' by chemical conjugation to flavonoid compounds [10]. A key question here will be whether individual activated complement components can be implicated in mediating immediate-type skin reactions, as suggested for example by the inhibitory effect of C3b-Inactivator on the Prausnitz-Küstner reaction [31].

Under conditions of natural exposure to both specific allergens and non-specific complement activating agents co-existing within the inhaled material, complement activation products may become physiologically relevant, for example by inducing local inflammatory reactions and exerting an adjuvant action. In this context, Francus et al. [32] have

already demonstrated that proteins complexed with the flavonol-glycoside rutin may exert a regulatory function on the immune response by suppressing the synthesis of immunoglobulin isotypes other than IgE. The possible synergistic action of immunogenic allergens and bystander adjuvants in allergic sensitization may in this sense represent an intriguing new field of investigation.

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