Chapter VI

PREPARATION OF ANTIGEN AND CLINICAL TEST TO EVALUATE THE ALLERGENIC POTENTIALITY

INTRODUCTION

Airborne fungal spores are well known to act as allergens to the susceptible individuals. The causation of allergy was realised as early as in 1873 when Blackley described an instance of allergy due to moulds in England. By inhaling *Penicillium* spores, he was down with "bronchial catarrh". In 1924, Cadham reported from Canada, that rust spores might be the cause of allergy to persons working in the wheat fields and he demonstrated for the first time the desensitization treatment with the extracts of airborne fungi. Hopkins *et al.* (1930) were the first to report the case of inhalent allergy caused by moulds like *Alternaria*. Brown (1932) described for the first time an extensive series of testing with moulds for allergenic significance. Since then a number of workers reported the allergenicity of different moulds. The causation of allergy by *Cladosporium, Alternaria, Aspergillus fumigatus, Chaetomium*, smuts and rusts were reported in 1930s and 1940s by several workers.

The occupational environments might be the significant sources of allergenic moulds. Prince *et al.* (1964) described the case histories of two patients, where moulds encountered in their occupational environments were the significant causes of bronchial asthma. Liebeskind (1965) reported that the prevalent incidence of asthma in patients was correlated with the increase in mould spores. Roth (1966) determined clinically the moulds as the most prevalent inhalent allergens.

In India, Bhargava *et al.* (1961) reported the results of skin tests with the antigens from *Alternaria, Helminthosporium, Hormodendrum* and *Fusarium* in 221 cases of various allergic disorders in Jaipur. In Delhi, Sandhu *et al.* (1964) reported the skin tests with the fungal extracts on patients with respiratory allergy. Shivpuri and Dua (1964) treated 250 patients sufferring from bronchial asthma by hyposensitisation with antigens from fungal spores of local origin in Delhi. Shivpuri and Agarwal (1969) recorded positive skin reactions with fungi found in the same area. Cases of aspergillosis and aspergilloma by species of *Aspergillus* and brain mycoses by *Cladosporium trichoides* were reported by

Mishra and Sandhu (1972). A survey on the occurrence of *Aspergillus* spp. in the respiratory tract of patients of bronchopulmonary diseases has been reported by Sandhu and Sandhu (1973).

Lacey (1973) studied the prevalence of actinomycetes in farm air and causation of health problems in human and animals. In 1976, Mullins *et al.* reported *Aspergillus fumigatus* with 6.6% positive prick test results in 1039 patients in Cardiff. Okudaira *et al.* (1977) studied 159 autopsy cases to estimate fungal flora in lung, where, 129 patients (i.e. 81.1%) showed the presence of filamentous fungi like *Aspergillus, Penicillium* and yeasts.

Among the recent works, the author has gone through the following literatures. Rantio-Lehtimaki (1988) has shown that yeasts (Rhodotorula) often gave positive provocation reactions. Hasnain *et al.* (1989) made a comparative study of scratch, intradermal and provocation tests in which *Alternaria* gave 56% positive reaction in intradermal test compared to 4.5% in scratch test. Cosentino *et al.* (1990) made an investigation of the fungal spores in the upper respiratory tract and in the homes of patients with allergic manifestations and recorded out of 391 patients, 28 persons (7.1%) showed positive skin prick reactions to fungal allergens. *Alternaria tenuis* and *Cladosporium herbarum* have been more frequently found to give positive reactions.

According to Frankland (1991) *Aspergillus fumigatus* causes various pathological conditions in man, animals and birds, of which allergic broncho-pulmonary aspergillosis (ABPA) is a complication seen in allergic asthmatics particularly in younger age group. During the studies in respiratory illness among the school children (8-11 years), Peat and Woolcock (1991) showed that along with 6 other allergens *Alternaria tenuis* had influenced the respiratory illness. Attapattu (1991) performed skin test, serological test, sputum examination and peripheral blood eosinophil count in 134 patients with severe bronchial asthma, and skin test sensitivity was demonstrated to be a screening test for the diagnosis of ABPA. Paris *et al.* (1991) purified the major allergen from *Alternaria alternata*. Jeng *et al.*

(1991) studied the relationship of Ig E, skin test, eosinophilia, eosinophil cationic protein and tumour necrosis factor production in allergic rhinitis. Among the 112 patients 86% showed positive skin test reactions to house dust mites and fungal allergens.

In 1992, Szánthó *et al.* studied the mould allergy in asthmatic children in Hungary. A study of 237 male workers at two flour mills and 71 controls was carried out (Fakhri 1992) to investigate the possible causes of hypersensitivity reactions to flour dust. Schwartz (1992) studied the vegetable dusts, mixed grain, corn and soybean dusts and extent of respiratory disease among the workers. Wickman *et al.* (1992) studied the indoor viable dust bound microfungi with relation to residential characteristics, living habits and symptoms in atopic and control children. Xia (1992) performed skin tests in 114 cases of asthmatic patients with fungal allergens and found the positive rate was 73.7%. Skin reactivity to histamine and allergens was studied by Niemeijer and de Monchy (1992) in a dutch asthmatic patient population from childhood to old age (4-75 years).

Hasnain (1993) reported that among the asthmatic patients only 5% gave positive immediate type of hypersensitive reactions to *Phoma* extract by skin prick testing. According to Crook (1994) in U.K. asthma and allergic alveolitis accounted for one third of the total cases of work related respiratory diseases. Apart from the chemical agents causing occupational asthma, biological agents in agriculture i.e. organic dusts and associated microorganisms have been encountered to cause asthma in significant numbers.

A study was therefore, undertaken with the fungal isolates from the air during threshing and harvesting operations for their allergenic significance since during these operations there is a heavy spore load in air to which the farmers are exposed.

MATERIALS AND METHODS

Schaffer *et al.* (1959) described a synthetic medium for mould cultivation having certain advantages over other media. This medium was used since the culture broth would

probably be a good source of allergen content than the mould pellicle itself. According to Blatt (1962) the media and the method employed by Schaffer *et al.* (1959) eliminates non-specific, allergenic and other interfering factors which might be present in other commonly used media. The extracts prepared were more specific, as evaluated by skin tests.

The method employed was according to Schaffer *et al.* (1959) with a slight modification. The fungi used were *Alternaria solani, Drechslera* sp. and *Helminthosporium oryzae*, the fungi which were very predominant during harvesting of potato, and threshings of wheat and "Aman" variety of paddy respectively. These fungi have already been reported as plant pathogens (Uddin and Chakraverty 1995; 1996).

In this method a specific synthetic medium i.e. Center mould medium no.1 was used, with the composition as follows :

Sucrose	40.0 gm.
Ammonium sulphate	2.0 gm.
Ammonium nitrate	1.3 gm.
Ammonium citrate	1.0 gm.
Sodium citrate	2.0 gm.
Potassium phosphate, monobasic ...	0.15 gm.
Potassium phosphate, dibasic ...	0.15 gm.
Ammonium phosphate, dibasic ...	0.20 gm.
Citric acid	1.00 gm.
Potassium citrate	0.40 gm.
Magnesium sulphate	0.25 gm.
Calcium carbonate	0.80 gm.
*Trace element stock solution ...	1.0 ml.
Distilled water q.s.	1000 ml.

pH adjusted to 7.0

*Trace element stock solution

Copper sulphate	...	...	...	5.0 gm.
Manganese sulphate	...	...	...	5.0 gm.
Potassium dichromate	...	...	...	2.0 gm.
Zinc sulphate	...	...	...	5.0 gm.
Ferric sulphate	...	...	...	1.0 gm.
Distilled water q.s.	...	...	...	1000 ml.

The above ingradients were added to distilled water and allowed to boil gently for half a minute and clarified by paper filtration and the pH was adjusted to 7.0. The medium was distributed in a number of Roux bottles, 125 ml. each, and autoclaved at 12 lbs pressure for 15 minutes. Roux bottles (1000 ml. capacity) containing medium, were inoculated with 1 ml of spore suspension of each of the above mentioned fungi and were incubated at room temperature (24-28^{o}C). After 6 weeks of full growth, the mould extracts were prepared from the mats as well as the culture broth. The mycelial mass left after filtration was dried and the respective weights were determined.

The hyphal mats were grounded with the help of mortar and pestle to pulverize the materials for efficient extraction. The grounded mycelial mass as well the separated culture filtrate were defatted separately with diethyl ether for efficient extraction of proteinaceous allergens and for obtaining a clear extract. Defatting was done by shaking the material with diethyl ether (4-6 volumes) and decanting the oily layer. The process was continued using fresh ether every time until no colour was visible in the supernatunt. The defatted materials were then filtered with filter paper and were allowed to dry at room temperature. After drying, the dry weight of the hyphal mat and culture filtrate were taken and preserved in air tight bottles and stored in a refrigerator for extraction and further use.

The active allergenic substances were extracted from the defatted powder of the mats as well as the culture filtrates with slightly alkaline phosphate buffered saline (PBS),

(pH 8.0) (Tilak 1987). The compositions of the buffer saline are as follows :

Sodium chloride A.R.	5.00 gms.
Potassium phosphate, monobasic A.R.	0.36 gm.
Sodium phosphate, dibasic (anhydrous) A.R.	7.00 gms.
Phenol (crystals)	4.00 gms.
Double distilled water	1000.00 ml.

To prepare 1 : 50 strength of allergenic extracts, 2 gm of defatted powder of mat or 2 gm culture filtrate was added in 100 ml. of buffer saline. The mixture was extracted for 72 hrs at room temperature with occasional shaking for a period of 30 minutes by employing a magnetic stirrer for 10-12 times. During the intervals, the flask was kept in the refrigerator.

A very small portion of the extracts were tested for the protein-nitrogen contents, by Lowry's method (Lowry *et al.* 1951) as the protein was supposed to contain the desired antigen.

The allergenic extracts thus prepared were filtered coarsely, passed through the a sintered glass filter No 5 (Bacteriological grade) and for absolute sterilisation the extracts were further passed through millipore filters (0.22 μm) and collected in sterile vials with screw cap, labelled and stored at $4^{o}C$ for skin testing.

Before skin testing, sterility was ascertained for each of the vials containing extracts. The skin testing was done by the kind courtesy of the Institute of Child Health, Calcutta.

RESULTS

The skin tests are employed for the diagnosis of mould allergy. The dry weights of mats as well as culture filtrates and protein contents are shown in **Table 41**. The purified allergenic substances were stored at 4°C before use. Our preparations were of 1:50 strength and therefore positive skin reactions could be noticeable. The skin tests were carried out in the patients whose previous history were known. The patients selected for skin tests were sufferring from allergic symptoms showing asthma or respiratory troubles. Skin reactions were interpreted according to Gupta Bhattacharya *et al.* 1994.

Table 41. Dry weight and protein contents of the fungal species

Sl. No.	Fungal Species	Dry weight (mg)		Protein Content (µg/ml)
		Mycelial Mat	Culture Filtrate.	
1.	*Alternaria solani*	8140	-	272
2.	*Alternaria solani*	-	1760	624
3.	*Drechslera* sp.	4371	-	208
4.	*Drechslera* sp.	-	2962	728
5.	*Helminthosporium oryzae*	5265	-	222
6.	*Helminthosporium oryzae*	-	2015	1260

All the mould extracts showed positive reactions in susceptible individuals (**Table 42**). The culture filtrates contain a comparatively larger amount of allergens than the hyphal mats itself. Again, *Helminthosporium oryzae* (culture filtrate) was found to be more allergenic (21.15%) than the other two fungi employed in the skin tests.

Table 42. Mould allergens and percentage of positive skin test reactions in the susceptible individuals

Source of Antigen (Organism)	Mycelial Extract	Culture Filtrate Extract	No. of susceptible Patients tested	No. of persons reacted positively	Percentage of positive reaction
1. *Alternaria solani*	Mycelial mat extract	-	47	8	17.0%
2. *Alternaria solani*	-	Culture filtrate extract	50	9	18.0%
3. *Drechslera* sp.	Mycelial mat extract	-	51	8	15.69%
4. *Drechslera* sp.	-	Culture filtrate extract	48	9	18.75%
5. *Helmin-thosporium oryzae*	Mycelial mat extract	-	46	7	15.22%
6. *Helmin-thosporium oryzae*	-	Culture filtrate extract	52	11	21.15%

DISCUSSION

It is evident from the above experimental results of the clinical tests of mould allergens that airborne moulds especially the plant pathogenic moulds under study are capable of producing reactions in the skin of susceptible subjects. The reactions are definitely similar to those of antigens and there is a possibility of moulds to stand the role of

causative agents of asthma with symptoms similar to that of hay fever.

There are at least half a dozen well known fungi whose abundant presence in air is observed during a particular season of the year with particular range of temperature, relative humidity and rainfall. It is significant that a specific condition of the atmosphere may sometimes become conducive to the abundant growth of moulds of certain types. The moulds are known to multiply at their sources, disseminated in air by the air current and the air becomes periodically surcharged with a highly increased frequency. Occupational environments are also rich with fungal load which resulted in hypersensitivity or allergic reactions to the working persons. Such causations have been proved by a number of workers (Fakhri 1992, Burge 1992, Schwartz 1992, Crook 1994). Farmer's Lung Disease, an extrinsic allergic alveolitis has been reported to be caused by huge inhalation of actinomycete spores in farm air (Lacey 1973, Crook and Lacey 1991).

Alternaria solani causing early blight of potato was found to appear in air during January while the potato plants were vigorously growing and the fungus was increased gradually with the peak at harvesting time, when the farmers working in the field were affected. Different species of *Alternaria* (*viz. A. humicola, A. tenuissima, A. brassicicola, A. tenuis* etc.) were mainly isolated during winter period from the air in wheat, "Boro" variety of paddy, mustard and winter vegetables especially at the time of harvest. The mould *Alternaria* is well recognised for its allergenicity in many parts of the world. *Alternaria solani* has given 17-18% positive skin reactions in the present study. Peat and Woolcock (1991) and Szantho *et al.* (1992) obtained the sensitivity of *Alternaria tenuis* and *A. alternata* respectively by prick skin tests to the children with respiratory illness. There are known cases of bronchial asthma and nasal rhinitis caused by this mould.

Apart from *Alternaria, Drechslera* sp. was found to be predominant in the air of wheat crop after flowering till harvesting and was released in huge amount during harvesting in the month of March every year. It is also an allergenic mould as evident from

the results of skin tests (about 16-19% positive reactions). Prahl (1992) reported, in Western Europe 5-30% of patients with asthma have a positive skin test to microfungal extracts. Only 5% patients gave positive reaction with the *Phoma* extract (Hasnain 1993).

Helminthosporium oryzae, a typical plant pathogen, is released in huge quantity in the air of crop fields in West Bengal, during harvesting and threshing of "Aman" variety of paddy in December of the year. The culture filtrate as well as the mycelial extract of the organism was found to be highly allergenic and was fully demonstrated by the skin reactions and obviously could cause ailments to the susceptible persons engaged in harvesting and threshing operations.

We have valid reasons to believe the possibility that the fungal plant pathogens not only cause the diseases of many economic crop plants year after year, these are similarly associated with the respiratory troubles among the farm workers. This is the reason for which the occupational farm workers sometimes suffer from respiratory allergy or other related problems caused by fungi.

The author is deeply conscious about his limitations to undertake a prolonged study of these antigens. Further investigations are therefore necessary in future to identify, to isolate, to characterise and to purify the allergens present in the mycelium itself as well as in the culture filtrates of the fungal organisms which have skin tested, through radio allergosorbent testing (RAST), immunoblotting, ion-exchange chromatography or other recent techniques like high performance liquid chromatography (HPLC) and recombinant DNA technology and their implications in mould related diseases in human beings.