CMLS, Cell. mol. life sci. 53 (1997) 275–278
1420-682X/97/030275-04 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Haemolytic complement consumption by *Parietaria* pollen extracts in relation to peptide-bound flavonoids

L. Berrens* and B. de la Cuadra López

Research Group C.B.F. LETI, S.A., Calle del Sol 5, Polígono industrial Norte, E-28760 Tres Cantos, Madrid (Spain), Fax +34 1 804 0919
Received 12 November 1996; received after revision 12 December 1996; accepted 17 December 1996

Abstract. Inhaled allergens from house dust, mites or animal danders activate human complement in vitro by engaging the C1-component through a non-antibody-dependent mechanism. These earlier findings are extended by showing that the allergenic components in extracts of *Parietaria* pollen are almost equally potent complement activators as those from house dust or mites. Spectroscopic evidence indicates that haemolytic complement consumption by the *Parietaria* allergens and their enzymic fragments is most likely related to post-translational side-chains comprising flavonoid derivatives. These adsorbed and/or peptide-bound tannin-like structures may also explain the exceptional stability of the high- and low-molecular mass allergenic components in *Parietaria* pollen extracts.
Key words. Complement; allergens; pollen; *Parietaria*; flavonoids.

Attention has been drawn in the past to the in vitro consumption of haemolytic complement in human serum (huC) by Type I inhaled allergens from house dust, human and animal epithelia, dust mites, moulds, etc. [1]. Complement inactivation at high serum dilution occurs mainly by engagement of the component C1 of the classical pathway [1], although in undiluted serum involvement of the alternative pathway through the C3-amplification loop has been observed as well [2]. The effect of allergens on C1 is known not to be mediated by antibodies, but the participation of unidentified serum proteins has been postulated [3, 4]. The fact that the complement consuming potencies in series of different allergens arrange themselves in a fairly constant manner independent of the serum source [5] has strengthened the opinion that allergen-complement interaction represents an immunologically nonspecific phenomenon. However, complement in individual human sera may differ in susceptibility to allergen-induced inactivation, the most sensitive sera in a statistical sense being those from so-called 'atopic' donors [2, 4].

The allergens extractable from house dust are by far the most potent complement activators, while those from animal danders, mites and moulds exhibit progressively less activity [3, 6]. At the lower end of the scale, the grass pollen allergens invariably failed to consume complement in a physiologically significant dose range [1, 5]. Recent studies have nevertheless confirmed some earlier observations on the activation of complement by extracts of ragweed pollen [2]. Since traditionally produced ragweed pollen allergens, like those from other weeds, differ from the grass pollen allergens in having a high proportion of protein-associated chromophores [7–10], we decided to probe the possible complement consuming properties of pollen extracts of the Mediterranean weed *Parietaria judaica*.

* Corresponding author.

Materials and methods

Dried and defatted pollen grains of *Parietaria judaica* (Biopol, Spokane, USA, lot #51414, collected in Europe in 1994, purity as stated less than 0.1% foreign pollen and about 6% plant parts) were extracted twice with distilled water for 2 h at room temperature. The combined and centrifuged extracts were dialysed in the cold from Visking tubing (nominal cut-off 10 kDa). Both the non-dialysable >10 kDa components (HMW-N) and the <10 kDa dialysate (LMW-N) were dried by lyophilization. Chromophoric substances physically adsorbed to HMW-N were then eliminated by repeated dialysis of solutions of HMW-N in 0.01 N HCl, followed by neutralization and freezedrying to give the 'depigmented' proteins HMW-D. The <10 kDa components thus removed at pH 2, representing 46% by weight of HMW-N, were also recovered by lyophilization after neutralization.

The allergenic activity of the preparations HMW-N and HMW-D was established by skin prick tests in *Parietaria*-allergic pollinosis patients. All preparations were also assayed for specific IgE-binding by inhibition assays in vitro, using the sera of *Parietaria*-allergic patients.

For enzymic fragmentation, a sample of HMW-D was dissolved in a suspension of pepsin immobilized on 4% cross-linked agarose (Sigma, USA) at a proportion of 2:1 w/w, and the mixture in HCl at pH 2 was stirred for 6 h at 37 °C. The reaction was stopped by neutralization

with NaOH and the enzyme particles were removed by filtration (0.2 μm). For eliminating salts and <1 kDa fragments, the digest was passed through a Minitan® tangential flow system equipped with a 1 kDa filter. The >1 kDa retentate was finally dialysed again by Minitan passage through a 10 kDa filter, thus separating the >10 kDa endo-fragments A and the >1 kDa/<10 kDa fragments B. Fragments A and B were dried by lyophilization, with a recovery from HMW-D of 57% and 26%, respectively.

Lyophilized preparations 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. Flavonoid reference compounds were purchased from Carl Roth AG (Karlsruhe, Germany).

Blood serum samples containing allergen-specific IgE-antibodies from patients clinically allergic to *Parietaria* pollen or to *Dermatophagoides pteronyssinus* were obtained from various hospitals in Spain. Individual samples were stored in small aliquots at −20 °C for no longer than 4 weeks before use.

Complement was measured in a CH50 haemolytic assay in microtiter modification of the method previously described [1, 4, 6]. 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 7.4×10^8 cells/ml. Dose-response curves were obtained by preincubating serial dilutions of reconstituted proteins and derivatives with patient serum diluted 1:30 to 1:50 in Ca^{++}/Mg^{++}-containing veronal-saline buffer (VSB^{++}) for 30 min at 37 °C. Residual haemolytic complement was evaluated in a second incubation step, using the EA detection system described above and the appropriate controls. Results from dose-response curves were expressed as percentage consumption of available haemolytic complement [1, 4, 6].

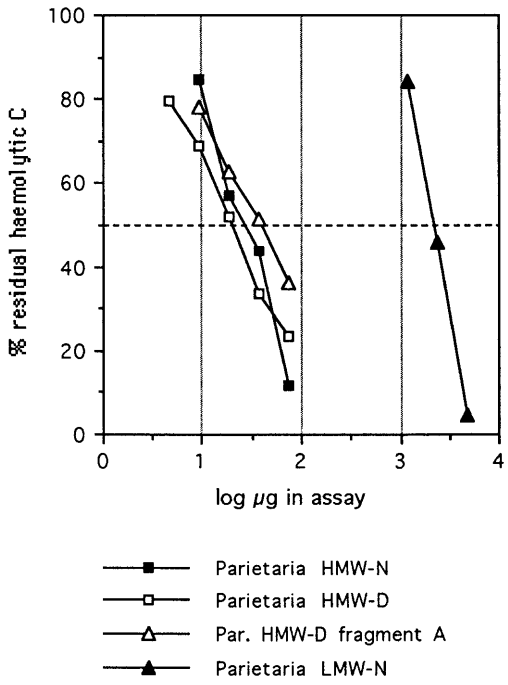

Figure 1. Dose-response curves for haemolytic C consumption by various *Parietaria* pollen preparations, detected after the pre-incubation step for 30 min at 37 °C, by microplate assay using 7.4×10^8 sensitized sheep red blood cells EA/ml for detection. The 1:50 diluted serum of a patient sensitive to *D. pteronyssinus* was used in the above example, but normal human serum and serum of *Parietaria*-allergic patients provided the same potency ratios.

Results

Using the serum of normal persons and of mite- or *Parietaria*-allergic patients we were able to confirm that purified house dust allergens are potent complement activators [1, 3, 4–6], whereas on a weight basis the grass pollen allergens (represented by *Lolium perenne* in fig. 1), as well as the natural <10 kDa *Parietaria* components, were the least active. Surprisingly, however, the >10 kDa allergens from *Parietaria* pollen extracts, either unheated or heated at 100 °C, were about as active on huC as the allergens of *D. pteronyssinus*, i.e. only 5–10 times less potent than a standard house dust preparation (HMW-N in fig. 1). Furthermore, the huC-consuming potential of the 'depigmented' *Parietaria* protein allergens HMW-D remained undiminished relative to HMW-N despite the removal of a large proportion of strongly adhering pigmented material. More important, prolonged digestion with pepsin, though causing appreciable hydrolysis of the allergenic proteins, left a pepsin-resistant core of >10 kDa components with undiminished huC-consuming power (fig. 1).

In trying to understand these findings, we first focused on a conspicuous common factor among the huC-consuming *Parietaria* protein preparations, i.e. the abundance of chromophores resisting elimination by physical or enzymic means (fig. 2). Comparison of the UV-absorption spectra with published data [7, 9, 10] and with those of pure flavonoids [11] suggested that the prominent 350 nm peak (at pH 2 or 7, shifting towards 395 nm at pH 10), is due to flavonol derivatives of the quercetin type (fig. 2). In agreement with this, the reaction of the *Parietaria* preparations with $FeCl_3$ for *o*-biphenols was positive, producing a deep-green colour. Flavonoids and other polyphenols from plant tissues are indeed known anticomplementary agents [12, 13]. However, the water-soluble natural LMW-N flavonol-glycosides were virtually inactive on huC (fig. 1), while the activity of the desorbed pigmented components similarly was low (not shown). It is likely, therefore, that the flavonols active on huC are in the form of their aglycones chemically bound to side-chains of proteins or peptides.

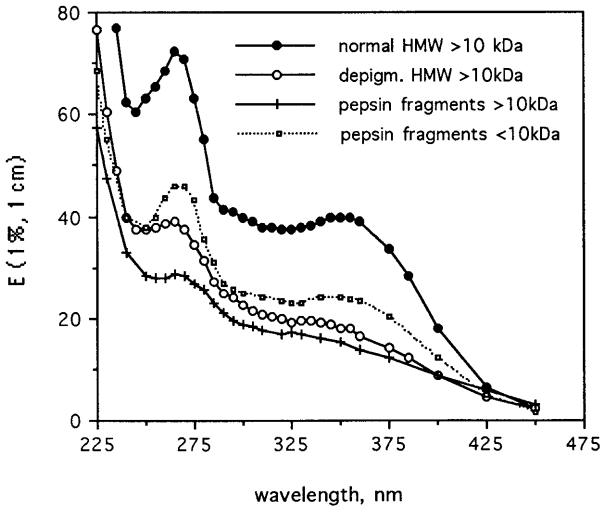

Figure 2. UV-absorption spectra of various preparations from *Parietaria judaica* pollen (see Materials and methods).

Discussion

A practical issue not encountered with other allergenic pollens is that, owing to morphological characteristics, the pollen of the *Parietaria* spp. cannot be collected free of green plant parts, i.e. from the anther. All published reports on *Parietaria* allergens mention a proportion as high as 25% of non-pollen contamination of the granules used for allergenic extract preparation. Pure pollen do not contain the enzyme polyphenoloxidase, whereas other plant parts do [14]. Together with the high content of flavonoid pigments in *Parietaria* pollen [8–10], the conditions during aqueous extraction therefore favour the oxidative generation of polycondensates of amino acids, peptides or proteins with chemically conjugated flavonoids, resulting in tannin-like derivatives with low to high molecular mass [15, 16]. Indeed, the reported tan colour, fluorescence and oxygen-scavenging properties of the *Parietaria* allergens persist even to the point of the firmly conjugated chromophores detected in the isolated 'major' allergen Par j1 [10]. Our results suggest that the property of huC-inactivation may be related to these flavonoid-peptide derivatives, but not to the free flavonoids or flavonoid-glycosides (LMW-N). In this respect, the situation is reminiscent of the skin-active protein-chlorogenic acid adducts from tobacco leaf and of the polyphenolic low-molecular allergen plicatic acid, both of which likewise engage complement through the C1 component [12, 17].

The observation that the activities of HMW-N, HMW-D and pepsin fraction A remained virtually unchanged despite the removal of a considerable proportion of pigments during their preparation, indicates that the desorbed or hydrolysable <10 kDa fragments, though less active on a weight basis, also contribute to the huC-consuming power of *Parietaria* pollen total extracts. The spectroscopic evidence indeed indicates that polyphenolic structures are found in a wide and diffuse range of high- and low-molecular mass components. It may therefore be presumed that it is the density of the conjugated flavonoids on the (poly)peptide carrier molecules which determines the complement-consuming power of any isolated fraction. Such a distribution pattern has in like manner been commented on in relation to IgE-binding and skin-reactivity [9, 15, 16], and was fully confirmed with respect to specific IgE-binding by our *Parietaria* pollen preparations during concurrent studies reported elsewhere [18].

Acknowledgement. The *Parietaria* peptic fragments were kindly made available by Dr. M. T. González Romano (C.B.F. Laboratories, Tres Cantos, Spain).

1 Berrens L., Van Rijswijk-Verbeek J. and Guikers C. L. H. (1976) Characteristics of complement consumption by atopic allergens. Immunochemistry **13:** 367–372
2 Hidvégi T., Schmidt B., Varga L., Dervaderics M., Lantos A., Gönczi Z. et al. (1995) In vitro complement activation by ragweed allergen extract in the sera of ragweed allergic and non-allergic persons. Immunol. Lett. **48:** 65–71
3 Berrens L., Guikers C. L. H. and Van Dijk A. G. (1979) Studies on serum factors mediating complement consumption by house dust allergens. Monogr. Allergy **14:** 150–154
4 Berrens L. (1975) Laboratory diagnosis of the atopic condition: Evaluation of a possible method based on human serum complement consumption. Acta Allergol. **30:** 169–183
5 Berrens L. and Guikers C. L. H. (1975) Value of fluid phase complement consumption as a means of in vitro allergen standardization. In: WHO-IABS Symposium on the Standardization and Control of Allergens administered to Man. Devel. Biol. Stand., vol. 29, pp. 235–248, Karger, Basel
6 Berrens L., Hénocq E. and Koers W. J. (1974) The specificity of animal dander allergens : a semi-blind clinical and experimental study. Acta Allergol. **29:** 10–24
7 Goldfarb A. R., Moore D. H., Rapaport H. G., Sklarosfky B., Gettner, H. H. and Abramson H. A. (1954) Preparation and properties of a purified ragweed extract. Proc. Soc. Exp. Biol. Med. **85:** 255–258
8 Serafini U. (1957) Studies on hay fever. With special regard to pollinosis due to *Parietaria officinalis*. Acta Allergol. **11:** 3–27
9 Geraci D., Oreste U. and Ruffilli A. (1978) Purification and characterization of allergens from *Parietaria officinalis* pollen. Immunochemistry **15:** 491–498
10 Polo F., Ayuso R. and Carreira J. (1991) Studies on the relationship between structure and IgE binding ability of *Parietaria judaica* allergen I. Mol. Immunol. **28:** 169–175
11 Markham K. R. and Mabry, T. J. (1975) Ultraviolet-visible and proton magnetic resonance spectroscopy of flavonoids. In: The Flavonoids, Ch. 2, Harborne J. B., Mabry T. J. and Mabry, H. (eds), Chapman and Hall, London
12 Koethe S. M., Nelson K. E. and Becker C. G. (1995) Activation of the classical pathway of complement by tobacco glycoprotein (TGP). J. Immunol. **155:** 826–835
13 Cimanga K., De Bruijne T., Lasure A., Van Poel B., Pieters L., Van den Berghe D. et al. (1995) In vitro anticomplementary activity of constituents from *Morinda morindoides*. J. Natural Prod. **58:** 372–378
14 Stanley R. G. and Linskens H. F. (1974) Pollen. Biology, Biochemistry, Management. Springer Verlag, Berlin, Heidelberg, New York
15 Bolzacchini E., Di Gennaro P., Di Gregorio G., Rindone B., Falagiani P., Mistrello G. et al. (1991) Allergenic fragments in *Parietaria judaica* pollen extract. Chromatogr. **542:** 337–344
16 Feo S., Cocchiari R. and Geraci D. (1980) Allergens of *Parietaria judaica* pollen. I. Purification and characterization of a hapten and a low molecular weight allergenic peptide. Mol. Immunol. **17:** 1019–1024

17 Chan-Yeung M., Giclas P. C. and Henson P. M. (1980) Activation of complement by plicatic acid, the chemical compound responsible for asthma due to Western Red Cedar (*Thuja plicata*). J. Allergy Clin. Immunol. **65:** 333–337

18 González Romano M. L., Gallego M. T. and Berrens L. (1997) Extraordinary stability of IgE-binding *Parietaria* pollen allergens in relation to chemically bound flavonoids. Mol. Immunol., in press

.