

Human sensitization to *Ganoderma* antigen

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Continuous air sampling with a Hirst volumetric spore trap over 3 yr has identified basidiospores of Ganoderma applanatum, a bracket fungus, as the most numerous fungal spores in two southern Ontario locations. The particle size is small and the calculated total spore mass approximates that of the spores of Cladosporium and Alternaria. Extracts of Ganoderma applanatum bracket fungus and spores in w/v, 1:10 concentration were prepared after collection of samples of the fungus from local woods. Skin prick tests with the extracts were performed in 294 consecutive children and adults attending two chest/allergy clinics. Of these patients, 182 (61.9%) reacted to 1 or more of the common inhalant allergen extracts and 24 (8.2%) reacted to Ganoderma antigen. There was no consistent relationship between reactivity to Ganoderma antigen and any of the common inhaled allergens. IgE-dependent sensitization to Ganoderma was confirmed by the radioallergosorbent test (RAST). Rabbit antisera to Ganoderma antigen preparations did not appear to cross-react with preparations of the various clinically important allergens. The findings indicate that Ganoderma antigen is commonly encountered, can induce human sensitization, and has unique antigenicity among common allergens of clinical importance.

Ganoderma applanatum is a fungus of the class Basidiomycetes, family Polyporaceae, bracket fungi. It is saprophytic, growing mainly on wood.¹ Basidiospores, derived from mushrooms, toadstools, and bracket fungi, have been identified in Britain in large numbers.^{2, 3} Bruce⁴ has demonstrated positive skin and inhalation tests using mixtures of basidiospores which included *G. applanatum*. Positive skin and inhalation tests to *G. applanatum* have subsequently been reported⁵ also in Britain. In North America, allergenicity and immunogenicity studies have been performed on cultured basidiomycetes,⁶ but these have not included *Ganoderma*, and no reports have examined local spore counts and sensitization.

Continuous sampling in 1972 in Hamilton, Ontario, with the Hirst automatic volumetric spore trap demonstrated large numbers of basidiospores not previously detected in Canada⁷—in particular, the species *G. applanatum*. In the present study, spore counts for a 3-yr period are reported. In addition, extracts of *G. applanatum* were prepared and used to test for sensitization of a clinic population. A preliminary examination for cross-reactivity with commonly encountered allergens was performed with the sera of rabbits immunized with the *Ganoderma* extracts.

MATERIALS AND METHODS

Spore counts

Hirst automatic volumetric spore traps were used to sample atmospheric fungal spores and pollen in Hamilton during 1972, 1973, and 1974, and in Toronto during 1974. The traps were located on the roof of McMaster University Medical Centre (39 ft above the ground) at the west end of Hamilton and on the roof of the Gage Research Institute (38 ft above the ground) in downtown Toronto. The operation of the traps and the method of counting has been described by Chatterjee and Hargreave.⁷ The Hirst suction trap is operated continuously 24 hr a day in all weathers. Counts are relatively unaffected by particle size or wind velocity. *Ganoderma* spores are recognized by their characteristic appearance among the colored basidiospores.

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Allergen extracts used for skin tests

Extracts of *G. applanatum* were specially prepared by Hollister-Stier Labs. of Canada (Mississauga, Ontario). The bracket fungus of *Ganoderma* was collected from nearby woodlands. Spores were allowed to fall out by gentle scraping of the sporulating surface of the bracket fungus. Separate extracts were made of the spores and the fungus itself in a w/v concentration of 1:10. Extraction was with Coca's solution in an equal volume of glycerin at 32° C for 3 days. The spore extract contained 28,000 protein nitrogen units (PNU) per milliliter and the bracket fungus extract contained 8,000 PNU/ml. These extracts were used in addition to 16 extracts of common allergens in modified prick skin tests⁸ on 294 consecutive patients referred to two ambulatory chest/allergy clinics in Hamilton. The results of subsequent additional skin tests, performed in the clinics for the purpose of "case finding," are not included in the 294 cases surveyed to determine the frequency of reactivity.

Common allergen extracts used in skin tests were *Alternaria*, *Cladosporium* (formerly known as *Hormodendrum*), cat epidermis, dog epidermis, and house dust, w/v 1:10 (Hollister-Stier Labs. of Canada, Mississauga, Ontario); *Aspergillus fumigatus*, w/v 1:20; *Dermatophagoides farinae*, w/v 1:100 (Bencard Allergy Service, Weston, Ontario); mixed feathers, mixed tree pollen, mixed grass pollen, ragweed pollen, egg white, milk, mixed nuts, and mixed shellfish, w/v 1:10 (Omega Labs., Toronto, Ontario).

Skin tests

In allergy skin tests by the prick (puncture) test method, the wheal diameter at 15 min was recorded as negative if absent, 1+ if 1 to 2 mm, 2+ if 3 to 5 mm, 3+ if 6 to 9 mm, and 4+ if over 9 mm in diameter. An arbitrary index of reactivity is used to categorize the patients; it is the resultant of the addition of all plus values in the series of skin tests with common allergens in each individual.

Radioallergosorbent test (RAST)

Direct RAST was performed using a modification of the technique described by Ceska et al.⁹ Cellulose discs were prepared from Whatman No. 1 filter paper and activated with cyanogen bromide. The *Ganoderma* fungus and spore extracts used were separately dialyzed against borate buffer and then concentrated with 30% polyethylene glycol. Protein concentrations were estimated by the Lowry technique¹⁰ with a bovine serum albumin reference standard. An initial 23 ml of bracket fungus extract was reduced to 10 ml containing 1.8 mg/ml protein. A 20-ml volume of the spore extract was concentrated to 5 ml with 3.8 mg/ml protein. Two hundred milligrams of washed activated discs (about 100) were incubated for 3 days at room temperature in a mixture of 0.75 ml fungus extract, 0.75 ml spore extract, and 0.75 ml 0.3 M NaHCO₃. The discs were then washed 3 times with 0.1 M NaHCO₃ 1 time with a mixture of equal volumes of 0.05 M ethanolamine and 0.1 M NaHCO₃ for 3 hr. Subsequently, they were washed with 0.5 M NaHCO₃ twice, 0.1 M Na acetate pH 4.0 twice, and twice

with the incubation mixture containing 0.9% saline and 0.5% Tween 20.

Discs were stored at 4° C in the incubation buffer. To perform the test, discs were incubated at room temperature with 50 μ l of test serum for 4 hr and then washed 3 times with incubation fluid. They were then incubated overnight with ¹²⁵I-labeled anti-IgE reagent in an amount producing 30,000 cpm. Following overnight incubation at room temperature, the discs were washed with incubation mixture and then counted in an automatic gamma counter.

The sera used in RAST were taken from 11 patients with positive *Ganoderma* prick tests and 6 otherwise allergic subjects who had negative skin tests to the *Ganoderma* antigen.

Specificity of rabbit antisera to *Ganoderma*

Antisera to the *Ganoderma* spore antigen preparation were prepared in 2 rabbits and antisera to the *Ganoderma* bracket fungus antigen preparation were prepared in 2 other rabbits. Immunization was by subcutaneous injection into each rabbit of an emulsion of 0.5 ml antigen extract (in the form supplied by Hollister-Stier Labs.) and 0.5 ml of complete Freund's adjuvant. At monthly intervals the rabbits received 2 additional injections of emulsion prepared with 0.5 ml antigen in 0.5 ml incomplete Freund's adjuvant. One month later there was a subcutaneous injection of 0.5 ml of aqueous solution of antigen. The animals were sacrificed by exsanguination 1 wk later. The antisera were tested for reactivity with the *Ganoderma* antigen preparation, and with various fungal extracts and other allergens of known clinical importance, by double-diffusion in 1.5% agar. Seven and one-half milliliters of agar was placed in disposable petri dishes 9 cm in diameter. Rabbit antisera were placed in central wells 8 mm in diameter and the antigen preparations, undiluted, and serial 1:2 dilutions were placed in peripheral wells of equal size with approximately 8 mm distance between the central and peripheral wells.

Antigens for precipitin tests with rabbit antisera, derived from clinical supplies, were dialyzed against phosphate-buffered saline and then concentrated as much as possible by dialysis against 30% polyethylene glycol prior to use. Final protein concentrations were estimated by the Lowry technique.¹⁰ The antigens, including protein content in milligrams per milliliter, were: *Alternaria*, 1.13; *Cladosporium*, 1.11; house dust, 0.48 (Hollister-Stier Labs., Mississauga, Ontario); *Aspergillus fumigatus*, 0.60; *Aspergillus niger*, 0.36; *D. farinae*, 0.31 (Bencard Allergy Service, Weston, Ontario); mixed feathers, 0.47; tree pollen mix, 0.41; grass pollen mix, 2.39; ragweed, 3.72 (Omega Labs., Toronto, Ontario); *Candida albicans*, 0.08; *Penicillium* mix, 0.41; *Alternaria*, 0.16; *Epicoccum*, 0.10 (Centre Labs., Port Washington, N. Y.); *Helminthosporium*, 0.31 (Beecham Pharmaceuticals, Clifton, N. J.).

An additional special group of mold antigen preparations was obtained specifically for in vitro tests with the rabbit antisera. The molds used and the protein concentrations of these preparations in milligrams per milliliter of the extracts were: *Alternaria tenuis*, 1.5; *Cladosporium herbarum*,

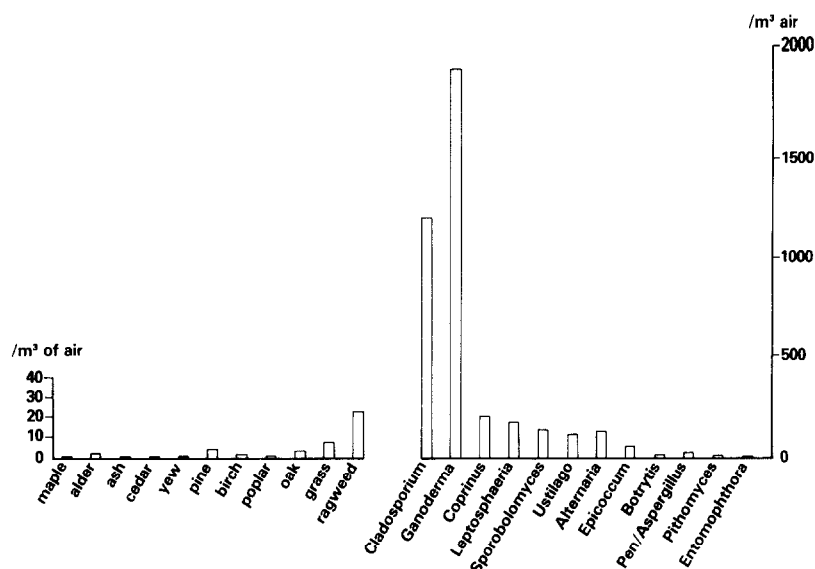


FIG. 1. Mean 24-hr numerical counts of airborne pollen grains (left) and fungal spores (right) in Hamilton in 1972.

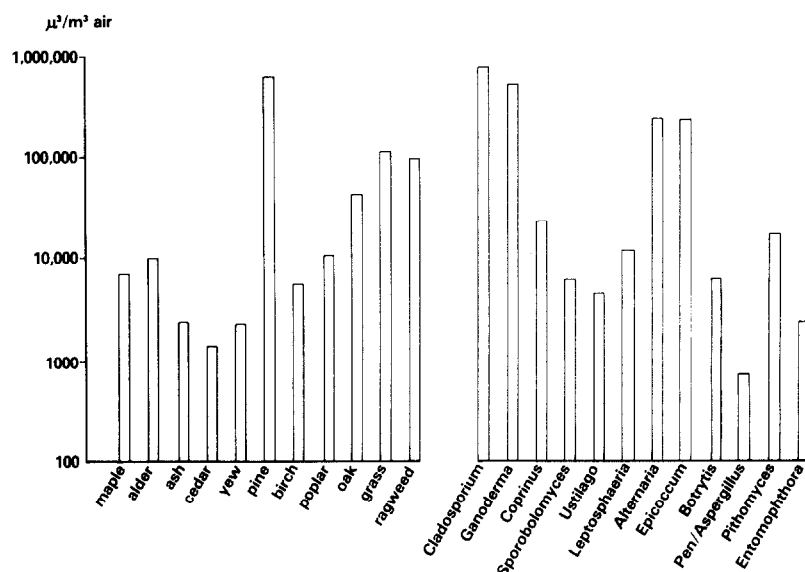


FIG. 2. Mean 24-hr calculated bulk of airborne pollen grains and fungal spores in Hamilton in 1972.

0.36; *Penicillium notatum*, 1.62; *Monilia albicans*, 3.17; *Fusarium moniliforme*, 1.10; *Botrytis cinerea*, 1.89; *Epicoccum purpurascens*, 0.71; *Rhizopus nigricans*, 0.76 (Greer Labs., Lenoir, N. C.); *Aspergillus fumigatus*, 4.96; *Aspergillus nidulans*, 4.61; *Aspergillus niger*, 24.83; *Aspergillus terreus*, 6.9; *Aspergillus flavus*, 7.8 (Mycological Reference Lab., The London School of Hygiene & Tropical Medicine, London, England).

The 1 rabbit antiserum to the *Ganoderma* spore antigen preparation and the 1 antiserum to the *Ganoderma* bracket fungus preparation which seemed to produce the best precipitin reactions in double-diffusion tests, performed as specified above, were used in radioimmunoassay to test for inhibition of binding of labeled *Ganoderma* antigen. The

2 rabbit antisera and a control serum from a nonimmunized rabbit were added to numerous 6-mm diameter wells, 1 cm apart in agar which contained goat antiserum to rabbit IgG. Following the development of ring precipitates, the slides were washed, and buffer or nonlabeled *Ganoderma* antigen or concentrated clinical allergen preparations (see above) were then added to the separate wells. The slides were then washed and ^{125} I-labeled *Ganoderma* antigen was added to all the wells. The *Ganoderma* antigen had been prepared by the mixture of concentrated spore *Ganoderma* antigen (3.82 mg/ml) with concentrated bracket fungus antigen (1.82 mg/ml) producing a concentration of mixed *Ganoderma* antigens of 2.9 mg/ml which was then labeled by the chloramine-T method.¹¹ The labeled antigen was allowed to

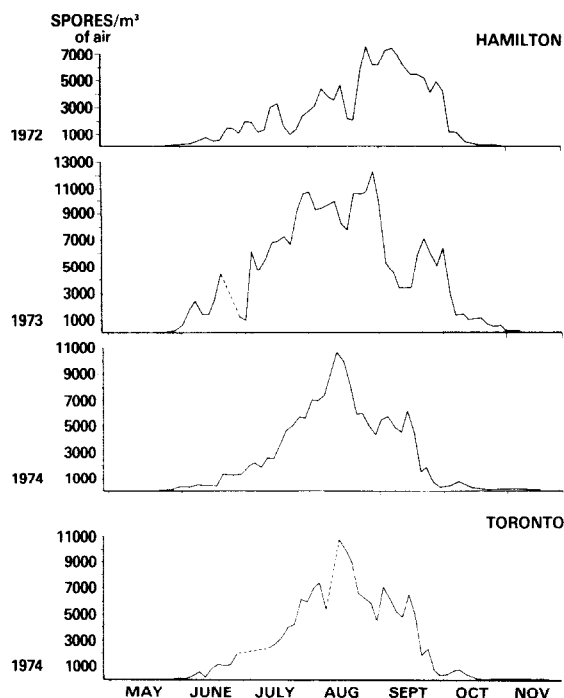


FIG. 3. Spore counts of *Ganoderma* in Hamilton in 1972, 1973, and 1974, and in Toronto in 1974, expressed as mean 24-hr counts for 6-day periods, plotted in 3-day segments.

diffuse through the ring precipitates containing rabbit IgG for 24 hr. The slides were then washed and examined by autoradiography.

RESULTS

Spore counts

Atmospheric pollen and spores in Hamilton in 1972 are expressed as mean 24 hr counts/m³ of air for the year in Fig. 1. *Ganoderma* basidiospores were the most numerous of the fungal spores; they were 1.5 times more numerous than *Cladosporium* and 10 times more numerous than *Alternaria*. When the average 24-hr bulk for the year was calculated, based on known mean volumes¹ of the individual spores, *Ganoderma* was slightly less than *Cladosporium* and slightly more than *Alternaria* (Fig. 2).

The mean hourly count per cubic meter of air over 6 days was tabulated for 3-day intervals for spores of *Ganoderma*, *Cladosporium*, and *Alternaria*; the results for 1972, 1973, and 1974 in Hamilton and 1974 in Toronto are plotted in Figs. 3 and 4. All types of spores predominated in the summer between May and November and peak values were between July and October. The counts for the 3 years in Hamilton and 1974 in Toronto are quite similar.

Hourly counts per cubic meter of air of *Cladosporium* and *Ganoderma* spores over 3 days in August, 1972, are plotted at 2-hr intervals in Fig. 5. There were obvious diurnal variations in the spore

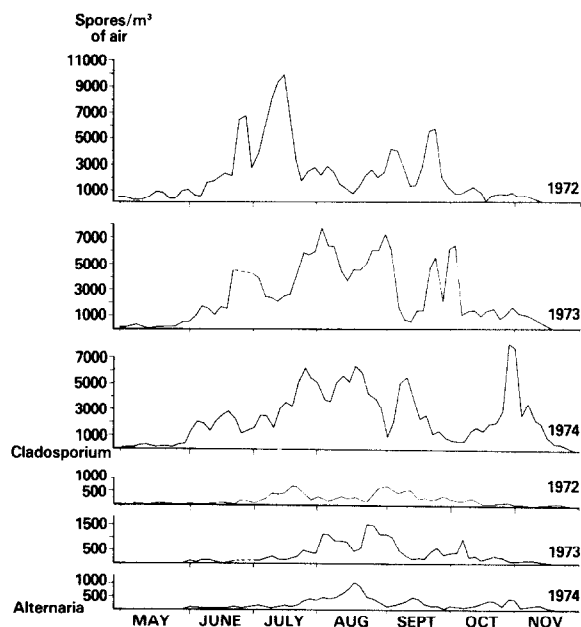


FIG. 4. Counts of *Cladosporium* and *Alternaria* spores in Hamilton in 1972, 1973, and 1974, expressed as mean 24-hr counts for 6-day intervals, plotted in 3-day segments.

counts; *Cladosporium* was more abundant during the afternoon and *Ganoderma* was more abundant in the night and morning hours.

Skin tests

The frequency of positive skin tests indicative of allergic sensitization to *G. applanatum* antigen(s) is illustrated in Table I. Twenty-four of 294 consecutive subjects (8.2%) reacted to *Ganoderma* antigen. Of the 294 subjects, 182 (61.9%) had positive skin tests to 1 or more of the common allergens. In skin tests on an additional group of subjects, 6 more *Ganoderma* skin test-positive subjects were identified. Twenty of the subjects had positive skin tests to both spore and bracket fungus extracts, 6 to spore antigen alone, and 4 to bracket fungus antigen alone. However, in those responding to only 1 *Ganoderma* extract, the response was generally weak; it was 1+ in 9 subjects and 2+ in only 1. All except 1 of the 30 *Ganoderma*-positive subjects had positive skin tests to other common inhalant allergens. However, careful examination of results in individual cases revealed no regular association between a positive test with *Ganoderma* and a positive test with any other allergen tested (Table II). In 2 subjects, *Ganoderma* was the only seasonal allergen which elicited positive skin tests. One of these gave a history of seasonal variation in symptoms, corresponding to the spore counts of *Ganoderma*.

In the subjects tested, a positive skin test with

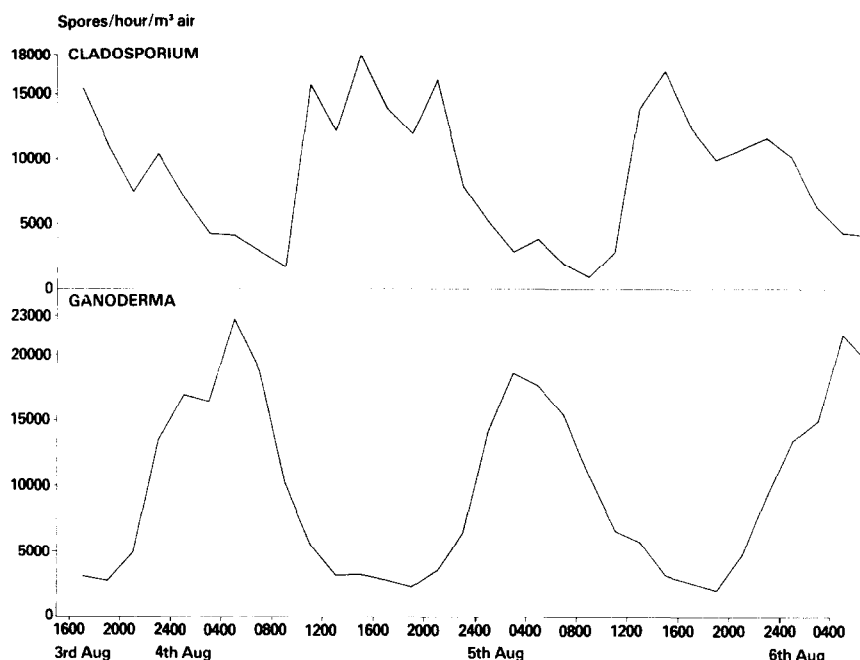


FIG. 5. Hourly counts of *Cladosporium* and *Ganoderma* spores plotted at 2-hr intervals to illustrate contrasting diurnal variations in these 2 types of spores.

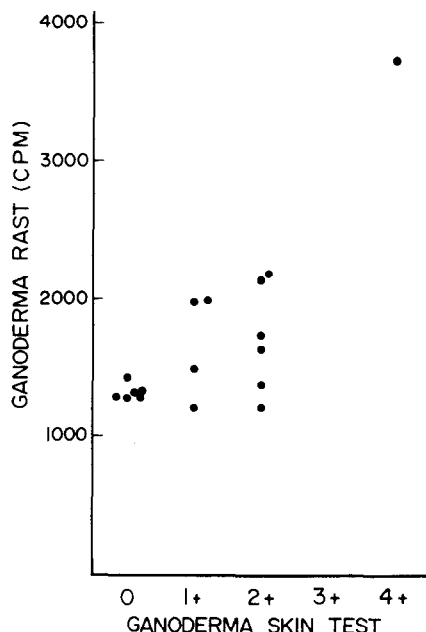


FIG. 6. *Ganoderma* RAST and skin test results. RAST values for normal sera expressed as mean $\pm$ SD were $1,326 \pm 55$ cpm. The RAST was clearly positive in some skin test-positive subjects.

Ganoderma (8.2%) was less common than with *Alternaria* (16.7%) and *Cladosporium* (11.2%) but more common than with *Aspergillus* (3.1%).

RAST with patient sera

The mean value for the sera of control subjects was 1,323 cpm. The RAST was considered positive for values greater than 2 SD (1 SD = 53 cpm) above the

TABLE I. Index of reactivity to common inhalant allergens in 294 consecutive patients

	0	1-5	6-10	11-15	16-20	Total
<i>Largest skin test response to Ganoderma cortex or spore antigen</i>						
0	111	75	41	28	15	270
1+	—	4	3	9	3	19
2+	1	—	2	1	1	5
3+	—	—	—	—	—	0
4+	—	—	—	—	—	0
Total	112	79	46	38	19	294

mean of 1,323 cpm. By this criterion, RAST confirmation of the presence of IgE antibodies to *Ganoderma* was obtained in 8 of the 11 skin test-positive subjects tested (Fig. 6).

Tests for cross-reactivity with rabbit antisera to *Ganoderma*

In double diffusion in agar, each of the 4 rabbit antisera produced a single major line and a minor of precipitation with each of the 2 *Ganoderma* antigen preparations. When the 2 *Ganoderma* antigen preparations were placed side by side (Fig. 7), there were continuous lines characteristic of complete identity (fusion). The rabbit antisera to *Ganoderma* were negative in double-diffusion tests with all the other clinical and special allergen preparations tested.

In autoradiography studies with the rabbit antisera, there was the expected binding of labeled *Ganoderma*

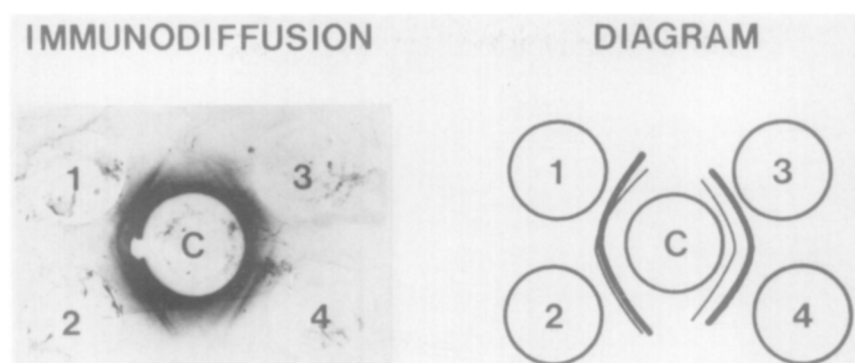


FIG. 7. Precipitin reaction between *Ganoderma* antigen preparations and rabbit antisera. The immunodiffusion reaction is represented on the left and a corresponding diagram of the reaction is on the right. The center well (C) contained rabbit antiserum to *Ganoderma* fungus. *Ganoderma* antigen extracts in the peripheral wells were (1) fungal antigen, w/v 1:10, as received; (2) spore antigen, w/v 1:10, as received; (3) fungal antigen dialysed and concentrated as specified in Materials and Methods; and (4) spore antigen, dialysed and concentrated.

The antiserum reacted with all antigen preparations and there were lines of complete identity (fusion) between the fungal and spore antigen preparations.

TABLE II. Skin responses to common inhalant allergens in 29 *Ganoderma*-positive subjects

Allergen	Response				Total
	1+	2+	3+	4+	
<i>Alternaria</i>	0	9	6	1	16
<i>Hormodendrum</i>	11	4	0	0	15
<i>Aspergillus fumigatus</i>	5	0	0	0	5
Dog	8	2	0	1	11
Cat	7	6	3	0	16
Horse	6	1	3	0	10
Mixed feathers	3	1	0	0	4
House dust	10	10	1	0	21
<i>D. farinae</i>	5	1	2	1	9
Tree	4	3	4	0	11
Grass	5	3	4	5	17
Ragweed	4	6	6	4	20

antigen to the IgG of the sera of *Ganoderma*-immunized rabbits but no binding to the IgG of the serum of a nonimmunized rabbit. The binding of label to the IgG of immune sera was completely blocked by the addition of nonlabeled *Ganoderma* antigens to the wells prior to the addition of labeled *Ganoderma*. None of the other clinical or special allergen preparations produced any noticeable inhibition of binding of the labeled *Ganoderma* to the IgG of antisera from rabbits immunized with *Ganoderma* extracts.

DISCUSSION

The Hirst automatic volumetric spore trap provides more accurate quantitation of atmospheric pollen and fungal spores than gravity samplers. Studies with the Hirst trap in 1972 led to the identification of high counts of basidiospores and ascospores in the Hamil-

ton region for the first time.⁷ *Ganoderma* was the basidiospore with the highest counts. In the present study, with continued sampling in Hamilton for 2 yr and Toronto for 1 yr, *Ganoderma* spores remained the most numerous. It was noted that they were absent in the winter and, when present, demonstrated a diurnal variation.

Previous studies in southern Ontario with gravity samplers¹²⁻¹⁴ identified spores of *Cladosporium* (previously called *Hormodendrum*) and *Penicillium/Aspergillus* species as the most numerous. However, studies elsewhere with the Hirst trap have described large numbers of basidiospores. Basidiospores have been grouped as "colored" and "hyaline."^{1,2} Actual counts of spores of *Ganoderma*, which are colored basidiospores, have not been reported. In the present study the mean 24-hr count for 1972 for *Ganoderma* in Hamilton was 1,200/m³ of air. This compares with colored basidiospore counts in a rural area in England during August and September of 1,000/m³. In Wales, reported counts have been 1,527/m³ in a coniferous plantation, 1,992/m³ in a deciduous woodland, and 294 and 530/m³ in consecutive years in the center of a city.¹⁵

About 8% of chest/allergy clinic patients were sensitive to *Ganoderma*. This was less than the frequency of sensitization to *Alternaria*, despite the higher counts of *Ganoderma*, and also less than the frequency of sensitization to *Cladosporium*, despite almost identical counts in 1972.⁷ In Wales, 16% of subjects were sensitive to basidiospores but this was a selected group with summer exacerbations of asthma.⁵

The relatively low incidence of apparent sensitization may be due to insufficient concentrations of rele-

vant allergen(s) in the extracts. At present, there is no information on the relevant allergenic components of *Ganoderma* extracts and no reference preparations for comparison. Alternatively, *Ganoderma* may indeed produce less sensitization than other aeroallergens; this could be due to its relatively small size or due to the peaking of spore counts during the night and early morning hours when exposure might be less.

Ganoderma sensitization in our patients was usually accompanied by positive skin tests to other common allergens. Nevertheless, there was no consistent relationship with sensitization to any other individual allergen. The RAST confirmed the sensitization. The counts with sera of *Ganoderma*-sensitive subjects were significantly higher than with sera of control subjects. Unfortunately, counts with the sera of sensitized persons did not exhibit a major increment above background and the "level of positivity" was considered insufficient to permit reliable RAST inhibition studies. The highly positive RAST result with the serum of the subject with a 4+ skin test was obtained at a time when insufficient *Ganoderma* antigen preparation remained to proceed with RAST inhibition studies. This would provide useful data on the possible occurrence of cross-reactivity with other allergens in man. The absence of cross-reactivity with other allergens would strengthen the argument that it was indeed exposure to airborne *Ganoderma* spores that led to the observed sensitization.

In vitro studies with rabbit antisera provided evidence of unique allergenic reactivity in the *Ganoderma* preparations, at least in relation to the various common clinically relevant allergens. Similarly, Lopez et al.,⁶ using double-diffusion techniques, found shared allergens among 7 cultured basidiomycete species (not including *Ganoderma*) but found no cross-reactivity with antigens of several common species of the *Fungi imperfecti*.

The clinical relevance of the findings reported here is still unknown. Of the 2 patients in our study who had positive skin tests to *Ganoderma* but negative responses to other seasonal allergens, only 1 gave a history of summer exacerbations of symptoms. In other subjects, perennial symptoms may have masked any effect of reactions to *Ganoderma*. The results of inhalation tests in Britain⁵ with *Ganoderma* antigen correlated poorly with skin tests. However, this is not surprising insofar as no attention was given to the level of nonallergic bronchial hyperreactivity which is now known to be an important determinant of airway responses to inhaled allergen.¹⁶

The demonstration of high counts of *Ganoderma*

spores in the air and in vivo and in vitro evidence of sensitization to *Ganoderma* antigen, together with the apparent unique antigenic character of the *Ganoderma* antigen vis a vis other clinical allergens, indicates that it should receive further consideration as a summer aeroallergen.

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