



## COMPLEMENT INACTIVATION BY ALLERGENIC PLANT POLLEN EXTRACTS

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### Summary

Dialyzed aqueous extracts of plant pollen are widely used in the clinical practice of allergy for diagnostic and therapeutic purposes. The present investigation shows that such allergenic extracts are capable of consuming complement in every human serum, independent of the clinical condition. Complement is engaged by way of the first component C1, but without the participation of allergen-specific antibodies. The capacity of distinct pollen extracts to inactivate haemolytic complement was found to depend on the plant species, the most potent extracts being from the pollen of the weeds and trees. Analysis by UV-spectroscopy of the flavonoids remaining firmly bound to the proteins gives rise to the proposal that complement inactivation by allergenic and non-allergenic pollen extracts is due to polyphenolic (flavonoid) structures complexed with, or chemically conjugated to, the pollen proteins.

**Key Words:** complement, pollen, allergens, flavonoids

Extracts of house dust, certain mites or animal epithelia used in the diagnosis and treatment of human allergies are known to consume complement (huC) in human serum *in vitro* (1,2). The mechanism of huC-consumption by these extracts in dilute serum mainly involves activation of C1, C4 and C2 of the classical pathway, though fragmentation of the C3 component has been observed as well (3-5). The engagement of C1 by allergenic extract components is not by direct interaction, but most likely is mediated by so far unidentified, non-antibody serum factors (3). In this respect, the mechanism resembles the action on huC of some polyphenols, like chlorogenic acid (6) or plicatic acid (7).

Although huC-activation by environmental dust and animal allergens has been confirmed by several authors, extracts of the pollen of the Gramineae family were not found to consume complement *in vitro* in any meaningful dose range (1,8). However, significant huC-consumption was observed in undiluted serum with ragweed pollen extracts through both the classical and alternative pathways (9). Here again, complement inactivation was shown to be antibody-independent and not to have diagnostic relevance for detecting specific allergen sensitization.

However, like in the case of house dust, it was clearly shown that the sera of predisposed, so-called 'atopic' patients were more susceptible in statistical terms to ragweed-induced huC-activation than non-allergic controls (9,10). Thus, complement (in)activation appears to be indicative of an intrinsic susceptibility of complement or of mediating serum factors to components in allergenic extracts, i.e. as a marker of the 'atopic' predisposition to develop clinical allergy, but not of immunologically induced specific allergen sensitization.

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The failure to inactivate (haemolytic) complement with extracts of pollen of the Gramineae, in contrast to the positive results reported with ragweed pollen extracts, prompted us to reinvestigate the phenomenon with a more extended series of pollen species.

### Materials and methods

#### *Pollen preparations*

Dried pollen grains were purchased from BioPol (Spokane, USA) and Greer Laboratories (Lenoir, NC, USA) and were defatted with acetone or diethylether. The air-dried powders were then extracted twice with distilled water for 2 h at room temperature. The combined and centrifuged extracts were dialyzed against distilled water in the cold from Visking casing (nominal cut-off 10 kDa). The non-dialyzable >10 kDa retentate components were dried by lyophilization. These preparations were manufactured in our facilities as skin-active test reagents for the *in vivo* diagnosis of pollen allergies. The freeze-dried materials were reconstituted in 0.01 M phosphate-saline buffer pH 7.4 and the electronic absorption spectra were recorded in a Philips UV-VIS Spectrophotometer in the range of 200-600 nm. In most cases, the *in vitro* potency was established by inhibition of specific IgE antibody binding in relation to an in-house reference standard. The tannic acid preparation (*ex* walnuts) was purchased from Sigma Chem. Corp. (St. Louis, USA).

#### *Complement assays*

Complement was measured in a CH50 haemolytic assay in microtiter modification of the method previously described (2,10). Sheep red blood cells (E) and rabbit amboceptor (A) were obtained from BioMérieux. The working dilution for amboceptor A was 1 : 800, sensitized cells EA were used at  $7.4 \times 10^8$  cells/ml. Dose-response curves were obtained by preincubating serial dilutions of reconstituted freeze-dried >10 kDa pollen constituents with patient's serum diluted 1:50 in  $\text{Ca}^{++}/\text{Mg}^{++}$  - containing veronal-saline buffer (VSB $^{++}$ ) for 30 min at 37 °C. Residual haemolytic C was evaluated in a second step, using the EA detection system as described above and the appropriate controls (10). Results from dose-response curves were expressed in % consumption of available haemolytic complement (2).

Since individual human sera may differ appreciably in complement sensitivity to a standard allergen preparation (10), all experiments were run side-by-side with the powerful huC-activator (# 72E) isolated from house dust as the standard reference (2,3,10). Because the quantitative relationship between the huC-activating potentials of distinct allergens is constant and independent of the serum (See ref. 1 and Results section, Fig. 2), potency calculations for the different pollen preparations refer to the 72E standard as unity, under identical conditions of assay. Since it was considered desirable to avoid any possible interference by IgG anti-pollen antibodies in the sera of hayfever patients, the serum of asthma patients mono-sensitized to the house dust mite *Dermatophagoides pteronyssinus* and lacking IgE-/IgG-antibodies to pollens was used throughout (courtesy of the Allergy Department of Hospital La Paz, Madrid). The serum samples were stored at -20 °C for no more than 4 weeks in small aliquots until use.

### Results

The nondialyzable > 10 kDa components in aqueous extracts of a series of different pollen species were examined for their capacity to inactivate haemolytic C in the serum of dust-mite allergic patients without the clinical signs of pollinosis. The examples of dose-response curves shown in Fig. 1 demonstrate that the inactivation of huC in dilute serum depends on the pollen species. In agreement with earlier studies (1-3, 10), a purified allergen preparation from house dust proved the most potent, whereas the pollen extract of *Lolium perenne* as representative of the Gramineae family was virtually inactive. However, several extracts of the pollen of weeds and trees were quite powerful activators, particularly those of the *Parietaria*, *Ambrosia* (Fig. 1) and *Artemisia* spp (Fig.3).

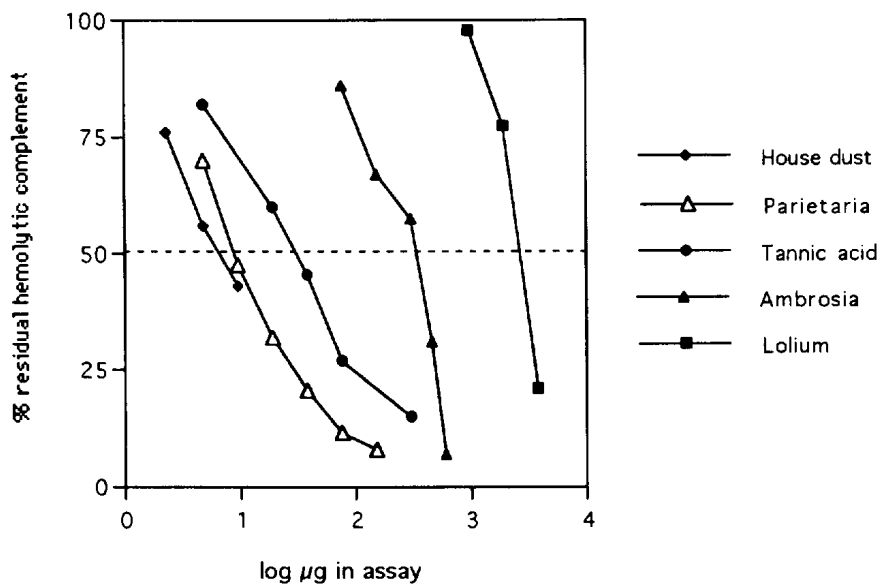


Fig. 1

Dose-response curves for huC- inactivation by the >10 kDa components in aqueous extracts of some representative plant pollen species and by (walnut) tannin, assayed in a standard haemolytic microassay, using the serum of a non-pollinosis asthma patient.

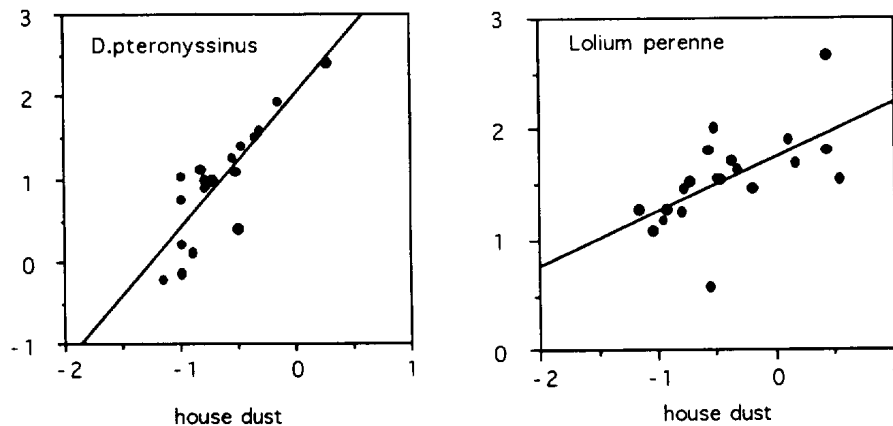


Fig. 2

Double logarithmic plots of doses in  $\mu\text{g}$  for 50% reduction of haemolytic complement (both axes) by (left): the >10 kDa components in aqueous extracts of *Dermatophagoides pteronyssinus* compared to highly purified house dust allergen (# 72E) in a standard assay using the sera of 19 individual mite-sensitive patients with allergic rhinitis or bronchial asthma but no pollinosis, and (right): the allergenic components in aqueous extracts of *Lolium perenne* compared to house dust allergen # 72E using the sera of 20 individual pollinosis patients with allergic rhinitis but no allergy to mites. The regression equations for the scatter plots were (left):  $y = 1.61x + 2.02$  for *D.pteronyssinus*/house dust ( $r = 0.85$ ,  $P < 0.0001$ ) and (right):  $y = 0.49x + 1.78$  for *Lolium perenne*/house dust ( $r = 0.61$ ,  $P = 0.005$ ).

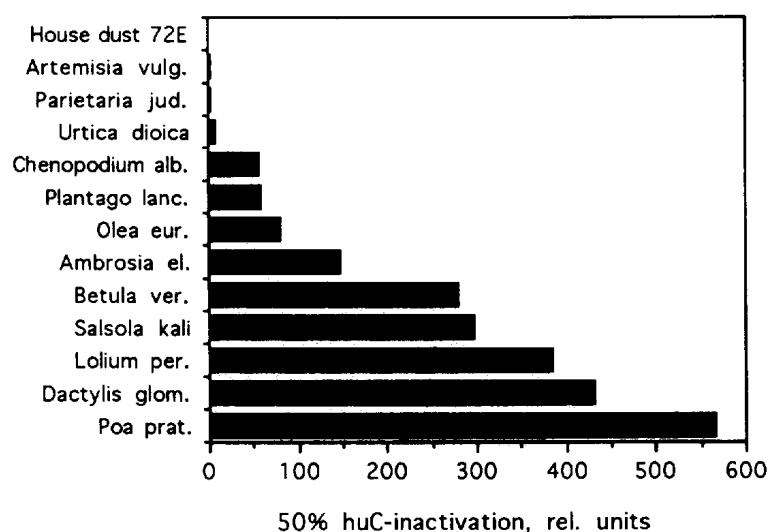


Fig. 3

Complement inactivating potential of the >10 kDa components in aqueous extracts of a series of plant pollen species in relation to a standard house dust allergen preparation, # 72E, determined in a standard haemolytic microassay system and using the same serum of a non-pollinosis asthma patient for all preparations.

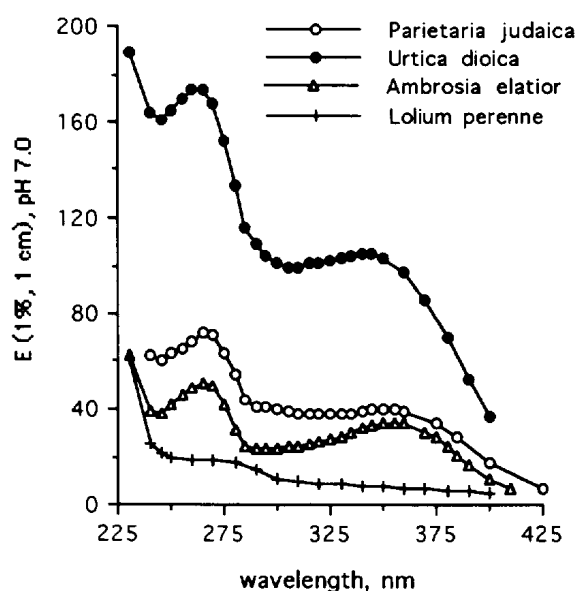


Fig. 4

Ultraviolet absorption spectra of the >10 kDa components in aqueous extracts of some representative pollen species. Solutions of lyophilized preparations made up in 0.01 M phosphate buffer pH 7.0.

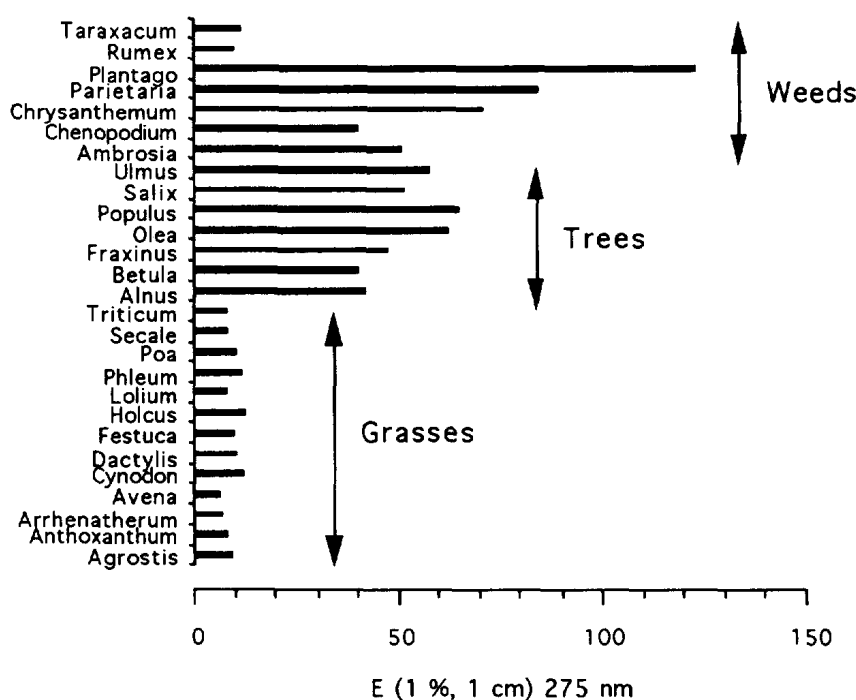


Fig. 5

Extinction coefficients  $E_{1\%,1\text{ cm}}$  at the main "protein" peak 275 nm of the >10 kDa components in aqueous extracts of various different species of plant pollen.

The ratio of the huC-inactivating potencies of these various preparations remained the same using normal human serum or the sera of individual patients with allergic disorders, including pollinosis, as illustrated with the examples in Fig. 2. The results therefore again point towards a non-antibody mediated phenomenon, which was corroborated by the remarkable C-inactivating potency of the non-allergenic *Urtica* pollen extract (11). The non-protein substance tannic acid was likewise found to be a potent C-activator (Fig. 1), as also described for the polyphenolic compounds chlorogenic acid (6), plicatic acid (7), rutin (6, 12) and several flavonols (13).

For the purpose of comparison, the relative potencies for 50% huC-consumption calculated from the dose-response curves for a series of different pollen products are collectively shown in Fig. 3. The diagram includes the complement-inactivating power of the >10 kDa constituents in pollen extracts of *Urtica dioica*, which is known to cause only few allergies in the Mediterranean area and not to crossreact with the pollen allergens of the botanically related *Parietaria* spp (11).

To understand these results, we first focused attention on the presence of pigmented material as a conspicuous common factor among most plant pollen extracts. More particularly, polyphenolic flavonoid pigments are known to abound among aqueous extracts of pollen, especially those of the weeds and trees, even after the usual processes of defatting with organic solvents and extensive dialysis through >10 kDa cut-off membranes (12,14,15). Indeed, despite the removal of free flavonoid aglycones and water-soluble flavonoid-glycosides by these procedures, the >10 kDa purified proteins from most pollen extracts continue to expose a high proportion of firmly complexed flavonoids. Such flavonoid components are revealed by their UV-absorption maxima in the 300 - 400 nm range, as illustrated with the examples of Fig. 4.

Dependent on their precise structure, polyhydroxylic flavonoids conjugated chemically or adsorbed physically to proteins not only give rise to new maxima in the 325-375 nm region (peaks II), but also contribute appreciably to UV-absorption at 270-280 nm, the main protein peak I (14,16). As a consequence, the determination of protein concentration in pollen allergenic extracts based on 275 nm peak I absorption often leads to overestimations, especially with the allergenic pollen proteins of the weeds and trees (17). Some pertinent examples of peak I distortion by complexed flavonoids are collectively shown in Fig. 5.

### Discussion

The present investigations demonstrate that the *in vitro* anticomplementary potential of the proteins in aqueous plant pollen extracts depends on the pollen species. Whereas we were able to confirm that the pollen of the Gramineae exhibit virtually no activity (1,8), we have now found that the >10 kDa proteins in extracts of *Parietaria*, *Urtica* and *Artemisia* pollen are almost equally potent complement activators as the commonly used house dust allergen preparations (2,3,10), while the proteins of other weed and tree pollens occupy intermediate positions (Figs 1 and 3). Similar to previous observations with non-pollen allergens (1,3,10), the ratio of the huC-inactivating potencies of individual pollen extracts remained independent of the human serum source (Fig.2), although in absolute terms complement sensitivity differed among individual sera (9,10). The same observations have been recorded using a series of the common allergens acting on complement in mouse serum (18). The phenomenon of haemolytic complement inactivation therefore seems to be neither related to allergenicity *in vivo*, nor to allergen-specific IgE- or IgG- antibody binding, other antigen-antibody complexes or aggregated IgG *in vitro* (1,3,8-10, 18). The point is further sustained by the observation that the pollen extracts of *Urtica dioica* and *Parietaria judaica* were almost equally potent C-activators (Fig. 3), whereas in the Mediterranean countries the former only rarely causes allergen-specific sensitization (11).

Comparison of the UV-absorption spectra of the >10 kDa components in the highly allergenic *P. judaica* extracts versus the non-allergenic constituents of *U. dioica* (Fig.4) suggests that the impressive huC-inactivating property of these preparations is related to the abundance in both extracts of polyhydroxylated flavonols and/or their tanninic polycondensates. Hydrolyzable or condensed tannins of polyphenolic composition (like the ellagic and tannic acids), as well as their constituent monomers indeed are known activators of both the complement (C1) and the contact phase (coagulation Factor XII) cascades (6, 7, 12, 13, 19). Such polyphenolic substances exhibit the characteristic tendency of forming tight complexes with proteins by hydrogen bonding and other physical forces (20). The most likely candidates for the observed property of huC-inactivation by plant pollen extracts therefore comprise the spectroscopically distinguishable flavonoids firmly complexed with, or chemically conjugated to, the constituent allergenic or non-allergenic pollen proteins (Figs 4 and 5).

Close inspection of the UV-absorption spectra of the individual pollen proteins reveals that the associated flavonoids are qualitatively dissimilar, despite almost equal potencies in huC-activation. Thus, the maximum at 355-360 nm observed with the *Parietaria* proteins (Fig.4) is consistent with the oxidation-prone 3',4'-biphenolic flavonols of the quercetin type, whereas the maximum at 340-345 nm in the *Urtica* extract indicates a prevalence of monophenolic 3'-flavonols of the apigenin type. In contrast, the powerful huC-activating protein components in the pollen extracts of *Artemisia vulgaris* are more likely to be combined with hydroxylated flavanones of the naringenin class with a peak II maximum at 325-330 nm. Hence, the precise chemical nature of the flavonoid conjugates may not be quite relevant for complement engagement. Rather, the phenomenon seems to depend on some non-immunological physicochemical interference with colloid stability among the protein complexes instrumental in the initiating phases of the complement (C1) and intrinsic coagulation systems (Factor XII-prekallikrein).

Even though the role of complement in clinically manifest allergic disease *in vivo* is still controversial, the conclusion that complement activation by allergenic pollen extracts *in vitro*

represents an immunologically unspecific event does not necessarily imply that the phenomenon has no physiological relevance. The complement and contact phase systems have been implicated in mediating histamine release and other allergen-induced *in vivo* mechanisms (21,22). In this connection, further studies are especially warranted of the generally higher *in vitro* allergen-sensitivity of complement in the sera of predisposed, so-called "atopic" individuals (9,10).

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