

Characterization of antigens and allergens of date palm (*Phoenix dactylifera*) pollen

Immunological assessment of atopic patients using whole extract and its fractions

Harfi HA, Kwaasi AAA, Tipirneni P, Parhar RS, Graff-Lonnevig V, Al-Sedairy ST. Characterization of antigens and allergens of date palm (*Phoenix dactylifera*) pollen. Immunological assessment of atopic patients using whole extract and its fractions. Allergy 1992; 47: 535–544.

Antigenic and allergenic components of date palm (*Phoenix dactylifera*) pollen were investigated to observe their effects on the skin test reactivity, lymphocyte blastogenesis and cytokine production in atopic and healthy individuals. Date pollen extracts were fractionated using SDS–PAGE and Sephacryl S-200 gel filtration. Western blotting of SDS–PAGE separated components with antiserum raised against whole pollen extract in rabbits revealed at least 22 immunoreactive bands ranging in molecular weight between 12 and 94 kD. The immunogenicity of the pollen extract was further confirmed by strong positive reactions in ELISA and Ouchterlony's double diffusion tests. Immunoblot analyses revealed IgG and IgE reactive components (14–94 kD for IgG and 12–90 kD for IgE) in the skin test-positive patients' sera against whole pollen extract. Fifteen of 60 atopics reacted positively to either whole or some fractions of date pollen extract when skin tested. In response to whole or components of date pollen extract atopic patients showed differential peripheral blood lymphocyte (PBL) proliferative response and cytokine (IL-2, IL-4) production when compared with PBL of normal subjects. Our findings strongly suggest that date palm pollen should be considered a reaginic component and should be included in the battery of allergens for determining the allergic status of atopic patients, particularly in those parts of the world where the date palm is grown commercially.

Clinical aspects

Our observation that date palm pollen extract can evoke strong allergenic reaction in atopic patients has important clinical implications. The patients who showed strongly positive skin test to whole date palm pollen extract, but were otherwise negative to other allergens, are yet another group of atopics requiring further study. The ability of whole date palm pollen extract to induce IL-4 production *in vitro* from atopic patient's sera implies its IgE-inducing potential *in vivo*. We hope a routine use of date palm pollen extract for skin testing in allergy patients will be clinically beneficial.

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Key words: allergens; cytokines; ELISA; immunoblot; *Phoenix dactylifera*.

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Accepted for publication 27 December 1991

Date palm (*Phoenix dactylifera*) is one of the most important indigenous plants in the Arabian Peninsula. Even though it is common knowledge amongst inhabitants of Saudi Arabia that date pollen is a major causative agent of asthma and seasonal allergic rhinitis, and clinicians in some allergy clinics have included date pollen from commercial sources in their allergy skin test panel, there is no published

reference to date pollen antigens or allergens. The plants are dioecious and are therefore hand pollinated, causing large amounts of pollen to be released into the atmosphere. Our aim was to use local date pollen extract to investigate its antigens and probable allergens. Crude pollen extracts were fractionated by SDS–PAGE and Sephacryl S-200 gel filtration and the immunogenicity of their fractions

was assessed by skin prick testing, enzyme-linked immunosorbent assay (ELISA), immunoblotting, lymphocyte proliferation assays, and cytokine production. Allergy clinic patients, date palm pollinators and non-exposed subjects were studied during the pollination months of 1990 and 1991.

Material and methods

All reagents used were of analytical grade and were purchased from Fisher (New Jersey, USA) or from other manufacturers as indicated in the text. New Zealand White Rabbits were bred by the Animal Facility Unit of King Faisal Specialist Hospital and Research Centre, Riyadh.

Date pollen was collected from about 50 male date palm trees during the pollination months (February to April) of 1990 and 1991. The pollen was sieved through a fine mesh to remove all large plant debris. Several samples were examined microscopically and suspensions were made in sterile saline solution (0.9%) and spread onto Czaped Dox Agar plates to assess the degree of contamination by fungal propagules. Batches free of contamination by fungi after 48 h of culture at 37°C were selected for the study.

All samples were weighed and cold acetone (American Scientific, IL, USA) added 1/10 (w/v), and they were incubated with shaking for 16 h at room temperature (25°C) to remove lipids. Acetone was removed by suction, and after drying by vacuum the pollen was washed twice with sterile distilled water. Phosphate-buffered saline (PBS, pH 8.0) was added to the defatted pollen 1/50 (w/v) and incubated with shaking at 4°C for 72 h to extract the proteins. The mixture was centrifuged at 15 000 *g* for 30 min at 4°C and the supernatants collected and pooled. Phenol was added to a final concentration of 0.3% (w/v) and the solution dialysed (MW cut-off 6000) against 6 changes of sterile distilled water for 48 h at 4°C. The dialysate was pre-filtered through Whatman No. 1 filter paper, sterilized through 0.45 µm filter (Nalgene, Rochester, USA) and then lyophilised and either it was reconstituted to 1:10 (w/v) in sterile distilled water and stored at -20°C, or equal amounts of sterile glycerine were added to the aqueous extracts (1/20 v/v) for skin testing.

Analytical procedures

The protein content of the pooled extract was determined by the method of Lowry et al. (7) using bovine serum albumin (Sigma St. Louis, USA) as reference protein. Total carbohydrate content was determined by the anthrone/sulfuric acid method (13) using D-glucose (BDH, Poole, UK) to construct a standard calibration curve.

Antiserum

New Zealand white rabbits, each weighing approximately 2–3 kg, were bled to obtain pre-immune serum samples and then injected subcutaneously at several dorsal sites with 15 mg of crude extract emulsified with Freund's complete adjuvant (FCA). Booster injections were given to the rabbits 2 weeks after the initial injections with 10 mg of extracts emulsified with Freund's incomplete adjuvant (FIA). A third injection was given to each rabbit with 10 mg of extract in PBS (pH 7.4) intraperitoneally. Antiserum was prepared from blood collected by venepuncture from the ears and tested against the whole pollen extract or its fractions by Ouchterlony's double diffusion (DID) method as adapted by Longbottom & Pepys (6). Antisera with satisfactory precipitation results were pooled and the immunoglobulin fractions isolated by the method described by Harboe & Ingild (2).

Human sera

Sera from 15 patients with weak to strongly positive skin test reaction to date pollen extract, 6 unexposed Western expatriate blood donors and 6 date palm pollinators who had been working with date pollen for between 2 and 9 years were prepared from peripheral blood collected by venepuncture during the pollination months and stored at -20°C. Normal cord sera were kindly provided by one of the authors (S.T. Al-Sedairy) from his tumour bank.

Sephacryl S-200 gel filtration chromatography

Sephacryl S-200, high resolution gel (Pharmacia, Uppsala, Sweden) was packed in a 1 m × 0.6 cm column (Pharmacia, Sweden) at a constant pressure of 30 cm water (10, 11). After washing and equilibrating the column in PBS (50 times bed volume), 2 ml of the crude pollen extract (approx. 2.5 mg protein) was applied to the column and elution carried out at a flow rate of 30 ml/h. Elution was monitored at A₂₇₉ (LKB Bromma 2212 Helirac, 2110 Recorder and 2238 Uvicord SII) and fractions (3 ml) collected and pooled as illustrated (Fig. 1a). Freeze drying and desalting was as described by Parsons et al. (10, 11).

SDS-PAGE

Pollen extracts or their separated fractions (500 µg/ml) in equal volume of sample buffer (0.124 M Tris hydrochloride, pH 6.8; 4 mM EDTA; 13.88 mM sodium dodecyl sulfate; 0.59 M sucrose and 0.57 M mercaptoethanol) were boiled for 3 min and separated on a 5% stacking and 12% separating sodium

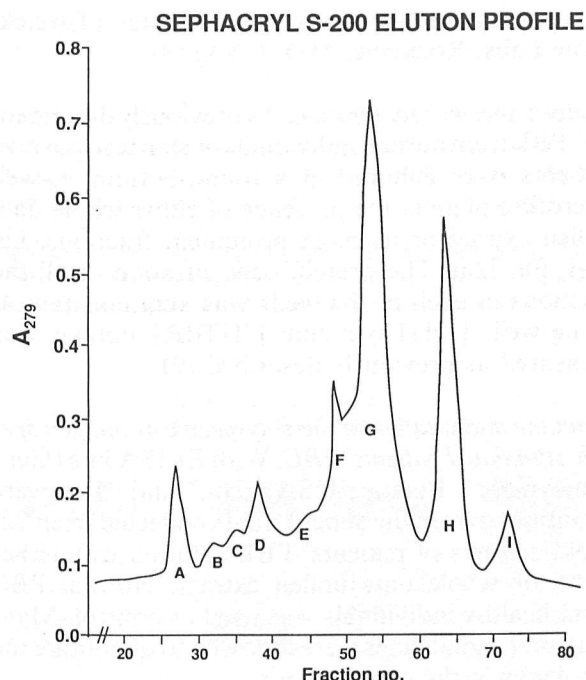


Fig. 1a. Sephacryl S-200 gel filtration profile of crude date pollen extract monitored at A_{279} . Fractions in each of the 9 peaks (A-I) were collected, pooled and characterized. Void volume was 75 ml.

dodecyl sulfate polyacrylamide gel electrophoresis using the discontinuous system described by Laemmli (5). Low molecular weight standards (Pharmacia, Uppsala, Sweden) were included to determine the MW of the separated pollen proteins. Electrophoresis was performed at 60 volts (constant voltage) for 16 h at 4°C. After electrophoresis, gels were either fixed in 7.5% (w/v) trichloroacetic acid in distilled water at room temperature (25°C) for 30 min and stained in 0.10% Coomassie brilliant blue (CBB) dissolved in methanol:acetic acid:deionized water (45:10:45) at room temperature for 4 h, or were used for Western blotting. All CBB-stained gels were destained by shaking in 7% acetic acid.

Western blotting

The protein components of date pollen were transferred onto nitrocellulose membrane (0.45 µm pore size, Bio-Rad, Richmond, CA) according to the method of Towbin et al. (12). The nitrocellulose membrane and 3 mm paper (Whatman, UK) were cut to the size of the gel and equilibrated in Towbin transfer buffer (25 mM Tris pH 8.3, 192 mM glycine and 20% methanol). Transfer was carried out at 30 volts (0.1 A) for 15 h at 4°C using the Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Richmond, USA). At the end of transfer the nitrocellulose sheet was incubated in 3% gelatin (w/v) in Tris-buffered saline (TBS, 20 mM Tris, 500 mM NaCl pH 7.5) for

1 h at room temperature (25°C) on a rocking platform to block non-specific binding sites. The membrane was washed (3 × 5 min) in TBS containing 0.05% Tween-20 (TTBS) and then cut into strips 0.5 cm wide for immunodetection studies. After transfer, all gels were stained with CBB and then destained to ascertain that complete transfer had occurred.

Immunodetection of blotted protein

The nitrocellulose strips were individually placed in 5 ml of the appropriate human or rabbit sera diluted in TTBS. Rabbit antiserum was diluted 1:500 but all other sera required 1:20-fold dilution for IgG and 1:5 for IgE detection. Strips were incubated for 2 h for IgG and 6 h for IgE at room temperature with shaking. After washing (3 × 5 min, TTBS) they were transferred into freshly prepared goat anti-rabbit or goat anti-human alkaline phosphatase labelled IgG or IgE antibody and incubated at room temperature for 2 and 4 h, respectively. Subsequent steps were carried out as described previously (11).

ELISA

The indirect method of Voller et al. (14) was followed: 96-well flat-bottomed polystyrene microtitre plates (Dynatech Laboratories Inc., VA, USA) were coated with 100 µl of crude date pollen extract (100 µg/ml) in carbonate buffer pH 9.6. The plates were kept in a humid chamber at 4°C overnight. After washing 3 times (5 min/wash) with PBS containing 0.05% Tween-20 (PBST) 100 µl of PBST containing 3% (w/v) bovine serum albumin were added to all wells and the plate kept at room temperature for 1 h to block all unbound reactive sites. The wells were emptied and washed as before and filled in triplicates with immune rabbit serum (1:500), sera from 8 date pollen-reactive patients, 6 date palm pollinators and 6 unexposed blood donors diluted (1:5) in PBST/1% BSA. Pre-immune rabbit serum and cord serum were included as negative controls. Plates were kept at room temperature in a humidified chamber for 3 h. All wells were washed and then filled with either anti-rabbit IgG peroxidase conjugate (11000), anti-human IgG (1:1000) or anti-human IgE (1:200) peroxidase conjugate (Sigma). Plates with IgG conjugates were kept at room temperature for 2 h but those incubated with IgE were kept for 4–6 h. All wells were washed with PBST/BSA and then filled with freshly prepared 0.4 mg/ml of o-phenylene diamine (Sigma Chemical Co., St. Louis, MO, USA), 0.02% H_2O_2 in citrate phosphate buffer pH 5.0 and kept in the dark for not more than 30 min at room temperature. The reaction was stopped by addition of 50 µl of 2N H_2SO_4 and

plates read at 490 nm in a Dynatech MR 600 automatic microplate reader (Dynatech Laboratories, Inc.) interfaced with an IBM AT computer which had been programmed to calculate the mean and standard deviation (SD) of all samples read. The mean and SD for the control sera were calculated, and those for the patients and gardeners were compared to the control sera values. Readings were classified on a scale of 0 to 5, and a positive score was considered to be any value above 1. For statistical analyses the reading for triplicate samples of each serum was compared to control sera and reagent blanks on the same plate to exclude plate-to-plate and day-to-day variations.

Allergenicity tests

Skin prick tests. Sixty of the patients that attended the local allergy clinic during the pollination season of 1990 and 1991 and consented to testing were skin tested with the whole crude pollen extract. Fifteen of these patients reacted positively to the extract and 6 of these 15 patients with strong skin reactions (≥ 6 mm wheal) were further tested with the purified fractions, all of which had been adjusted to the same protein concentration (25 $\mu\text{g}/\text{ml}$). PBS and 50% (v/v) glycerol in PBS were included as negative controls and histamine HC (1 mg/ml) as positive control. The diameters of the resulting wheals were measured after 15 min and any erythema and pseudopods noted.

Lymphocyte proliferation tests

Preparation of peripheral blood lymphocytes. 20 ml heparinized peripheral blood was collected from normal healthy young individuals. PBL were isolated from the whole blood by subjection to Ficoll-Hypaque gradient fractionation (9). The PBL were suspended in RPMI 1640, Hepes 25 mM with L-glutamine (Flow Labs, VA, USA) supplemented with 10% heat-inactivated FCS, penicillin (100 U/ml), streptomycin (100 $\mu\text{g}/\text{ml}$) and fungizone (25 $\mu\text{g}/\text{ml}$). Cell counts and cell viability were judged by the blue dye (0.02%) exclusion method. PBL with at least 98% viability were used in the experiments.

PBL activation with phytohaemagglutinin and interleukin-2. 10^5 PBL either from normal subjects or date pollen skin test-positive patients were cultured in quadruplicate in a 96-well microtitre plate in a final volume of 250 μl RPMI-1640 medium containing 5 $\mu\text{g}/10^6/\text{ml}$ of PHA or 100 U IL-2 (Genzyme, MA, USA) for 72 h. The proliferation response was measured from the [^3H] thymidine [$^3\text{HTdR}$] uptake after an 18 h pulse (1 $\mu\text{Ci}/\text{well}$ sp activity 6.7 mCi/mM) as given by the β counts of

cultures harvested with a cell harvester (Titretek, Flow Labs, Rockville, MD, USA) (9).

Antigen-specific proliferation. As previously described, 10^5 PBL from normal individuals or skin test-positive patients were cultured in a round-bottom 96-well microtitre plate in the presence of either whole date pollen extract or its most prominent fractions, i.e. A–I, for 72 h. The protein concentration of all the fractions in each of the wells was kept constant at 25 $\mu\text{g}/\text{well}$. [^3H]Thymidine [$^3\text{HTdR}$] uptake was measured as previously described (9).

Cytokine quantitation in the polyspecific or antigen specific stimulated patients' PBL. With ELISA kits (Genzyme Corp., Boston, USA), IL-2 and IL-4 were quantitated from the supernatants collected from 72-h-old cultures of patients' PBL cultured with either PHA or whole date pollen extract. Normal PBL from healthy individuals was used as control. Manufacturer's guidelines were followed to quantitate the cytokines in the supernatants.

Statistical evaluation

To evaluate the significance of differences in PBL proliferation, cytokine production or ELISA one-way analysis of co-variance was employed. Student's t-test was used to calculate the differences between means of data under the different experimental conditions. A difference was considered significant at p values ≤ 0.05 .

Results

The pollen grains that were viewed microscopically appeared homogeneous and had typical date pollen morphology (8). There were no contaminating fungal propagules since no contaminated batches were used. After extraction, dialysis and lyophilisation of 100 g date pollen yielded 13.8 g (dry weight) of crude extract. This extract when reconstituted to 1/10 (w/v) in distilled water contained 336 $\mu\text{g}/\text{ml}$ protein and 439 $\mu\text{g}/\text{ml}$ of total carbohydrates. Even though many of the water-soluble extract components could have been lost during the washing procedure, there was good recovery of protein and carbohydrates. Details of other components were not determined, and since all batches were pooled before analysis, batch-to-batch variation was not assessed.

Sephacryl S-200 fractionation gave 9 distinct fractions (A–I) and a well-defined profile which was reproducible in all experiments (Fig. 1a). The pooled fractions contained different amounts of proteins as detailed in Table 1. The area of the fraction peaks appeared to have no quantitative correlation with the total protein contents of the pooled fractions.

Table 1. Protein and carbohydrate content of Sephacryl S-200 separated fractions

	Fractions ¹								
	A	B	C	D	E	F	G	H	I
² Protein conc. (µg/ml)	70.3	110.5	171.5	128.4	244.0	116.2	29.0	25.5	20.3
³ Carbohydrate conc. (µg/ml)	22.5	3.0	37.5	30.5	150.2	200.2	137.5	117.5	85.3

¹ Note: All fractions were pooled and readjusted to the same volume for this determination.

² Protein concentrations of pooled fractions expressed as µg/ml of fraction (bovine serum albumin equivalent).

³ Total carbohydrate concentrations of pooled fractions expressed as µg/ml of fraction (D-glucose equivalent).

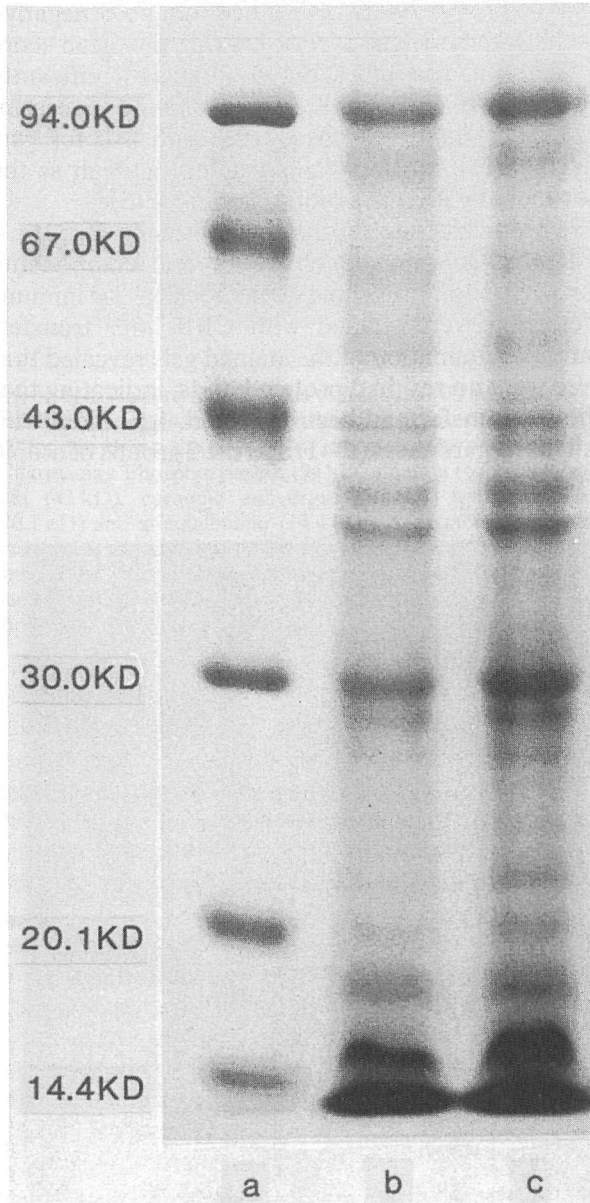


Fig. 1b. SDS-PAGE protein of date pollen extract and molecular weight markers separated on 12% gel and stained with Coomassie brilliant blue (a) low molecular weight markers (Pharmacia): Phosphorylase-b (94 kD), albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), trypsin inhibitor (20.1 kD) and α -lactalbumin (14.4 kD); (b) 50 µg and (c) 75 µg of date pollen extract.

The SDS-PAGE results of the whole extract (Fig. 1b) and the Sephacryl S-200 fractions (Fig. 2) indicate that the extract contained proteins ranging in molecular weight from 12 kD or below to over 94 kD; 22 distinct bands as well as several faint intermediate bands are seen. The protein profile of the fractions (Fig. 2), judged from their apparent molecular weights, shows that good size exclusion was achieved. Fractions A and B comprise only proteins with molecular mass of 37 to 94 kD or higher. Apart from 2 (65/67 kD) peptide bands (probably an α and α or an α and β subunit of one of the larger proteins in fraction A) which were common to all fractions, fraction C contained bands of mainly intermediate mol. wt. (30 to 50 kD), but fraction D contained peptides of between 12 and 37 kD. Apart from the "ubiquitous" 65/67 kD proteins, fractions E, G and H contained only proteins of 37 kD and below; most of them barely discernible with CBB staining (Fig. 2). Fraction F and I rarely resolved any distinct protein bands with CBB stain

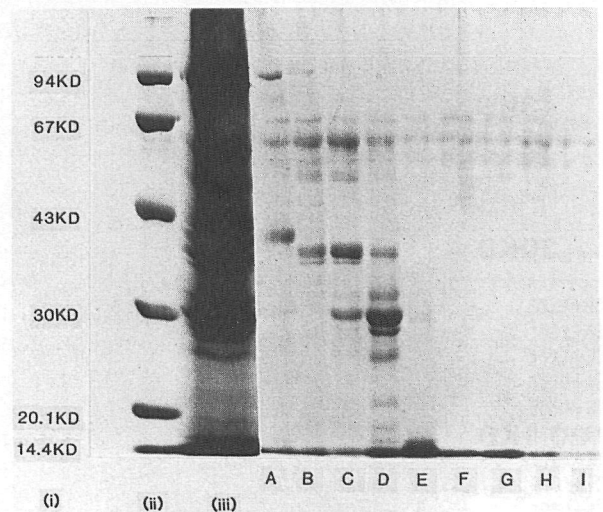


Fig. 2. SDS-PAGE of whole crude date pollen extract and Sephacryl S-200 fractions. (i) description of molecular weights (ii) Coomassie brilliant blue stained low molecular weight marker (Pharmacia): phosphorylase-b (94 kD), albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), trypsin inhibitor (20.1 kD) and α -lactalbumin (14.4 kD); lane (iii) is a Coomassie brilliant blue stain of SDS-PAGE of whole date pollen; A-I are CBB stains of fractions.

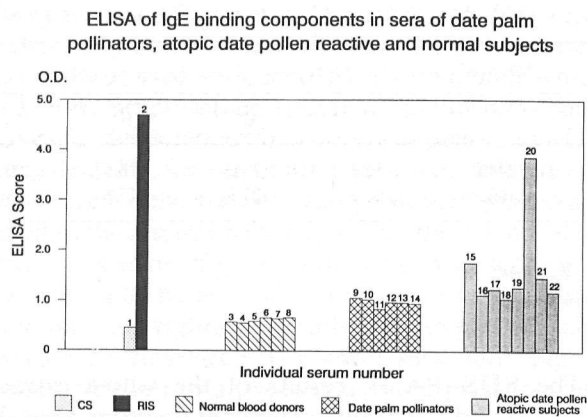


Fig. 3. Histograms of IgE ELISA scores for cord serum; (1) immune rabbit serum, (2) 6 normal blood donors, (3–8) 6 date palm pollinators (9–14) and 8 of the 15 skin test-reactive atopics (15–22). Group mean of the normal blood donors was 0.57, and 0.90 for the date palm pollinators, as compared with 1.57 for the atopics ($p < 0.0001$).

except that there was a 12 to 14.4 kD band which appeared on the dye front, indicating that these fractions either contained lower mol. wt. peptides that cannot be resolved on a 12% gel or might require special stains. This 12 to 15 kD band appeared in the whole extract as well as in fractions A–I.

Double immunodiffusion results with antiserum raised in rabbits to the whole extract indicated that the pollen extract and some of its Sephacryl S-200 fractions (A to E and G) are immunogenic (results not shown). ELISA results (Fig. 3) with sera from 8 randomly selected skin test-positive allergic patients, 6 sensitized date palm pollinators, and 6 normal blood donors gave differing degrees of ELISA results. Unexposed and cord sera reacted negatively (0–1), as did pre-immune rabbit serum (not shown), which was a pool from all the rabbits before they were immunized. Apart from 7 of the patients' sera (15, 16, 17, 19, 20–22), all other sera gave negative results and the 8th a score of 1. In all, the mean score (1.57) for the patients' sera (15–22) was significantly higher than all other sera tested (0.45 for cord serum, 0.57 for normal unexposed sera and 0.90 for gardeners' sera), but this value was not as high as the score for the immune rabbit serum (4.75).

Western blotting results are presented in Figs. 4 and 5: all gels that had their protein components transferred to nitrocellulose membranes for immunodetection were stained with CBB after transfer. Careful examination of the stained gels revealed that there were no residual protein bands, indicating that efficient transfer had been achieved. IgG blots with individual gardeners (D–I) and the 2 groups of atopic

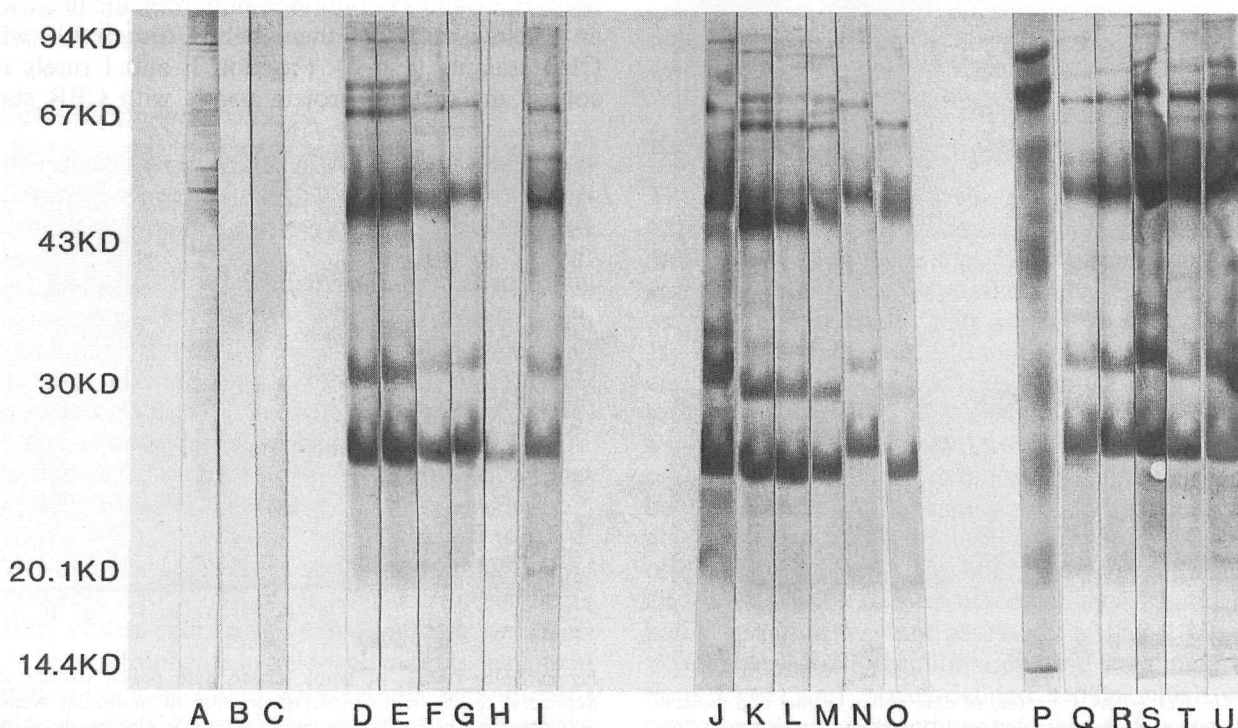


Fig. 4. Immunoblots of date pollen-specific IgG components: in left lane is position of low molecular weight markers (Pharmacia): phosphorylase-b (94 kD), albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), trypsin inhibitor (20.1 kD) and α -lactalbumin (14.4 kD). A–U are individual immunoblotted strips treated with: A = immune rabbit serum; B = cord serum; C = normal unexposed non-atopic serum pool; D–I = individual gardeners' sera; J–O = atopic individuals with weak skin reaction, and P–U = atopics with strong skin reaction sera.

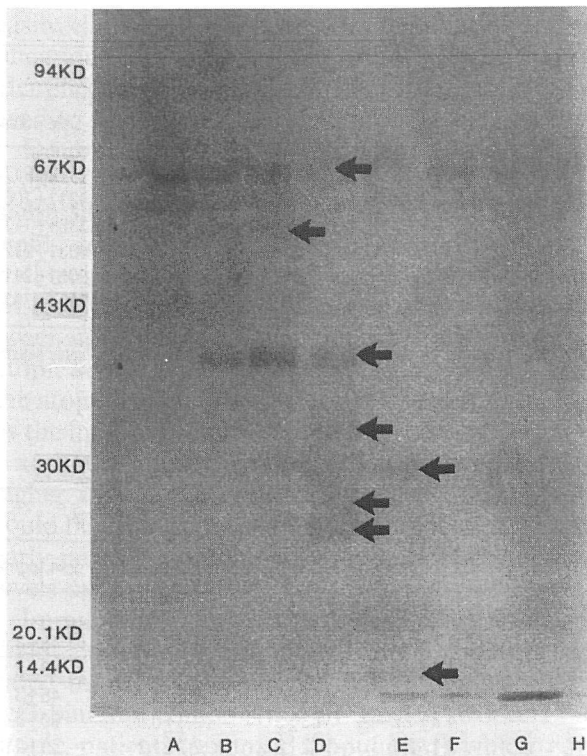


Fig. 5. IgE Western blot analysis of fractions A-I using a pool of sera from 6 atopics with strongly positive skin test reaction. The left margin shows the position of low molecular weight markers (Pharmacia): Phosphorylase-b (94 kD), albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), trypsin inhibitor (20.1 kD) and α -lactalbumin (14.4 kD). A-H indicates immune bands with the sephacryl S-200 fractions. Arrows indicate where some of the reactive bands appeared. G was a particularly intense band of about 12 kD. No bands were seen with fraction I (not shown).

sera (J.-O. & P.-U.) gave several bands which differed in numbers and intensity; one band in one of the gardeners (H) to as many as 18 for one of the atopic patients (J). There appeared to be no correlation between atopy or exposure with number of IgG-reactive bands, and the intensity of any specific bands. Overall, atopic sera often gave bands which were more diffuse, but some gardeners' sera gave more discrete but faintly staining bands in most experiments.

IgE blots with pooled positive atopic patients' sera were done to determine which of the Sephacryl S-200 fractions contained the most IgE reactive bands (Fig. 5). Approximately 12 or more IgE reactive bands can be identified at 12–15, 18–20, 25, 28–30, 32–35, 37, 40, 52, 58–60, 65–67, 72 and 94–97 kD, with fraction D containing the greatest number of reactive bands. This fraction also contained 5 of the most intense IgE staining bands. The 12–15 kD band which appeared in all fractions only gave IgE bands in fractions E–I, with that in fraction C being the most intense. Some of the IgE bands appeared to have been obscured by the unavoidably high background that ensues due to the longer reaction time and low dilution (1/5) with human serum for IgE blots.

In all, the molecular masses of the most intensely stained IgE-reactive bands were 12–15, 25, 28–30, 32–35, 37–40, and 65–67 kD. It was consistently observed that the group of bands between 25 and 35 and 65 and 72 were always the first to appear on all IgE blots. All the IgE staining bands appeared to be reactive on most of the individual IgG blots of atopic patients and gardeners but there were many IgG-reactive bands that did not bind IgE.

Table 2. Effects of whole date pollen extract and its fractions on skin reactivity and PBL proliferation

Serial No.	Fractions	Skin test score*		PBL proliferation:** ³ HtdR uptake (CPM $\times 10^{-3}$)	
		Pt (n=6)	Normal (n=2)	Pt (n=6)	Normal (n=8)
1	Whole extract***	4+	1+	36575 $\pm$ 2107	3329 $\pm$ 903
2	Fraction A	2+	0	13031 $\pm$ 1907	1521 $\pm$ 430
3	B	3+	0	27081 $\pm$ 2190	1137 $\pm$ 370
4	C***	4+	0	34935 $\pm$ 1103	1270 $\pm$ 483
5	D***	4+	1+	32121 $\pm$ 985	2250 $\pm$ 370
6	E***	4+	0	35917 $\pm$ 827	1731 $\pm$ 645
7	F	2+	0	11311 $\pm$ 1213	1105 $\pm$ 251
8	G	2+	0	13071 $\pm$ 1021	2107 $\pm$ 531
9	H	1+	0	7073 $\pm$ 831	1080 $\pm$ 431
10	I	1+	0	3621 $\pm$ 921	1271 $\pm$ 671
11	PBS	0	0	785 $\pm$ 251	1007 $\pm$ 280
12	Glycerol	0	0	991 $\pm$ 357	1121 $\pm$ 385
13	Histamine	4+	3+	ND	ND

* Skin test results were recorded as follows: wheal size ≤ 3 mm=0, 3–4 mm=1+, 4–5 mm=2, 5–6 mm=3+, and ≥ 6 mm=4+.

** PBL proliferation is expressed as CPM mean $\pm$ SE of 6 pts and 8 normal individuals when tested against either whole or various fractions of date pollen.

*** Test samples giving wheal sizes comparable to the positive histamine control (≥ 6 mm), some with pseudopods.

Table 3. Lymphocyte proliferation response $^3\text{HtdR}$ uptake ($\text{CMP} \times 10^{-3}$)

	Atopic patients			Normal		
	PHA	IL-2	Date pollen	PHA	IL-2	Date pollen
1.	31312 $\pm$ 3017	37321 $\pm$ 3111	43203 $\pm$ 3014	48037 $\pm$ 2311	39211 $\pm$ 1171	2312 $\pm$ 917
2.	49391 $\pm$ 2171	27921 $\pm$ 2941	39211 $\pm$ 1678	59311 $\pm$ 3217	28911 $\pm$ 1091	4317 $\pm$ 706
3.	59321 $\pm$ 2717	38912 $\pm$ 3211	29221 $\pm$ 2701	50327 $\pm$ 3311	39241 $\pm$ 1170	3385 $\pm$ 1121
4.	39211 $\pm$ 3417	29301 $\pm$ 2811	33011 $\pm$ 3901	39819 $\pm$ 2807	36001 $\pm$ 1709	4031 $\pm$ 2121
5.	44832 $\pm$ 4211	31376 $\pm$ 2643	42070 $\pm$ 4370	49799 $\pm$ 2911	31852 $\pm$ 970	3011 $\pm$ 211
6.	36341 $\pm$ 2374	23411 $\pm$ 1312	32374 $\pm$ 2372	57017 $\pm$ 3719	41011 $\pm$ 3219	2921 $\pm$ 792

Lymphocyte proliferative response to PHA or IL-2 stimulation showed no significant differences between atopic and normal controls ($p=0.086$ PHA; $p=0.080$ IL-2). A highly significant difference ($p=0.0001$) was observed between the atopics and normal controls when PBL was stimulated with whole date pollen extracts.

Results of skin testing (6 patients and 2 normals) and PBL proliferation (6 patients and 8 normals, $^3\text{HTdR}$ uptake) with whole extracts and fractions are summarized in Table 2. PBL from normal individuals ($n=8$) failed to respond to either whole extract or its various fractions. These results coincided with the negative skin test response of 2 normal subjects. The 6 patients reacted very strongly, some with pseudopods when skin tested with either whole extract or fractions B–E, but fractions A, F, G, H, and I also gave lower and varying degrees of reactivity. Similarly, the results of PBL proliferation also revealed that patients' PBL proliferated to a much higher degree with either whole date pollen or its fractions compared to normal individuals (Table 2).

The results of the PBL proliferative response to PHA, IL-2, or date pollen (Table 3) revealed no significant difference between atopic and normal controls when stimulated with PHA or IL-2 ($p=0.086$ for PHA and $p=0.080$ for IL-2), whereas date pollen-stimulated cultures showed highly significant differences ($p<0.00001$) between atopic and normal controls. Cytokine quantitation in the supernatant from PBL stimulated by PHA or whole date pollen revealed a differential production of IL-2 and IL-4 (Table 4). Patients' (Pt) PBL, when stimulated with PHA, produced more IL-2 and IL-4, as compared with normals': Pt mean 3.7 U/ml vs 2.35 U/ml of IL-2 for normal ($p=0.011$), and Pt mean 60 pg/ml vs 48.3 pg/ml of IL-4 for normal ($p=0.00012$). The stimulation of patients' PBL with whole date pollen induced 7 U/ml vs 2.81 U/ml of IL-2 from normal subjects ($p=0.00014$). The amount of IL-4 produced (120.83 pg/ml) by patients' PBL was highly significant ($p=0.0001$) when compared to normal subjects (48.16 pg/ml).

Discussion

The present study was designed to investigate antigens and allergens of the date palm pollen, particularly its clinical importance as an allergen. Though preliminary, the results of our study appear to indi-

Table 4. Cytokine production

	Atopic		Normal	
	IL-2 (U/ml)	IL-4 (pg/ml)	IL-2 (U/ml)	IL-4 (pg/ml)
PHA				
1.	3.2 $\pm$ 1.7	53 $\pm$ 3	1.9 $\pm$ 1.7	48 $\pm$ 3
2.	4.7 $\pm$ 1.3	60 $\pm$ 4	2.3 $\pm$ 0.3	49 $\pm$ 8
3.	2.3 $\pm$ 0.9	58 $\pm$ 6	1.3 $\pm$ 0.7	45 $\pm$ 3
4.	4.3 $\pm$ 1.2	63 $\pm$ 3	2.3 $\pm$ 0.9	50 $\pm$ 5
5.	3.1 $\pm$ 0.7	72 $\pm$ 8	2.7 $\pm$ 1.2	51 $\pm$ 4
6.	4.6 $\pm$ 1.7	58 $\pm$ 6	3.6 $\pm$ 1.1	47 $\pm$ 2
RPML-1640				
whole date pollen				
	0	0	0	0
1.	5.7 $\pm$ 1.8	113 $\pm$ 11 ^a	2.7 $\pm$ 1.3	45 $\pm$ 3 ^b
2.	7.2 $\pm$ 1.3	123 $\pm$ 23 ^a	2.9 $\pm$ 0.7	48 $\pm$ 6 ^b
3.	6.4 $\pm$ 2.1	131 $\pm$ 9 ^a	2.9 $\pm$ 1.01	47 $\pm$ 4 ^b
4.	9 $\pm$ 2.4	118 $\pm$ 7 ^a	1.7 $\pm$ 0.6	49 $\pm$ 3 ^b
5.	6.7 $\pm$ 1.9	109 $\pm$ 12 ^a	2.8 $\pm$ 0.9	51 $\pm$ 2 ^b
6.	7 $\pm$ 1.3	131 $\pm$ 7 ^a	3.9 $\pm$ 1.3	49 $\pm$ 4 ^b

Highly significant ($p=0.00014$ and $p<0.0001$) differences were observed between atopics and normal controls in the productions of IL-2 and IL-4, respectively in response to date pollen extract. A less significant ($p=0.11$) for IL-2 ($p=0.0012$ for IL-4) difference was observed with PHA stimulated PBL from atopics and normal controls.

cate that some cases of pollinosis, especially during the date pollination months of February, March and April (in Saudi Arabia), could be attributed to date pollen.

At least 22 distinct as well as several faintly staining bands are seen on SDS-PAGE gels stained with CBB. The number of bands and their estimated mol. wt. are similar to those described for other pollen components (1, 3, 4), except that there is a dark staining band on the dye front which might reveal even lower allergenic bands with a higher percentage or gradient gel: an important aspect which requires further study. Fractionation by Sephacryl S-200 size-exclusion chromatography of the crude extract helped to separate the extracts according to their mol. wt. and to determine which of the fractions are the most likely candidates for further purification and detailed study. Even though there were some areas of overlap between adjacent fractions, their problem can be

easily eliminated by repeated fractionation and purification (11). The most important fraction, D, was identified and has proved useful in all experiments used to assess the allergenicity of the extracts.

Double immunodiffusion (not shown) and ELISA results (Fig. 3) indicated that date pollen extract is highly immunogenic: antisera raised in rabbits were highly reactive to components of whole extract as well as its size-exclusion chromatography fractions. ELISA results revealed differences in reactivity between normal non-atopic, date palm pollinators and atopic sera; even though the ELISA score for one of the atopic patients' sera (No. 18) was almost as low as the highest scores in 2 of the gardeners' sera (Nos 9 and 10). The atopic population mean was clearly higher than all negative controls. This low value could be because the serum was collected during the early part of the pollination season when antibody levels are lower.

Immunoblot analyses with sera from normal individuals, atopics and date palm pollinators indicated that there are circulating date-pollen-specific IgG-binding components in garden workers and atopic patients (exposed population) which could not be seen with the control unexposed serum pool. Specificity of these bands and details of differences between atopic and exposed non-atopic population will be the subject of a later study, since the presence of these bands in date palm pollinators, though not pathognomic at the time of the study, could be indicative of the onset of a probable occupational allergy. There appear to be more IgG bands in 2 of the gardeners (D and E) who have worked with date pollen for a longer period than the rest. This could indicate the cumulative nature of date pollen-specific antibodies as well as their low clearance *in vivo*. Immune rabbit serum (A) bound almost all the bands (lower bands are diffuse and indistinct due to fading of stain) that were seen on SDS-PAGE gel as at all mol. wt. ranges, an indication of the successful immunization and high antigenicity of date pollen. Bands were more defined because background binding was not as high as in the atopic sera.

Interesting results were obtained when pooled sera from the atopic skin test-positive patients were used to detect IgE-binding components in the Sephacryl S-200 separated pollen fractions. A higher IgE-binding activity was displayed by 5 components of fraction D than was evident in all other fractions. This fraction also showed high mitogenicity, skin test reactivity and IL-4 production (unpublished observation). The strong IgE reactivity in fraction D coupled with high IL-4 production is highly suggestive of disease/atopy-specific antigens in this fraction. However, this fraction contained 50% less protein and 80% less carbohydrate than fraction E. Fractions E to I contained a band of approx. 12/

14.4 kD which appeared to stain IgE, but this band was intensely stained only in fraction G and less so in the other fractions. Detailed assessment of any cross-reactions and the prevalence of major IgE-binding components in atopic sera are not reported here due to the small sample size. More sera from patients with different degrees of symptoms and skin test reactivity need to be assessed before the major allergens can be identified with certainty.

What our studies show, however, is that both non-atopic and atopic individuals are capable of producing antibodies to date pollen, and that prolonged exposure to this pollen in non-atopics, such as date palm pollinators, can cause the production of IgG antibodies. The ability of date pollen to evoke frank allergic and mitogenic response and to induce IL-2 and IL-4 production from atopic patients emphasizes its role as a potent allergen.

Acknowledgements

We wish to thank Hani Arufsha, Head of the Grounds Maintenance of KFSH & RC Riyadh, and his staff for collecting date pollen and donating blood for this work. The excellent secretarial assistance of Ofelia Frias, Delia Bonifacio and Jennifer M. Almazan is acknowledged. The help and encouragement of George Kessie in the preparation and review of this manuscript are greatly appreciated.

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