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ISOLATION AND CHARACTERIZATION OF A MAJOR CROSS-REACTIVE GRASS GROUP I ALLERGENIC DETERMINANT

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Abstract—A series of peptides has been isolated from the Group I (GpI) allergen from five species of grasses currently under study in this laboratory. These peptides were generated by performing a limited tryptic digestion on each extensively reduced and alkylated GpI allergen and fractionating the digest by molecular sieve, ion-exchange and HPLC reverse-phase chromatography. Material in the elution profiles was assayed with a monoclonal antibody known to cross-react with all GpI allergens in this study. In addition, this particular monoclonal antibody has been previously shown to inhibit the ability of human IgE to bind to these GpI allergens. The peptides identified and isolated in this way were found to be highly homologous to one another.

INTRODUCTION

The antigenic structure of grass GpI antigens is being investigated to establish the molecular basis of cross-reactivity and to characterize epitopes which account for the antigenic and allergenic properties of these molecules. Previous reports of the antigenic structure of GpI antigens isolated from five grass pollen species have demonstrated that these molecules possess both cross-reactive and unique epitopes (Esch and Klapper, 1986). These epitopes were identified using panels of hybridoma derived monoclonal antibodies (mAbs) raised against the five GpI antigens. Anti-GpI mAbs, which block the binding of human IgE antibodies ("blocking mAbs"), were used to identify allergenically important sites on the GpI molecules. One of these sites, designated site A, was localized to a 15,000 mol. wt fragment of GpI generated by CNBr cleavage (Esch and Klapper, 1989). In addition, site A was determined to be a cross-reactive antigenic determinant, since the anti-site A mAbs reacted with the five different GpI antigens. Furthermore, the anti-site A mAbs each blocked approximately 25% of human IgE binding to the five GpI antigens. The potential importance of this site in terms of the allergenic structure of GpI antigens was the rationale for aiming to define this epitope more precisely. In this manuscript, the localization of site A on immunoreactive GpI peptide fragments generated by controlled trypsin digestion is reported. The amino acid sequences of homologous peptides from the GpI antigens were determined and comparisons of the

sequences confirm earlier evidence (Esch and Klapper, 1989) that site A represents a highly conserved region of the GpI molecules.

MATERIALS AND METHODS

GpI antigens (the 29,000–31,000 mol. wt major allergen) from *Festuca elatior* (*Fes e I*), *Lolium perenne* (*Lol p I*), *Poa pratensis* (*Poa p I*), *Anthoxanthum odoratum* (*Ant o I*) and *Agrostis alba* (*Agr a I*) were purified by a combination of gel filtration and ion-exchange chromatography as previously described (Esch and Klapper, 1986).

Enzymatic cleavage

GpI antigens were reduced and alkylated prior to trypsin digestion. Extensive reduction was carried out in 7 M guanidine-HCl, 0.1 M Tris, pH 8.6, and 0.05 M dithioerythritol at 37°C for 1 hr. Alkylation was carried out using excess iodoacetic acid in the dark for 1 hr at room temp. The reaction mixture was dialyzed against 0.5% ammonium bicarbonate, aliquoted and lyophilized. Citraconylation was carried out on the reduced and alkylated GpI antigens using the procedure described by Atassi and Habeeb (1972). Reduced and alkylated or citraconylated GpI antigens were reconstituted in 1% ammonium bicarbonate, pH 7.8, and digested with trypsin (TPCK treated; Worthington), at an enzyme:protein ratio of 1:50, for 4 hr at 37°C. The reaction was terminated by the addition of soybean trypsin inhibitor (Sigma, St Louis, MO).

Sera

Mouse antibodies specific for each of the five purified GpI antigens were prepared by immunizing at least four female BALB/c mice (Jackson Labora-

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tories, Bar Harbor, ME) i.p. with 100 µg of GpI antigen in Freund's complete adjuvant. The mice were boosted at 3- to 5-week intervals with the same material and bled 3-7 days after each booster injection. The sera obtained were pooled and the titers determined by direct binding ELISA.

Monoclonal anti-site A mAbs

The preparation and properties of the monoclonal antibodies were described previously (Esch and Klapper, 1986, 1989). MAb MF21.4 was produced from a hybridoma cell line derived from a fusion using spleen cells from a BALB/c mouse hyperimmunized with *Fes e I* and mAb PR16.9 from a mouse hyperimmunized with *Lol p I*. Both mAbs were derived from fusions employing 45.6TG1.7 BALB/c plasmacytoma cells (a gift of D. Scott, Rochester University, Rochester, NY) as the spleen cell fusion partner. The monoclonal antibodies were purified from hybridoma cell culture supernatants by ion-exchange chromatography (Klapper *et al.*, 1986).

ELISA

Experiments for evaluating the immunoreactivity of GpI antigen cleavage products were performed by the ELISA using techniques described by Voller *et al.* (1980) and Esch and Klapper (1989). In the direct binding ELISA, digests of GpI antigens or isolated peptide fractions were diluted in coating buffer and allowed to adsorb to PVC microtiter plate wells for 6-16 hr. The wells were washed three times with PBS-Tween and a dilution of an anti-GpI antibody preparation was added to the wells. An alkaline phosphatase-labeled goat anti-mouse IgG (Sigma, St Louis, MO) was used as the second antibody. After 3 hr of incubation and washing the wells with PBS-Tween, the enzyme substrate, *p*-nitrophenyl phosphate, was added in diethanolamine buffer. The absorbance was read at 405 nm using a Biotek automated ELISA reader (Biotek, Burlington, VT). In the competitive inhibition ELISA, various dilutions of GpI digests or isolated peptides were pre-mixed with an anti-GpI antibody preparation and added to microtiter plate wells coated with native GpI antigen. Antibody binding was detected using an alkaline phosphatase-labeled goat anti-mouse IgG, as described above, and the percentage of inhibition of binding was calculated as follows:

$$\% \text{ inhibition} = [(A - B) / A] \times 100,$$

where A is the absorbance at 405 nm in the absence of inhibitor and B is the absorbance in the presence of inhibitor.

Isolation of site A peptides

Tryptic fragments possessing site A were isolated from the GpI antigen digests by sequential gel filtration, anion-exchange and C18 reverse-phase HPLC procedures. Prior to fractionation, the citraconyl blocking groups were removed by lowering

the pH to 4.5 using acetic acid and incubating at 37°C. Deblocking of lysines was monitored by the recovery of antigenic reactivity as measured by competitive inhibition ELISA. Maximal recovery of antigenicity was achieved within 8 hr. The treated digest was lyophilized and stored at -20°C. The deblocked peptides, reconstituted with 0.5% ammonium bicarbonate, pH 7.8, were applied to a small (1.6 × 30 cm) Sephadex G50F column (Pharmacia, Piscataway, NJ) and eluted with the same buffer. Fractions containing cleavage products reacting with the anti-site A mAbs were pooled and applied to a small (0.6 × 10 cm) DEAE-Fractogel column (EM Sciences, Hawthorne, NY). Elution was carried out using an *N*-ethylmorpholine pyridine formic acid gradient as previously described in detail (Klapper and Capra, 1976). Site A peptides were further purified on a 300 Å pore size C18 reverse-phase HPLC column using a 20-50% acetonitrile gradient in 0.01% TFA (see Figs 2-4).

Amino acid sequence determination

Citraconylated tryptic peptides which retained antibody binding activity were sequenced using an Applied Biosystems (Foster City, CA) model 470A protein-peptide gas-phase sequencer with standard protocols. PTH amino acids were identified using an on-line Waters (Milford, MA) gradient HPLC with NOVA-PAK C18 reverse-phase column and detection at 254 nm. Amino acid analysis (Waters Pico-Tag method) of the peptides prior to sequence analysis suggested that the peptides were closely related and the sequence and composition agreed very well.

RESULTS

Figure 1 shows the immunoreactivity of *Fes e I* tryptic digests, as judged by the ability of the digests to inhibit the binding of two anti-site A blocking mAbs to solid-phase native *Fes e I*. Complete trypsin cleavage of the molecule led to the total loss of inhibitory activity. This result is consistent with earlier findings by Johnson and Marsh (1966b) who demonstrated the susceptibility of *Lol p I* antigens to trypsin digestion. In contrast, protection of lysine residues from the action of trypsin by citraconylation generated fragments recognized by the anti-site A mAbs. Although the fragments were capable of greater than 90% inhibition of antibody binding, 3- to 10-fold molar excesses relative to the native MF GpI were required.

The GpI antigen fragments generated by the controlled trypsin digestion procedure were fractionated by sequential Sephadex G50F, DEAE-Fractogel and C18 reverse-phase HPLC steps. Figure 2 shows a representative experiment with *Fes e I*. Immunoreactive fractions were identified in direct binding ELISAs using both mouse polyclonal anti-GpI antibodies and the anti-site A mAbs. The Sephadex

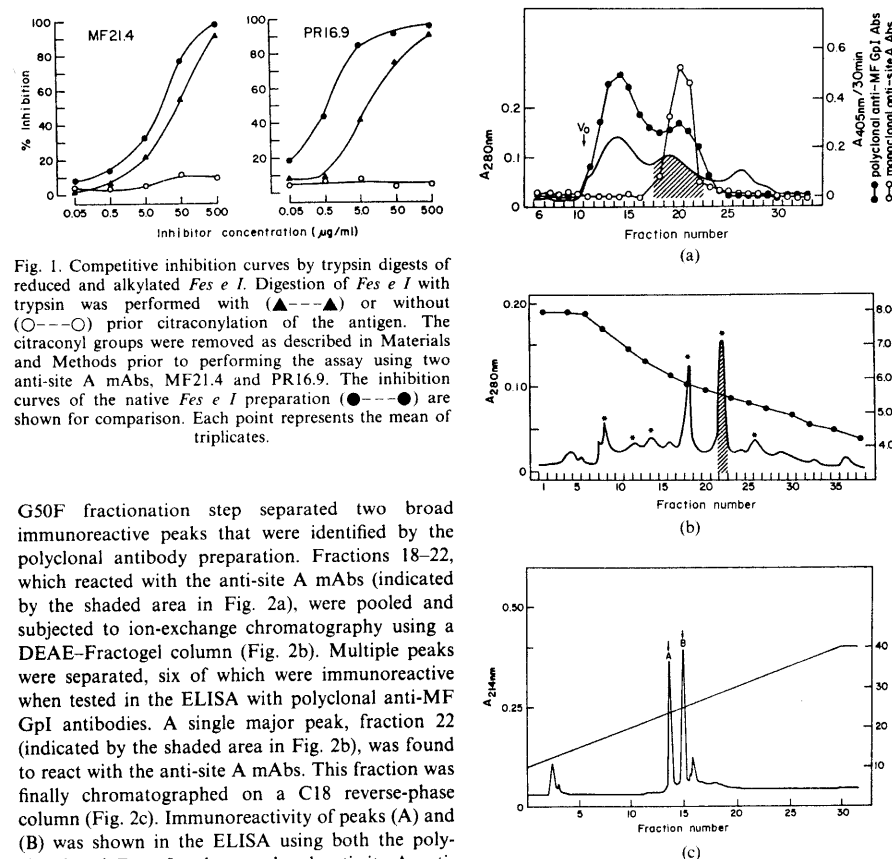


Fig. 1. Competitive inhibition curves by trypsin digests of reduced and alkylated *Fes e I*. Digestion of *Fes e I* with trypsin was performed with (▲---▲) or without (○---○) prior citraconylation of the antigen. The citraconyl groups were removed as described in Materials and Methods prior to performing the assay using two anti-site A mAbs, MF21.4 and PR16.9. The inhibition curves of the native *Fes e I* preparation (●---●) are shown for comparison. Each point represents the mean of triplicates.

G50F fractionation step separated two broad immunoreactive peaks that were identified by the polyclonal antibody preparation. Fractions 18-22, which reacted with the anti-site A mAbs (indicated by the shaded area in Fig. 2a), were pooled and subjected to ion-exchange chromatography using a DEAE-Fractogel column (Fig. 2b). Multiple peaks were separated, six of which were immunoreactive when tested in the ELISA with polyclonal anti-MF GpI antibodies. A single major peak, fraction 22 (indicated by the shaded area in Fig. 2b), was found to react with the anti-site A mAbs. This fraction was finally chromatographed on a C18 reverse-phase column (Fig. 2c). Immunoreactivity of peaks (A) and (B) was shown in the ELISA using both the polyclonal anti-*Fes e I* and monoclonal anti-site A antibody preparations. The corresponding site A peptides from *Lol p I*, *Poa p I*, *Ant o I* and *Agr a I* were also isolated using an identical protocol. As with *Fes e I*, two anti-site A reactive peptides were isolated from *Lol p I*. In contrast, a single peptide was obtained from *Poa p I*, *Ant o I* and *Agr a I*.

The complete amino acid sequence of the site A peptides from *Lol p I*, *Agr a I* and *Ant o I* and partial sequences of *Fes e I* and *Poa p I* peptides are presented in Table 1. Comparison of the sequences

Fig. 2. Isolation of peptide fragments possessing site A from a tryptic digest of citraconylated *Fes e I*. The site A peptides were sequentially fractionated by (a) Sephadex G50F, (b) DEAE-Fractogel and (c) C18 reverse-phase HPLC as described in Material and Methods. The shaded areas in chromatograms (a) and (b) represent fractions which react with the anti-site A mAbs and were used in the subsequent fractionation step. The asterisks in chromatogram (b) represent peaks reactive with a polyclonal anti-*Fes e I* antibody preparation. The peaks designated (A) and (B) in chromatogram (c) represent the purified site A peptides which were subsequently sequenced (see Table 1).

Table 1. Amino acid sequences of site A peptides isolated from *Lol p I* (CT-PR26A and CT-PR26B), *Fes e I* (CT-MF81A and CT-MF81B), *Agr a I* (CT-RT76), *Poa p I* (CT-J85) and *Ant o I* (CT-SV31)

Peptide	1	10	20	28
CT-PR26A	Y T T E G	G T K S E	V E D V I	P E G W K A D T S Y S A K
CT-PR26B	---	---	F	---
CT-MF81A	---	---	A	---
CT-MF81B	---	---	---	---
CT-RT76	---	A	A	---
CT-J85	---	A	---	V
CT-SV31	---	K	V	A

*IUPAC single letter abbreviations for the amino acid residues are used.

†Dashes represent residues which do not differ from those of the CT-PR26A peptide.

showed a high degree of homology among the peptides, the differences being detected at position 7, 9, 11, 21 and 26.

DISCUSSION

In this study of the antigenic structure of grass GpI antigens, site A, which has previously been demonstrated to be an allergenically important site (Esch and Klapper, 1989), is investigated in some detail. Site A is an epitope defined by two mAbs (MF21.4 and PR16.9) which inhibit the binding of human IgE antibodies to GpI antigens. In the previous study (Esch and Klapper, 1989), site A was characterized as a "continuous" or "sequential" antigenic site and localized to a 15,000 mol. wt CNBr fragment of *Fes e I*. Site A has now been more precisely localized by examining smaller fragments of GpI antigen which still bind the anti-site A mAbs. Site A was lost after complete trypsin cleavage of the GpI molecule as shown by a competitive ELISA (Fig. 1). Complete tryptic digests were also incapable of inhibiting the binding of polyclonal mouse anti-GpI antibody preparations and human IgE antibodies from grass allergic individuals. These results suggested that virtually all of these cleavage fragments lacked immunoreactivity. Reversible citraconylation was employed to prevent cleavage by trypsin at the lysyl peptide bonds. Specific cleavage at arginyl peptide bonds by trypsin of the acylated GpI antigens was accomplished and, after removal of citraconyl blocking groups, the cleavage products retained a significant proportion of the original antigenic activity, as determined by a competitive ELISA using anti-GpI antibody preparations. Most importantly, immunoreactivity toward the anti-site A mAbs was preserved (Fig. 1). These experiments suggested that peptide fragments possessing site A were generated by the controlled tryptic digestion and that isolation and characterization of the active peptides could be pursued. The site A peptides derived from five grass species were successfully isolated by sequential gel filtration, ion-exchange and C18 reverse-phase HPLC procedures. At each fractionation step, column fractions containing immunoreactive peptides were identified by direct binding ELISA using anti-GpI antibody preparations. The *Fes e I* and *Lol p I* antigens each yielded two cleavage products which bound the anti-site A mAbs, while the *Ant o I*, *Agr a I* and *Poa p I* antigens yielded a single immunoreactive fragment. The complete primary sequences of the site peptides from *Lol p I*, *Agr a I* and *Ant o I* and partial sequences of the corresponding peptides isolated from *Fes e I* and *Poa p I* have been determined (Table 1). The destruction of site A by complete trypsin digestion could be explained by the existence of lysine residues within the site. Three such potential trypsin cleavage sites are found in *Fes e I*, *Lol p I*, *Poa p I* and *Agr a I* sequences and four are found in the *Ant o I* peptide sequence. It is of interest

to note that one of these lysines at position 8 falls within a putative antigenic determinant predicted by a Hopp and Woods hydrophilicity index plot (Hopp and Woods, 1983).

Comparison of the sequence data revealed five residues at which substitutions occurred (residues 7, 9, 11, 21 and 26). Residue 11 was the site where differences were noted between the two peptides isolated from either the *Fes e I* or *Lol p I* antigens. It is not yet possible to explain the existence of these pairs of peptides. One possibility is that site A is a repeated site and the pairs of peptides represent different portions of the *Fes e I* or *Lol p I* molecule. The inability to isolate more than one reactive peptide from the *Ant o I*, *Agr a I* and *Poa p I* antigens might be explained by the presence of repeated structures giving rise to a pair of identical peptides. Alternatively, substitution by an arginine residue within the hypothetical second peptide could have made it susceptible to trypsin cleavage and thus undetectable by immunoassay. A second possibility is that the peptides arise from different GpI molecules produced by the same plant. The two distinct peptides may represent corresponding portions of two different GpI molecules encoded for by distinct GpI genes. The first possibility appears unlikely since site A is not a repeated site, based on double bind ELISA results (Esch and Klapper, 1989). There are at least four "isoallergenic" forms of *Lol p I* antigens, differing in pI. The published amino acid analysis of two *Lol p I* isoallergens, I-B and I-C (Johnson and Marsh, 1966a), showed minor differences. It is possible, therefore, that the different peptides isolated from *Fes e I* and *Lol p I* might reflect differences between GpI isoallergens. This question can be answered only after more precise analysis with the purified GpI isoallergens and characterization of the GpI gene structure.

Knowledge of the antigenic structure of allergens is critical for an understanding of their biologic activities and may answer questions about the human immune allergic response (Benjamin *et al.*, 1984). Studies employing mAbs to define the antigenic structures of allergens are only beginning to appear in the literature (Ekramoddoullah *et al.*, 1984, 1986; Olson and Klapper, 1986; Singh and Knox, 1985; Chapman *et al.*, 1985; Standing *et al.*, 1987). As shown in this paper, as well as others (Esch and Klapper, 1986; Singh and Knox, 1985; Chapman *et al.*, 1985; Standing *et al.*, 1987), mAbs are ideal tools to probe the molecular relationships between allergens derived from different sources. The design of appropriate mAb-based immunoassays has also allowed for the identification of allergenically important sites (Esch and Klapper, 1989; Olson and Klapper, 1986). The information gained from these types of studies can be used to define more clearly the immunochemical and molecular basis of cross-allergenicity and should allow for a definition of allergy, based on hypersensitivity to particular epitopes.

Of considerable interest are reports on the use of synthetic peptide vaccines and on carrier-conjugated haptens or univalent fragments in immunotherapeutic regimens (Emini *et al.*, 1983; Chow *et al.*, 1985; Sehon, 1982). The delineation of antigenic determinants of grass GpI allergens is the first step for the implementation of these approaches in treating grass pollen allergy. Additionally, the existence of cross-reactive determinants may allow for a reduction in the number of peptides required for effective treatment. The site A peptides described in this paper are ideal candidates for such studies. Results of scanning polyclonal and monoclonal anti-GpI antibodies on GpI antigenic fragments indicate that several antigenic peptides can be separated (Fig. 2 and unpublished observations). These fragments may also prove useful in future work on GpI antigen structure.

To summarize, an allergenically important site, designated site A, has been localized on GpI antigen fragments generated by controlled trypsin cleavage. Corresponding fragments possessing site A were isolated from GpI antigens derived from five different grass species. The primary sequence of these peptides were compared and found to be highly conserved. Since anti-site A mAbs which bind these cross-reactive peptides are effective blocking antibodies, it is possible that these peptides can be effective in an immunotherapeutic strategy based on the induction of protective blocking antibodies. Studies to evaluate the feasibility of such an approach are presently being investigated.

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