FUNGAL AER0ALLERGENS IN ZARIA ATMOSPHERE: THE STUDY OF POSSIBLE ALLERGENICITY OF CRUDE ANTIGENS PREPARED FROM FUNGI ISOLATED FROM ZARIA ATMOSPHERE.

.'By:

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A thesis submitted to the Postgraduate School Ahmadu Bello University, Zaria, in partial fulfilment of the requirements for the award of the degree of Master of Science.

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November, 1990

THESIS APPROVAL

This thesis by OLONITOLA, OLAYENI STEPHEN meets the regulations governing the degree of Master of Science of Ahmadu Bello University and is recommended for acceptance in partial fulfilment of the requirements for the degree.

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DECLARATION

I hereby declare that the work reported in this thesis was carried out by me under the careful supervision of Doctors J.D. Dada, M. Galadima and Mrs. L.E. Odama in the laboratory of the Department of Microbiology, Ahmadu Bello University, Zaria.

No part of this work has been submitted for any degree or qualification before. The works of other workers were referred to during this work and they are duly acknowledged.

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CERTIFICATION

This thesis entitled "Fungal aeroallergens in Zaria atmosphere: The study of possible allergenicity of crude antigens prepared from fungi isolated from Zaria atmosphere" meets the regulation governing the degree of Master of Science of the Ahmadu Bello University, Zaria and is approved for its contribution to scientific knowledge and literary presentation.

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ACKNOWLEDGEMENT

My sincere gratitude goes to Doctors J.D. Dada, M. Galadima, and Dr.(Mrs.) L.E. Odama whose assistance and advice were generously given throughout this work. I appreciate Dr. Dada's gesture in not only allowing me the free use of his laboratory, but also his research facilities. I am greatly indebted to these beautiful people for sacrificing their time and energies to ensure the success of this work.

I am grateful to the authorities of the Ahmadu Bello University, Zaria for giving me the opportunity to undertake this work in the institution. I am also very grateful to the Head, Dr. A.A. Ahmad for his concern, encouragement and financial assistance during the research.

I appreciate the moral support received from my colleagues: Akpulu, Akinyemi, Inabo, Ocholi, Kolo, Gimba, Ya'u, Zakari and Addo. The same goes to my friends: Musa, Peter, Funke, Safia, Taiwo, Kenny, Ike, Oladunjoyes, Aishat and a whole lot of them that space will not allow me to mention.

There were those who assisted in one way or

the other during this study. I am particularly grateful to Mr. Olonitola Alfred (Vet. Pathology and Microbiology), Mr. Akubo (Biochemistry) Mr. Adekunle (A.T.C.), Mallam Hussain Diko (W.T.C), Mr. Ugochukwe (Vet. Pathology and Microbiology), Mr. Augustine (Vet. Pathology and Microbiology), Mr. Osasona (Physics), the Head, Irrigation Research Programme, I.A.R., A.B.U., Zaria, the Head, Records Office, A.B.U. Teaching Hospital and several others who could not be mentioned here.

I wish to thank Mr. Garba Stephen (Department of Microbiology) who patiently typed this work and Mrs. Ogundare Snr. whose personal concern and financial commitment ensured the successful production of this thesis. I should also like to thank Mr. Batholomew and Mallam Bawa. I should like to thank a few individuals whom God used to raise my arms above my head in the course of this study: Mr. Oladunjoye Sam, Mr. Osadumo, Dr. Abi, Mr. Babarinsa, Mr. Yakubu and a lot of other people who could not be mentioned here.

Finally, I wish to thank my parents and guardians for their untiring support. I am particularly grateful to my elder brother Levi and elder sister Bola. To them I say God bless. But my greatest gratitude goes to God Almighty, not only

for giving me the opportunity, but the strength and desire to accomplish this programme. I can do all things through Him who gives me strength (Philippians 4:13). For with God nothing shall be impossible (St. Luke, 1:37).

OLONITOLA, O.S.
NOVEMBER, 1990

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ABSTRACT

A survey of fungal aeroallergens in Zaria atmosphere was carried out by air sampling at various points, namely open field (high and low altitudes) homes of asthmatic and non-asthmatic individuals, and an irrigation farm. A total of 38 genera of fungi were isolated from the open field, ,21 from irrigation farm, 18 from the homes of asthmatic patients and 10 from the homes of non-asthmatic individuals. The most frequently encountered genera of fungi in the open field were: Fusarium (13.99%), Curvularia (12,7%), Saccharomyces (10;58%)". Others were Oliocladium (9.15%), Aspergillus (8.14.1%), Penicillium (8.01%), Helminthosporium (5.914-%). The least commonly encountered genera were Syncephalastrum, Spegazzinla, Pcstalotia, Dreschslera, Cunninghamela, Chaetomium, Stylopage, Monllla and Blastomyces. Gliocladium, Curvularia and Penicillium dominated the fungal spor& of the irrigation farm site accounting for 22.7%, 16.16% and 10.92% of total farm isolates respectively, while Aspergillus, Penicillium and Saccharomyces dominated the homes of asthmatic and non-asthmatic individuals - 20.98%, 20,6% and 12.31% respectively. The level of fungal aorospora in the homes of asthmatics and non-asthmatics was not significantly

different p • 0.05. The highest level of fungi in the air was found to occur during the months of January, November, December, May and February (26%, 22.14.%, 21.1%,, 25.83% and 19.89% respectively). This finding correlates well with the records of asthma attendance at the Ahmadu Bello University Teaching Hospital (Asthma Clinic). In an experimental study of possible allergenicity of some of these fungi (Curvularia, Rhizopus and mixtures of antigens prepared from the two fungi) to rabbits, it was found that antisera from animals #iven intravenous route of inoculation caused visible precipitin bands in agar gel, and at the same time the route caused more basophil degranulation than nasal and oral routes.' Curvularia caused a rise of about 120% in the level of serum antibody compared to a rise of about 30% caused by Rhizopus. The route of inoculation did not influence the differential blood counts when compared with the normal, control animals, but affected the onset and duration of symptoms. The concentrations of each of the 3 antigens required to cause 50% basophil degranulation were as follows: Curvularia (63.8ug/ml), Rhizopus (59ug/ml) and mixed antigen (73.9ug/ml). During the second challenge of the animals with the same antigens, the concentrations

required to cause *$0%* basophil degranulation were as follows: Curvularia (101.5lug/ml) Rhizopus (96.!?1ug/ml) and the mixture of Curvularia and Rhizopus (1l5.l6ug/ml). The physical reactions of the animals to the first antigen challenge included scratching of nose and mouth, resting posture, increased breathing rate, stretching and rolling on the floor, inability to stand on fpet etc. During the second.challenge some of the animals died within a week. None of the animals inoculated through the oral route died. One animal each died following intravenous inoculation with the antigens during the second challenge, while all the animals given a second challenge with Rhizopus through the nasal route died. The choi'b'e of Rhizopus antigen for further immunologic studies was based on its consistent isolation in the homes of asthmatic patients.

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CHAPTER ONE

INTRODUCTION

Components of the air spora possessing such qualities as buoyancy, abundance, and allergic toxicity have been termed aeroallergens (Gregory, 1973). These components of the air spora cause allergy in many people but are completely harmless in others; and some may provoke an allergy in animals but not in man (Benveniste, 1978).

Fungi were first recognized as an important cause of allergy by Feinberg in 1935 (Hosen, 1978). According to this author, Morrow, Lowe and Prince shortly thereafter, did extensive work in the field of allergy and were largely responsible for clarification of the role played by fungi in the field of respiratory allergy. Allergic reactions vary widely and have been categorized by various workers. For example, Frazier (1980) broadly classified allergic reactions into Eczematoid, bronchial or nasal, nervous system, gastrointestinal, urticarial or anaphylactic forms; and observed that each or combinations of these may be brought about by inhalation of moulds, dusts and the like; contact with fabrics, plants, metals, drugs and infection with bacteria, viruses, fungi and

parasites. Similarly, contact with irritants and toxins, injection of drugs, serum, ingestion of foods, drinks and drugs as well as stings of insects could bring about allergy. On the other hand, Benveniste (1978) categorized the most important allergens as follows:

a. Inhaled antigens. These include pollens, house dusts, hairs and danders from domestic or laboratory animals such as horses, dogs, cats, guinea pigs, rabbits, etc. (Bach and Salmon, 1978).

b. Ingested allergens: The most frequently encountered are of milk, eggs, and fish obtainable from the environment and especially when these have been chemically preserved.

c. Drugs: Examples are Penicillins, Aspirin, Digitoxin, Quinine, Novobiocin, Propythiouracil, Sulphonamides, Ampicillin etc. (De Weck, 1972).

d. Fungi, bacteria, and their products.

e. Other allergenic substances which may include vegetable fibres.

A number of factors are involved in the development of allergy and these factors have been classified by Frazier (1980) as follows:

1. Genetic predisposition - if allergy runs in one side of a person's family, that person has about 50% chance of developing an allergy himself. If it runs in both sides, the chances for the person being allergic to something increases to around 75%. Furthermore, the allergic disease may not be the same as those of the forebears, nor may the allergens one in hypersensitive to be those of one's relatives,

2. Nature of the allergen itself - protein, carbohydrates, lipid etc.

3. Amount of allergen.

4. Duration of exposure and frequency of exposure. All these factors play a role in the development of allergy.

Gregory (1973) has attributed a number of allergic conditions to aeroallergens. These include:

a. Hay fever (pollent rhinitis) caused by inhaling pollen.

b. Asthma, which may follow rhinitis or may occur without rhinitis.

c. Allergic alveolitis (Pneumonitis) in which allergens are carried by small, deeply - penetrating particles; now exemplified by farmer's lung disease - which is known to result from inhaling air-borne dust from mouldy hay.

d. Other kinds of alveolitis caused by true fungi including Aspergillus funigatus, A. clavatus, Cryptostroma corticale, Alternaria, Cladosporium, Ustilago, Chaetomium spp. and many others.

Airborne fungal spores and mycelial fragments have been established as etiologic agents in allergic respiratory diseases, and numerous surveys have demonstrated their ubiquity (Salvaggio and Seabury, 1971). Gregory (1973) observed that knowledge of air spora is needed for a variety of reasons in allergy. These include:

1. Need for clues to the possible identity of unknown allergens.

2. Need for avoidance of known allergens by precise information on the time of day, season, and locality of occurrence. Some of the methods of reducing allergic diseases in the past include:

   i. reduced exposure to the allergen
   ii. cutting weeds or spraying herbicides
   iii. removal of cats from the home
   iv. avoiding feather pillows and
   v. control of house dusts (Reed et al., 1986).

   Practical application of the strategy of avoidance requires information about the quantity of allergen in the air at different times and places.

3. Need for precision in choice of material for treatment by hyposensitization.

The knowledge of the air spora gained by continuous sampling has led to a widening of the range of allergens known from the restricted group of pollens and moulds formerly recognized. The abundance of basidiospores found in the air immediately suggested that they should be examined as potential allergens (Gregory and Hirst, 1952). The precise knowledge of the fungal aeroallergens in the atmosphere is very important as a first step towards a useful monitoring

of allergic diseases in the environment. Information(s) on aeroallergens in Nigeria is highly desirable. Although asthma is a very common problem in Nigeria, there is not much information on the possible role of fungal aeroallergen as the aetiological agent of asthma. Such information when available, could lead to better patient management by health workers.

The objectives of this work shall therefore include:

1. to investigate the fungal air spora in Zaria atmosphere and to determine their patterns;

2. to find out if there is any correlation between the fungal air spora at various times of the year with the records of asthma cases within Zaria in the same period;

3. to observe possible allergic reactions in laboratory animals challenged with antigens prepared from some of the fungi that have been incriminated in fungal allergy.

CHAPTER TWO

REVIEW OF LITERATURE

2.1 Fungal aeroallergens:

Compared to pollens which have been widely studied as aeroallergens, fungal spores though known to be present in concentrations considerably in excess of pollen grains have been given far less attention (Lopez, et al., 1976; Lehrer, et al.,1986). This observation has been attributed by these workers to reasons such as:

1. Seasonal basis of the presence of allergic symptoms relating to pollens, whose seasonal presence in the atmosphere makes identification of such symptoms easier. On the other hand, fungal spores are often present in the atmosphere over longer, less defined time periods.

2. So many different species of fungi release spores into the atmosphere which makes it almost impossible to identify a particular one causing allergic symptoms without extra work-load being incurred.

3. It has been relatively easy to harvest the large quantities of pollen grains necessary to prepare extracts for the testing of patients, whereas pure samples of fungal spores/fragments are more difficult to obtain; particularly in amounts necessary for study.

Many of the fungi imperfecti are known to induce respiratory allergic reactions in sensitized individuals, leading to the observation that most studies of fungi as aeroallergens have been limited to the fungi imperfecti resulting in little information being made available with regard to allergenicity of other fungal classes (Lopez et al., 1976). The present awareness of the potential importance of basidiospores as aeroallergens could be attributed to Gregory and Hirst (1952). Although these workers were interested in isolating members of the class Deuteromycetes - particularly Alternaria and Cladosporium, they observed a steady high atmospheric concentrations of basidiospores in the open field within a period of three months (between June 1 and September 30, 1951).

The availability of fungal particles in the environment depends on a number of factors which Frazier (1980) summarized as follows:

a. Temperature - Fungi grow well at temperature of 70° to $90^{\circ}F$, some can endure freezing temperature and will remain dormant until thawed out, while some are thermotolerant. Heat and dryness generally affect them.

b. Climate - Release of spores into the atmosphere and even the growth of these spores may be seasonal. Moulds grow best in the presence of oxygen under warm temperature and humid conditions. This explains their greater prevalence in warm, humid climates. In cold areas, moulds seasonally occur in late spring, summer and early fall. In warmer areas, moulds are perennial in nature but tend to be more prevalent in the spring and fall when humidity is higher (Hosen, 1978).

c. Weather - This may also affect spore production and dispersal. It may also affect distances spore can travel, their abundance in the at atmosphere and their distribution.

d. Environment - This depends on varying ecological factors. For example, forest fungi differ from grass and cropland fungi. Frazier (1980) further observed that fungi inhabit wide range

of habitats such as factories, farms, homes, outdoors, forests-in rotting logs and stumps, among the leaves, on the lawn, in the garden, compost pile, flowers or potted plants, in mattress or upholstered furniture, humidifier and air conditioner, on paints, wall paper paste, wood, leather and various other fibres. It may contaminate food (for example, bread), it may be deliberately invited to food as an essential ingredient such as in beer. It may also be found in grains - rusts and smuts; cheese-washer's lung (by Penicillium moulds); bagassosis from mould encountered in handling of bagasses; sequoiosis from moulds in sawdust etc.

All known classes of fungi, including the Myxomycetes are probably important as aeroallergens. Lynch and Poole (1979) listed thirteen occupational allergic diseases and various classes of fungi were responsible either partly or wholly for eleven. Similarly, Salvaggio and Seabury (1971) isolated fungal spores comprising as many as twenty three genera of fungi from the atmosphere over a period of six days. The isolates included species of Cladosporium, Aspergillus, Bispora, Humicola, Hormodendrum, Fusarium and Mucor. Others were Monosporium,

dematiaceous. Penicillium, Helicosporium, Sepedonium, Phoma, Sporobolomyces, Oospora, Alternaria, Graphium, Curvularia, Paecilomyces together with some unidentified genera. Some of the occupational allergic diseases discussed by Lynch and Poole (1979) include: Mushroom picker's lung, Grain dust allergies (both are of immediate allergy). The others are of delayed allergy and include: Farmer's lung, Malt workers lung, Mushroom worker's lung, Bagassosis, Suberosis, Cheese washer's lung, Farmer's lung - like syndromes and Humidifier's lung. All these are caused by diverse groups of fungi.

2.2 The Atmosphere and its spora:

The atmosphere is distinguishable into various layers of which the nearest to the earth is the troposphere. This extends to about 11km in the temperate regions and 16km in the tropics. The troposphere is the layer of the atmosphere where high concentrations of microorganisms are constantly discharged from the soil, vegetation and other sources), (Campbell, 1977). Microorganisms usually occur in air in small numbers compared with soil or water and they are very rarely metabolically active because of the low water and nutrient levels. Also, microbial components of the outside air spora is dominated by fungal spores. Being parts of living organisms, spores

have to be liberated into the environment in which the living organism bearing them are located. This is probably why the air spora comes mainly from species which are highly adapted towards using wind energy for dispersal (Gregory, 1973). Blowing of soil microbes by dust, sea spray and many of man's activities may be responsible for the initial take-off of the airborne microbes (Campbell, 1977).

The observation that the viability of a microbe is not required of an allergen as evidenced by the fact that an allergenic microbe is still active when dead, so long as it is not too far denatured chemically, (Gregory 1973), seems to favour fungi which generally can serve as aeroallergens due to their ability to produce significant percentage of airborne spores as well as the spores and mycelial fragments of most species of the fungi imperfecti (Lehrer, et al., 1986). In other words, the ability of both the spore and mycelial forms of most fungi to serve as aeroallergens is of great significance in the overall effectiveness of fungi as aeroallergens. In the study of New Orleans asthma, Lopez et al. (1976) observed that the late summer and fall months were associated with high total spores and basidiospore catches. From the foregoing, it becomes apparent that any factor or factors which favour aerial dispersal of fungal spores

and/or fungal mycelial fragments will most probably favour an upsurge in the incidence of aeroallergen-induced allergies.

2.3 Variations in Atmospheric Spora

According to Campbell (1977) the density of the air spora varies on an annual and a daily basis. The annual variation is mainly caused by the climate, so that in temperate countries the lower winter temperature are less favourable and there is also increased wash out by rain. In tropical conditions, the temperature may be so high as to cause desiccation resulting in low levels during both prolonged dry conditions and monsoon periods. According to Lacey (1979), fungus spores are almost always present in the air, but their numbers and types vary with time of day, weather, season, vegetation and geographical location. This author noted that spores of Cladosporium, Alternaria, Epicoccuum, Erysiphe, Puccinia and Ustilago spp. predominate on a fine summer day in rural England air, and that these are replaced by spores of Sporobolomyces, Tilletiopsis, Basidiomycetes and Ascomycetes at night.

2.4 Patterns of Spore release

Based on the characteristic day and night

populations of airborne spores, five patterns of spore release have been reported by various workers as follows:

2.4.1 Daytime Release

a. Post-dawn: Liberation mechanisms depend on decreasing or rapidly changing relative humidity, e.g. Phytopthora infestans, Dieghtoniella, Toruloides.

b. Middle day: associated with warmest period when wind speed and turbulence are greatest, e.g. Cladosporium, Alternaria, Ustilago, Erysipe spp.

c. Double peak: reasons for two daytime maximal is uncertain e.g. Helminthosporium, Curvularia, Cladosporium spp. and bacteria (Lacey, 1979).

2.4.2 Night Release:

a. Post-dusk maximum in late evening, reasons are uncertain, e.g. Ustilaginoidea viren, 'tetraspore type; Tilletia spp.

b. Night: Water is required for release, e.g. Sporobolomyces spp. ascospores, basidiospores. These patterns of release will no doubt ensure

the persistence of fungus spores in the atmosphere (Lacey, 1979).

2.5 Seasonal Flourish of agarics and polypores

The orders Agaricales and Polyporales belong to the subclass Homobasidiomycetes, and to the class Bisidiomycetes. The basidiospore is produced by the most advanced class of all fungi, having up to 25,000 species distributed worldwide with extremely high seasonal atmospheric concentrations (Santilli, Jr. et al., 1985). It has been suggested that the steady high concentrations of basidiospores associated with the seasonal flourish of agarics and polypores be investigated in connection with asthma (Gregory and Hirst, 1952). A recent clinical study highlighting the possible relevance of basidiospores in allergy showed that up to 68% of asthmatic subjects in New Orleans area showed immediate skin test reactivity to basidiomycete metabolic and somatic antigens (Lopez, et al, 1976).. Many species of these fungi are found in widely separated geographical areas ranging from open field, city lawns, wooded areas to dead wood-logs or cut lumber from spring to fall (Alexopoulos, 1962).

Moore - Landecker (1972) separated the Agaricales into two major groups. The first group - the "boletes" - belongs to the family Boletaceae in the

temperate regions are commonly encountered in the late spring, summer of fall and are especially abundant on the group under conifer trees in the fall. The second group is the "agarics" of the family Agaricaceae. The group includes the mushrooms and toadstools. They are found in wide variety of habitats such as lawns, forests and fields. The polyporales on the other hand, include the "bracket" or "shelf" fungi. The members occur predominantly on living trees and on dead woody plant parts such as tree trunks and stumps. A few occur as saprophytes on soil; the majority of these bracket fungi are annual, but the species of Fomes are perennial. In a study of selected members of the Agaricales and Polyporales, wide seasonal occurrences were recorded (Lehrer, et al, 1986). They were also found to inhabit a wide variety of habitats.

2.6 General Observations on Allergens:

Under natural conditions of exposure, atopic persons become sensitized on repeated challenges with extremely small doses of allergens, especially inhalants (King, 1976). It is being speculated that allergens are possibly different from antigens, but there are evidences - based on chemical that they are not different from antigens. Some of the evidences reviewed by King (1976) indicate that allergens are

fundamentally similar to antigens. For example, the inhalants, foods or insect venoms are all multi-allergen systems with each source having one or more major allergens to which the majority (~90%) of allergic persons are highly reactive, and several minor allergens to which a smaller group of persons (10-50%) is also highly reactive. This individual variations indicate that the allergenicity depends both on the chemical nature of allergen and on the response of the host.

Furthermore, the major allergens from inhalants and foods are all acidic proteins while the minor allergens can be acidic or basic proteins. All are globular proteins as indicated by their chromatographic properties on porous gals and by their diffusion coefficients. Some allergens contain carbohydrates, and some do not. Some are proteins of a single polypetide chain (ragweed antigens $C_{3,4}$, and $_5$; Codfish allergen M; Serum albumin) while others are proteins of two non-identical polypetide chains (Beta-lactoglobulin). The amino acid compositions of most of the allergens do not show any unusual chemical features. As evidenced by the loss of allergenicity on denaturation, the dominant antigenic determinants of the major allergens are dependent on both the primary structure and the conformation of the mloecules. This property is shared with other globular proteins.

Apart from natural allergens, such as pollen, mould, ragweed, mite, danders etc evidences now show that other proteins can stimulate IgE responses in atopic or normal individuals. Salvaggio, et al (1964) demonstrated this fact in bovine pancreatic ribonuclease. Other workers have demonstrated the fact with various other proteins. For example, Kuhns (1962) demonstrated it with diphtheria and tetanus toxoids; Pepys et al, (1969) with laundry detergent additives, proteases, and amylases from Bacillus subtilis. The widely different biological functions as well as origins of these protein allergens also argue for the unlikeliness that they may have certain structural features particularly suited for the induction of IgE production. As observed by Tada and Ishizaka (1970), IgE-forming plasma cells are predominantly located in the respiratory and gastrointestinal tracts, but most allergens are absorbed on inhalation or ingestion through the respiratory or gastrointestinal tracts and since there will be an upper size limit to the solutes that can be rapidly absorbed through the mucosal membrane, the immunogenicity (allergenicity) potency of a protein will obviously depend on its molecular complexity. Immunogenic potency also depends on the degree of foreigness of the protein and the genetic make up of the immunized host (Bellanti, 1978). King (1976) argued

that the combination of permeability and immunogenicity requirements may be the explanation to why proteins of molecular weights of 20,000 to 40,000 are better allergens than proteins of other sizes. This is because, they are not too large, so they can not be readily absorbed through the mucosal membrane of atopic individuals. Similarly, they are too small so that they can be sufficiently complexed to be good immunogens.

Various animal model studies have brought to light, the facts that:

a. Atopic persons differe from normal persons in their ease to respond immunologically to minute doses of antigen.

b. Low-dose immunization favours persistent and boosterable IgE than IgG production, and the reverse is true for high-dose immunization for atopic persons.

c. Both IgE and IgG responses to specific antigens are under the control of histocompatibility linked immune response genes. According to a review by King (1976), the presence of an IgE - regulating gene in man has been postulated, and this gene regulates the serum level of IgE. The IgE - regulating gene is believed to play

an important role together with immune response gene in the expression of atopic allergy in man.

2.6.1 Uses of Purified Allergens:

Attentions have been focused on the possibilities of relieving or treating allergic conditions in sensitized individuals. One of the various possibilities is the use of hyposensitization therapy. One important use for purified allergens is in in the standardization of allergen extract; which are important reagents for the diagnosis and treatment of a number of allergic disorders. Other uses of purified allergens are as reagents for studying the immunological mechanism of immunotherapy and for the development of chemically modified derivatives better suited for immunotherapy.

It has been observed (King, 1976) that on immunotherapy, there is usually an initial rise in the serum level of allergen-specific IgE followed by a slow decrease, but the serum level of allergen specific IgG continues to rise during therapy, usually reaching a plateau. The best clinical improvement has been associated with high doses of allergen extracts, high serum levels of allergen-specific IgG, and lower levels of specific IgE. It has been suggested that these changes could form a feedback suppression of

IgE by IgG antibody. It could also be as a result of IgG antibody being able to serve as blocking antibody for IgE antibody (Benveniste, 1977; Barber, 1977).

2.7 Allergy:

Allergy is a term originally used to describe altered immunological reactivity to a foreign material (Barber, 1977) and thus could involve either delayed or immediate types of hypersensitivity. In clinical medicine, however, allergy refers to a medical subspecialty and research area dealing with diseases of immediate hypersensitivity (Lichtenstein, 1972). Allergic reactions are triggered when allergens to which an individual has become sensitized combines with cell-fixed IgE antibody against it. This union initiates a complex multistep response; modulated by cyclic Adenosine Mononucleotide Phosphate (AMP) which leads to the extracellular secretion of intracellular granules containing the mediators of the reaction. The union can take place in diverse locations at very low antigen and antibody concentrations. The sequence of events leading to the release of mediators can be influenced in several ways. For instance, pharmacological agents (or endogenous hormones) can break the cycle at several different stages, leading to a failure of mediator

release, or the effects of the released mediators can be blocked, leading to the absence of a physiological effect.

2.7.1. The Allergens:

A variety of materials can cause allergic states in man. Such materials as simple polysaccharides (dextran), complex proteins, many material acting as haptens (penicillin, attached to a schleppers) all act as allergens. According to Lichtenstein (1972), the most clinically important allergens are derived from pollens of grasses, and trees and the allergenic extracts used for skin testing and immunotherapy are crude aqueous extracts of pollens, moulds, danders and others such as dust - which can be made by extracting the debris from a crumbling mattress or from vacuum cleaner sweepings into buffer. Studies have shown that the major allergen in these natural substances are simple proteins with a molecular weight of 30,000 to 40,000, and with no unusual structural features or prosthetic groups (King, 1976). Other allergens are known. For example such materials as codfish allergen M, serum albumin (which must be artificially extracted), diphtheria and tetanus toxoids, insulins detergents, enzymes (such as proteases and amylases from Bacillus subtilis etc) have been shown to be

allergenic (Salvaggio, et al, 1964; Kulns, 1962; Pepys, et al. 1969).

2.7.2 The Antibodies:

According to a review by Lichtenstein (1972), reaginic antibodies were first described in 1921 by Prausnitz and Kustner who demonstrated that cutaneous allergy could be transferred to a normal recipient by the intradermal injection of serum from an allergic individual. It was later observed that the reaction was absolutely antigen-specific, could occur at high dilutions of serum, was obviated by heating the serum at 56°C for a few hours and did not have any relation to the antibody content of the serum as measured by standard serological tests. The works of Ishizakas climaxed the attempts of allergists to isolate and characterize reagins. Ishizaka and Ishizaka (1968) showed that the reagin antibodies belong in a unique class of immunoglobulin which they called IgE. Lichtenstein has argued that the claim by some investigators that some or all reagins are IgG is not widely accepted and that while these claims are possible, IgG reagins - at best-play little or no role in human allergic reactions. According to him, measurements of total IgE levels in the serum have shown that atopics have higher levels than normal individuals, but that the

range in each is great. For example, the levels of IgE in serum could not be used to diagnose atopy because the highest levels are not found in atopics, but in individuals with intestinal parasites, in whom the IgE concentrations may exceed 10,000 ug/ml. This may be due to the location of IgE-producing cells in the submucosals (Tada and Ishizaka, 1970). Lichtenstein, also observed that there is concensus on the fact that IgE antibodies are produced by the lymphocyte/plasma cell series as are the other immunoglobulins, and that the location of these IgE - producing cells are quite distinct. For example, they are sparse in the spleen, and in most sets of regional lymph nodes, but more represented in tonsillar tissues, peyer's patches and, in general, in proximity to the respiratory and gastrointestinal mucosal surfaces. Tada and Ishizaka (1970) have made similar observations.

2.8 Atopy:

The origin of the term 'atopy' was traced to Cooke and Vander veer who in 1916 described human protein sensitization in 621 patients suffering from asthma due to animal exposure, seasonal hay fever due to pollens, urticaria and angioneurotic oedema and acute gastroenteritis due to certain foods (Pepys, 1975). According to Pepys, these workers found a familiar association of these diseases, and the

inherited factors. Coca and Grove were reported to later introduce the term atopic reagins to describe the specifically reactive substances in the serum of atopic individuals. They found atopic reagins in the blood of all the subjects with hay fever and asthma who gave cutaneous reactions to the atopen. This reagin according to Pepys- were found to be non-precipitating, heat-labile, specific and long-lasting in their passive sensitization. Many challenges have been thrown at the concepts of atopy as it was originally construed. For example, atopy is not confined to man since similar manifestations have been observed in other animals (Patterson, 1960; Ishizakas, 1971).

In Pepys opinion, a single defining characteristic should be agreed for the term 'atopy'. This definition should not carry implications such as clinical (that all rhinitis and asthma is atopic) nor immunological (that all evidences of reagin IgE antibody, is necessarily clinically relevant). The essential feature of atopy, Pepys believes, is that form of immunological reactivity of the subjects in which reaginic antibody is readily produced in response to ordinary exposure to the common allergens of the subject's environment.

The essential feature in atopy is 'natural' sensitization in which the route of entry of the allergen is through mucosal surfaces (Pepys, 1975). This observation was supported by the results of the works done by Leskwitz et al., (1972). These workers compared the effects of intranasal administration or injection of purified protein and polysaccharide materials in atopic and non-atopic subjects. In all cases, they observed that the atopic subjects were more readily sensitized by the intranasal route to give type I skin test reactions than the non-atopics. On the other hand, there were no differences in the responses of either group in the case of sensitization by injection. Thus suggesting some abnormality of the mucosal surfaces in atopic subjects - possibly genetic abnormality.

Another possible factor that can influence the development of atopy is deficiency of secretory IgA. Decreased secretory IgA activity in atopics has been shown by some workers. For example, a lower incidence and weaker response of secretory IgA was found in atopic than in non-atopic individuals who were given intranasal administration of tetanus toxoid (Salvaggio et al., 1973).

Subjects classified as immunologically atopic - and without clinical implications are those with

multiple sensitivity and are said to be of high atopic status, while others with limited or isolated sensitivity are of low atopic status. According to Pepys (1975) the manifestation of atopic immunological status depends on the extent of allergenic exposure, and that individuals who develop IgE antibody solely in response to peculiarly potent allergenic exposures, for example, in Ascaris infestation means that the subjects showed reactions which are reaginic in nature but could not be classified as atopic, since they usually do not show sensitivity to the common allergens encountered in the ordinary way. According to this investigator, the earlier the age of onset the higher is the atopic status, with those starting below the age of 10 having 90% positive reactions and a large proportion reacting to many allergens.

Johansson, (1967) found elevated serum IgE in asthma and in about two years later, similar findings were reported in hay fever in Berg and Johansson (1969). These findings provided the earlier evidences for the role of IgE in atopic allergy. Raised serum IgE levels have been found in 40%-60% of patients with extrinsic asthma, whereas in intrinsic asthma normal IgE values are regularly found (Johansson and Foucard, 1978). According to these

investigators, an increase of the IgE concentration occurs when the patient is exposed to allergen to which he is sensitive, and patients with multiple allergies have a higher IgE level than patients with a single allergy. IgE antibody to inhalant allergens can rearely be detected in serum from healthy people. This fact is supported by the observation that only one positive RAST reaction was found out of about 600 tests carried out on donated bloods selected for test of their freedome from allergy symptoms using ten common allergens. (Berg and Johansson, 1974) in contrast to the observation that almost all patients with a clinically manifested inhalant allergy of the immediate type have detectable amounts of IgE antibody in serum.

2.8.1 Immunotherapy of Atopic Disorders:

Hyposensitization is the commonest treatment device for allergic diseases especially in areas where avoidance of the allergen is not possible and it has been in use for a very long time (Noon, 1911); and Aas (1971) who used prolonged treatment with house dust extracts have confirmed the efficacy of this form of treatment. Similarly, Oehling (1977) stated that hyposensitization therapy had been employed in his clinic for over 20 years and that when compared with various other methods, hyposensitization

was considered to be the most efficient and gave the best results. Many attempts have been made of explaining the mechanism of hyposensitization therapy. It has been reported that there is early rise in specific IgE antibody with hyposensitization, followed by a gradual decrease as hyposensitization continues (Berg and Johansson, 1971b). The production of blocking antibodies which reduce reactions in passive transfer tests have also been reported (Maunsell, 1968). Associated changes in both IgE and IgG antibodies may be related to clinical improvement, and a possible explanation for this association could be the suppression of reagin synthesis by blocking antibodies (Pepys, 1975).

There could also be cellular mechanisms in hyposensitization. Lichtenstein et al. (1971) found that ragweed treated subjects showed a decrease in Leukocyte histamine release three times as frequently as untreated subjects and that this was associated with clinical improvement. The duration and dosages of treatment are quite important since prolonged treatment with maximum tolerated dosage of grass pollen extract gave the best clinical results. Similarly, it has been observed that total dosage is important, even if given over a short period of time (Sadan et al., 1969).

2.9 Epidemiology and Natural History of Asthma:

The knowledge of the epidemiology of a disease and what sets the stage for its development is basic to a search for its causes, and understanding the natural history of disorder is basic to therapeutic judgement (Smith, 1978). Whereas, the natural causes of asthma have been studied extensively in children, those in adult life have been studied little since 1978, and according to Smith (1978), the following possible factors influence the development of the disease:

1. Family history - Asthma is frequently transmitted within families, and according to Frazier (1980) if allergy runs in one side of a person's family, that person has about 50% chance of developing an allergy himself. If it runs on both sides, the chances for the person increases to about 75%.

2. Age - It is believed that youth is the most usual time to develop allergy, but it is possible to manifest symptoms of extrinsic asthma at any age (Smith, 1978). The other factors as enumerated by Smith include sex, race, infection, pollution, Psychological factors, social class, and exposure to allergens.

Reed and Townley (1978) quoted the joint committee of the American Theracic Society and the American College of Chest Physicians as having defined asthma based on clinical findings as a disease characterized by an increased responsiveness of the airways to various stimuli and manifested by showing of forced expiration which changes in severity either spontaneously or with treatment. The term asthma may be modified by words or phrases indicating its etiology, factors provoking attacks, or its duration.

The following list of classes of recognized allergens are able to induce asthma: Tree pollen, Grass pollen, Weed pollen, Animal hairs or feathers, Moulds or fungi, Insects (not insect sting), occupational dusts, chemicals (not acting as irritants), House dusts, foods etc. (Reed and Townley, 1978). These workers have also enumerated the stimuli that provoke asthma and these are: allergens (as listed above), aspirin and related substances, infections with such agents as bacteria, respiratory viruses (like respiratory syncytial virus and parainfluenza virus, rhinovirus and influenza virus). (Simon and Jordan, 1967; Horn and Gregg, 1973; McIntosh, et al., 1973; Minor et al., 1974; Minor et al., 1976). There are other associations between viral infections and the incidences of asthma. For instance, children with

asthma have more viral respiratory infections, especially rhinovirus, than their healthy siblings (Minor, et al., 1974). Other possible stimuli include: situations and emotional responses, exercise and irritants. Reed and Townley (1978), observed that the heterogeneities in pathogenesis and pathophysiology is directly responsible for heterogenous responses to therapeutic agents.

2.10 Implications of Spore Release into the Environment:

One of the major implications of the continuous release of fungal spores into the atmosphere as aeroallergens is the continuous exposure of the populace to these allergens. About 10% of the population have been shown to have a predisposition to becoming sensitized due to ordinary exposure to normal environmental concentrations of allergens, often leading to multiple sensitization to a variety of substances (Gregory, 1973). This group of people are said to be atopic (Bach and Salmon, 1978). Atopy is defined by Pepys (cited by Bach and Salmon, 1978; Gregory, 1973) as an abnormal form of immunologic reactivity in which immunoglobulin E(IgE) reagins results from ordinary exposure to common environmental allergens; particularly when the allergen is inhaled or ingested. Bach and Salmon (1978) further stated that atopy may range from single sensitization to a given allergen to

polysensitization. Thus, the more diverse the spore varieties in the atmosphere, the worse for the atopic individuals. Also, the observation by these workers that genetic and other factors such as certain parasitic and bacterial infections may augment IgE production and reinforce atopy is of significance with respect to the 90% non-atopic members of the population.

The central roles of IgE in allergic reactions have been well studied. Benveniste (1978) observed that other immunoglobulins do not play an important role in the onset of allergic manifestations in man; although the situation may be different in animals. He also pointed out that both effector cells and mediator substances are needed in immediate hypersensitivity reactions to which allergies no doubt belong. The effector cells are basophils and mastocytes. This may not be unconnected with the fact that although they have different origins, both cells contain numerous, highly osmophilic granules that contain heparin and proteins which act to support various immediate hypersensitive mediators (Chase, 1965). These mediators include: Histamine, Serotonin, slow-reacting substance of anaphylaxis (SRS-A) and the platelet-activating factor (PAF) (Benveniste, 1978; Chase, 1965). The interaction between the

antigen (allergens) and IgE on the mastocyte and basophil surface is believed to initiate a series of very complex intracellular events. The cyclic Adenosine monophosphate - Guanine monophosphate (CAMP-GMP) system regulates this response. IgE fixation on basophils and mastocytes and the response of these IgE-coated cells in the presence of antigens may lead to the release of the mediator substances, which in most cases are stored in the effector cells as granules from which it is released during anaphylactic degranulation (Benveniste, 1978).

2.11 Methods of Sampling Aeroallergens:

2.11.1 Sampling Instruments:

Study of airborne microbes necessitates their separation from the air, cultivation, identification, enumeration and classification. Of the different sampling techniques for trapping microorganisms, filtration, impingement into liquids and impaction on solid surfaces are probably the most important (Lynch and Poole, 1979). Dransfield (1966) however observed that the two fundamental methods of sampling air-spora are deposition by gravity and by impact from an air stream. Three general methods of estimating the quantity of airborne allergens include:

1. Counting morphologically identifiable allergenic particles, such as pollen grains and moulds spores.

2. Culturing viable particles such as fungi and bacteria.

3. Assaying chemicals such as diisocyanate by sensitive specific chemical analysis (Reed et al., 1986). The inability of these methods to measure amorphous, non-viable airborne allergens prompted these workers to device a system for measuring airborne antigens by collecting some on a 20 x 25cm fibre-glass filter paper sheet by the use of a high-volume air sampler. The antigens were eluted from the filter paper by descending chromatography into a collecting beaker, and then assayed by a radioimmunoassay inhibition technique.

Of the various air sampling instruments, two have passed into routine use. These are:

a) Hirst's automatic volumetric spores trap (a power-driven trap designed for operating continuously in the field). Burge et al. (1977) used a wind-oriented variety in their work, as well as Gregory and Hirst (1952) during their investigation into the possible role of

basidiospores as air-borne allergens. Salvaggio and Seabury (1971) favoured this instrument because it has been reported to yield more quantitative data than conventional gravity samplers. Therefore its greater efficiency, known air sampling volume, and larger collected quantities allow for greater statistical reliability.

b) Anderson Sampler - Air is sampled at the rate of 28.3 litres per minute (Gregory, 1973). Burge et al., (1977) used this instrument along with the Burkard (Hirst) sampler to compare the recoveries of airborne fungus spores by viable and non-viable modes of volumetric collection. These workers noted that non-viable techniques require only that particles have distinct form while germination and growth potential, which govern viable recoveries, are multifactorial variables and often defy accurate prediction. Salvaggio and Seabury (1971) used timed exposure of agar plates along with the rotoslide samplers. However, only plates exposed during certain epidemic intervals were examined since "it soon become apparent that plate counts did not aid in presumptive identification of the large numbers of spores collected on 24 hour rotoslide sampler."

Similarly, the use of timed exposure of agar plates depends largely on gravitational forces. This is only effective if the spores are large enough to respond to gravitational pull at the prevailing atmospheric condition.

According to Southworth (1974), no one sampler does everything, and inspite of the fact that gravity slide and culture plates are not quantitative, they are widely used by physicians. Gravity slides and culture plates are equally favoured for various other reasons. For example, Dransfield (1966) made use of these instruments because, according to him "no apparatus of the Hirst spores trap or its equivalent was available in Northern Nigeria" when he was carrying out his investigations. In their studies on the airborne fungi during harmattan in Zaria, Nigeria, Lawande and Onyemelukwe (1984) used culture plate method "based on the exposure of plastic petridishes containing sabouŕand's dextrose agar medium." Samplings were done at 10 metres and 1.5 metres above ground level. No considerable differences in the counts at these heights were noticed. These workers were able to isolate as many as thirteen fungal genera and a total of one thousand, one hundred and ninety one (1,191) viable colonies over a period of four months between November 1980 and February 1981. The fungi reported by them were Fusarium (19.39%), Alternaria

(19.22%), Penicillium (13.09%), Cladosporium (11.83%) Apergillus (10.91%) and Helminthesporium (7.8%). Others included Candida (6.21%), Curvularia (4.78%), Chaetomium (2.18%), Epicoccum (1.67%), Geotrichum (1.59%), Mucur (0.75%), Trichoderma (0.5%) etc. Although these workers were mainly interested in viable airborne fungi in Zaria, any investigation about allergenicity of airborne particles must of necessity embrace both viable and non-viable particles, because both can be allergenic (Gregory, 1973). The non-viable aeroallergens can be estimated by use of gravity slide method (Southworth, 1974). This can be done by finding the differences between viable counts (on exposed culture plates) and the total count on grease-coated, exposed gravity slides. Leventine and Hordwitz (1978) observed more than eleven thousand (11,000) colonies and fifty-four (54) genera while undertaking a one year survey of the airborne moulds of Tulsa, Oklahoma. In their studies, the plate culture technique was used. Plates containing Potato Dextrose agar with 0.1% Streptomycin were exposed for ten (10) minutes at a time. Five plates were exposed at each location each month. In a related investigation, a survey of allergenic airborne pollen and spores in the Fair Banks area of Alaska was carried out by Anderson (1984). This investigation involved about 13 collaborators in 13 different stations. Between 14 and 25 genera of

fungi were recovered at each station. The gravity plate method was employed "in order that airborne flora could be identified following growth on nutrient agar surfaces," Two plates were exposed for 15 minutes at each station, twice a month. It was observed during their investigation that many species of fungi settled on the exposed plates in slime masses, clumps of spore heads, single cells, fragmented mycelia of many cells and as non-viable flora. It was considered that any mould or yeast encountered was a dominant and a potential aero-allergen.

2.11.2 Elevation and Particle Collection:

Burge et al. (1977) carried out their samplings for fungal spores on the roof of a building about 46 metres above ground. There was no parapet, and the site was unobstructed. Simultaneous particle collections were made between 08.30 and 09.30 hours on weekdays under a variety of weather conditions. A total of 124 paired samples were used for analysis. Unlike Burge and his co-workers, Salvaggio and Seabury (1971) placed two samplers in a central business district; approximately 150 feet (about 46 metres) above ground level on top of a students' dormitory. A second station with a single sampling device was located 8 feet (about 2.4 metres) above

ground level on a golf course near the geographic centre of the city. Samples were collected twice daily, in most cases at approximately 7.00 a.m. and 6.00 p.m. In their studies, concerned primarily with fungi pathogenic on crop plants, Gregory and Hirst (1952) sampled the air at the height of 2 metres above a potato plot continuously for three months. Their choice of site "in an arable field adjacent to grassland with trees" clearly demonstrated that these workers and the others earlier cited, chose sites at which free movement of air was not obstructed.

2.11.3 Treatment of Samples:

Salvaggio and Seabury (1971) used a rotoslide tetrabar containing four sampling slides. Two slides were stained with cotton blue stain for spore identification and quantitation. The other two slides were stained with a modified Calberla's solution for pollen quantitation. The Sabouraud's dextrose agar plates were incubated at room temperature for three days, wrapped in sealed plastic bags, and stored at $4^{o}C$ for variable periods prior to subculture and colony counting. Burge et al (1977) on the other hand, cut the particle-bearing tapes into 24 segments after removal from the Burkard trap, each segment was then mounted on a 1 x 3 inch microslide in hard

glycerine jelly. Particles counts were made at 312.5 x from 50-60 fields (each 464 micrometres in diametre) inspected, using a graduated mechanical stage. In the Anderson sampler, Malt extract agar was used. Carefully timed sampling periods of 30 seconds to 10 minutes were employed to prevent overloading on prevailing spore levels. Following exposures, the plates were incubated in high humidity at 22°C (for seven days and all growth examined microscopically). Gregory and Hirst (1952) had the spores impacted on a 'Vaseline' - coated slide. The resulting 24 hours trace were then scanned under the microscope. Other workers have employed these methods in their works (Dranshied, 1966; Lawande and Onyemelukwe, 1984).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Preparation of Reagents and Media:

a. Czapek Dox Broth

	grams/litre
Sodium nitrate	2.0
Potassium chloride	0.5
Magnesium glycerolphosphate	0.5
Ferrous sulphate	0.01
Potassium sulphate	0.35
Sucrose	30.0

The contents were well mixed and sterilized by autoclaving for 15 minutes at 103.35KPa (121°C)

b. Physiological Saline

Sodium chloride	0.9g
Distilled water	100ml

Sterilized by autoclaving for 15 minutes at 103.35 KPa (121°C).

c. Lactophenol cotton blue stain

Phenol	20g
Lactic acid	20ml
Glycerine	40ml
Distilled water	20ml
Cotton blue	0.05g

Materials were first heated gently under hot water to dissolve before cotton blue was added.

d. Giemsa Stain

Powdered stain	1g
Glycerol	66ml
Methanol	66ml

1g of the powdered stain was weighed into a conical flask. 66ml of glycerol weree added and the mixture was heated at 56°C for 120 minutes in a water bath. 66ml of methanol were then added and after thorough mixing, the solution was allowed to stand for 7 days at room temperature before filtered and diluted to 1:15 v/v ratio in phosphate buffered saline (pH 6.8).

e. Agar gel

Iron agar	1g
Distilled water	100ml
Sodium azide (0.1% v/v)	1ml

f. Phosphate Buffered Saline (pH 6.8)

	grams/litre
Sodium chloride	8.00
Potassium chloride	0.2
Di-sodium hydrogen phosphate	1.18
Potassium di-hydrogen phosphate	0.2

The materials were dissolved in 1000ml of distilled water and stored at 4°C prior to use.

g. Potato Dextrose agar (Lab. made)

Potato Extract	100ml
Dextrose	20g
Agar	15g
Distilled water	900ml
Tartaric acid (1M)	10ml

The mixture was sterilized by autoclaving for 15 minutes at 15lb/sq inch (121°C), allowed to cool down to about 45-50°C prior to pouring.

3.2 SAMPLING SITES

The samplings of fungal spora were carried out at the following sites: open fields, irrigation farm site and indoors (homes of allergic asthma and non-asthmatic individuals). Two sites were used for a year-round sampling of fungal spora in the open field, and these were designated as high altitude and low altitude respectively (Larsen, 1981; Al-doory, et al., 1980; Lawande and Onyemelukwe, 1984).

High altitude

This site was the top of a water tank, about one and half metres high and at least, 0.5 metres above the parapet of a two-storey departmental building. The site stands at between 12 and 15 metres above ground level and was not altered

throughout the investigation. There were no buildings higher than this point from all directions and therefore, no obstructions to the free air-movement from any direction. The reason for chosing this site was to ensure that fungal spores and mycelial fragments with light weights and poor responses to the force of gravity are adequately collected for qualitative and quantitative analysis.

Low altitude

This site was the top of a platform, about 1.5 metres (breathing level) on the university main sports field. This site was relatively open with the closest building being over 200 metres away from the sampling site; consequently there were no obstructions to the free air movement. The reason for choosing this site was to ensure that fungal spores and mycelial fragments with heavy weights, and with good responses to the force of gravity are adequately collected for qualitative and quantitative analysis.

Irrigation farm site

A raised stump, about 1 metre high was used as the sampling site; and the site was maintained for the entire sampling period on the farm site. Farmers are known to be highly disposed to particle

sensitization as a result of constant exposure. Moreover, little evidences are available on the aerospora of farm sites (Sneller and Roby, 1979). The reason for choosing this site was to determine the fungal spora of a farm site to which farmers could be having prolonged exposure.

Indoor samplings

The homes of asthmatic patients and normal individuals were sampled for viable fungal spora. Sampling sites were on top of the bed pillows of the individual patients (Sneller and Roby, 1979). The reason for choosing this site was to determine the most abundant fungal spora of the homes of these individuals since it has been reported by Roby and Sneller (1979) that in almost all instances there is greater percentage correlation of positive skin test with indoor colony forming units.

3.3 Methods of Sampling

TIMING: All samplings were done between 9.00 hours and 10.30 hours on each samply day (once weekly). However, in the case of farm site, samplings were done within the same time period, but on different week-days. Samplings in the homes were done when no

activities of inhabitants were going on. This is to ensure that particles were not unnecessarily discharged into the air during sampling. If any form of human activities were going on, especially on the farm and the field, samplings were deferred for about 2 hours or shifted to the next day between the chosen hours. Similarly, samplings were not done on days when there were rainfall before the sampling hours.

Viable Sampling

Standard petridishes (15 x 100mm) containing either Saboraud's dextrose agar or Potato dextrose agar (acidified with 1% v/v tartaric acid) were employed for sampling throughout the investigation. The plates were exposed for exactly 10 minutes and immediately labelled. A time of 15 minutes was allowed in the case of indoor air sampling. For every sampling session, an unexposed plate of Potato Dextrose agar was used as a control to detect contaminations. After exposure, the plates were returned to the Laboratory where they were incubated at room temperature (Dransfield, 1966; Al-doory, et al., 1980; Larsen, 1981; Reed, et al., 1986; Lawande and Onyemelukwe, 1984). This temperature was considered to be optimum growth temperature for saprophytic fungi (Meyer, et al., 1983). After satisfactory

growth (3 - 10 days), colonies were counted and purified by subculturing on to fresh, sterile agar media.

Total fungal particle sampling

Thoroughly cleaned and sterilized glass slides (25 x 75mm) were thinly coated with petroleum jelly (Petroleum B.P. Chesebrough - Ponds Inc.) and exposed side by side, inside a petridish (to counter the effects of the raised edges of the petridishes above the agar level). The dishes were immediately covered after 10 minutes exposure and promptly labelled. Particle count were made at 100 x objective, following gentle flooding of the slides with lactophenol cotton blue. Counts per field were taken as mean of 60 fields scanned (Burge, et al., 1977). Total counts were determined using the formula:

$$\text{Total fungal particle} = \frac{\text{Average count/field} \times \text{area of plate}}{\pi r^2}$$

where r is the radius of the microscopic field which was taken as 0.08mm (Collins and Lyne, 1976).

3.4 Mould and yeast identification

All viable mould isolates were identified using the conventional methods of microscopy and slide

culture preparations. The schemes of Wolf, et al., 1975; Smith, 1969; Beuchart, 1978; Collins and Lyne, 1976 etc were employed for the identification of the yeast isolates, while those of Barnett and Hunter (1972) were employed for the identification of the mould isolates.

3.5 Meteorological data

Meteorological data were taken for the entire sampling period at the Samaru Weather Station (Irrigation Research Section), Institute for Agricultural Research, Ahmadu Bello University, Zaria. These data were later used to study the influence of the various elements of weather on fungal air spora level.

3.6 Annual flourish of agaricales and polyporales

Fungal forrays were conducted in search of members of the Agaricales and Polyporales in Zaria area throughout this study. The samples were collected during the raining season between the months of June and September. Searches were directed mainly to open field, refuse dumps, dead stumps, tree trunks, dead woods, under the shade of taller trees, animal dungs, gardens and farm sites where these organisms are commonly encountered (Alexopoulos, 1962). When

encountered, they were identified in sittu, and harvested to the laboratory for further identification. Identifications were based on the schemes of Pilat and Usak (1961) and those of Bon, et al. (1987).

3.7 Immunological studies

3.7.1 Preparation of fungal antigen for animal inoculation

Selected mould species were grown at room temperature in Czapek Dox broth for 21 days on Orbit environ-shaker 18 (Lab-line instruments, Inc. Illinois) - to discourage sporulation (Lopez, et al. 1976). Filtrates were kept for further possible use. The mycelial mat were harvested by filtration with Whatman No. 1 filter paper, washed twice in sterile distilled water and concentrated by centrifugation at 5,000 rpm for 15 minutes in Bench centrifuge (Baird and Tatlock (London) Limited). The concentrated mass were then slow-dried at $40^{o}C$ for 48 hours in a drying oven (Gallenkamp England). The dried fungal mass were then crushed into very fine powder in a clean mortar. The powder was designated "crude fungal antigen."

Before use, the fungal antigens were suspended in physiological saline and filtered through glass fibres. Dry weight of the suspension was determined

as follows: A cleaned crucible was weighed, 10ml of the antigen suspension was then pipetted into the crucible, weighed and allowed to dry up at 100°C in a drying oven (Gallenkamp England). The crucible was then allowed to cool and re-weighed. The dry weight of the suspended antigen was taken as:

(Weight of crucible + antigen suspension) - (weight of crucible + dry antigen) = weight of water. Weight of antigen = (weight of crucible + antigen suspension) - (weight of crucible + weight of water). The antigen suspensions were then diluted with sterile physiological saline so that they contained 125 ug/ml of antigen for animal inoculation.

3.7.2 Animal inoculation

For the purpose of animal inoculation, 21 rabbits were divided into 7 groups of 3. Each group comprised 2 test animals and 1 control - appropriately marked with dyes. The animals were allowed to adjust to the laboratory environment for a week, after which a set of blood samples was collected for differential blood cells count, Basophil degranulation test and for the preparation of sera. Primary inoculation was done 3 days later.

3,7.2 (i) Primary inoculation

The first 3 groups of rabbits were challenged with fungal antigens prepared from Curvularia lunata as follows: Aliquots (0.1ml) of physiological saline containing 50ug of the antigen were inoculated into 2 animals each intravenously, orally and intranasally. Intravenous inoculation was done through the marginal ear veins. In each case, a third animal which was not challenged served as control. The next 3 groups of rabbits were similarly inoculated with antigens prepared with Rhizopus stolonifer. The last one group comprising 3 rabbits were inoculated intravenously with mixed antigens prepared by mixing Curvularia antigen with Rhizopus antigen in the ratio of 1:1 w/w and then suspended in physiological saline at a concentration of 125ug/ml.

Seven days later, a second set of blood samples were collected for differential blood cells count, Basophil degranulation test and for preparation of sera.

3.7.2(ii) Secondary inoculation

The animals were inoculated a second time with antigens as described under section 3.7.2.(i).

Another set of blood samples was collected 4 days after the second inoculation. 2 more sets of blood samples were collected at 4 days interval after the second inoculation. Following each inoculation, the physical reactions of the rabbits were observed and recorded.

3.7.3 Differential blood cells count

Blood samples for differential blood cells count were drawn into heparinised microhaematocrit tubes (Bilbate Ltd, Devartry, England) until smears were made. To make the smear, a drop of blood sample was placed in the centre line of a slide (2cm from one end). The spreading slide with smooth edge and narrower width than the slide on which the film was to be made was placed at an angle about 45^{o} to the slide and moved back to make contact with the drop of blood. The drops spread out quickly along the line of contact of the spreader with the slide. The film was then spread, by a rapid, smooth, forward movement of the spreader (Dacie and Lewis, 1968). The smears were air-dried and fixed for 4 minutes in absolute methanol. The fixed smears were allowed to air-dry and then stained for 25 minutes in Giemsa stain. The differential blood cells count records were based on the first 100 cells counted; by the longitudinal strip method (Dacie and Lewis, 1968).

3.7.4 IN VITRO BASOPHIL DEGRANULATION TEST

This test was based on the method described by Benveniste (1977). About 5 millilitres of blood were collected from each rabbit into test tubes containing Ethylene diamine-tetraacetic acid (EDTA). Concentrations ranging from 15ug/ml to 1h0ug/ml of each of the 3 different antigen suspensions were separately put into microtitre plate wells and allowed to dry. 0.1ml of blood was added to each well and aggitated, followed by 10 minutes incubation at 37°C. The first well in each series contained no antigen and thus served as control wells. After the incubation period, 0.9ml of Giemsa stain was added to each well, aggitated and the blood cells allowed to stain with the Giemsa solution. The content of the individual well (i.e. mixture of antigen, blood cells and Giemsa stain) was transferred into haemocytometer chamber in succession with dropping pipette. All normal basophil cells cytoplasms will be stained and can be counted, while degranulated basophil cells' cytoplasm will not stain and can therefore not be counted but are estimated as percent basophil degranulation using the formular:

$$\frac{\text{No. of basophils in control wells} - \text{No. of basophils in test wells}}{\text{No. of Basophils in control wells}} \times 100$$

(Benveniste, 1977).

The amounts of antigen in ug giving 50% basophil degranulation were determined by extrapolation (on graphs of % basophil degranulation vs concentration of antigen); and the degranulation unit, expressed as:

$$\frac{1}{\text{ug of antigen causing 50\% degranulation}} \times 100$$

(Benveniste, 1977.

3.7.5 Immunogenicity of fungal antigens based on route of inoculation

Immune precipitation method, based on the Ouchterlony agar gel diffusion technique was used. 1% (w/v) ion agar was prepared and stabilized with 1ml of 0.1% (v/v) sodium azide. 20ml of the molten agar were pipetted warm into standard petri dishes and allowed to gel. 4 wells were then punched out of the agar gel by means of type 5 cock borer (about 5mm diameter); consisting of a central well, surrounded by 3 other wells - 1.8cm apart. 0.25ml of antigen suspension (diluted two-fold) was pipetted into the central wells, while the same amount of undiluted antisera were pipetted into the surrounding wells.

Immunogenicities were determined based on the formation of visible precipitin bands. The test was repeated with the other antisera and with sera from the various animal challenges. This was a preliminary

test to see if antibody has been produced against the various antigens under study.

3.7.6 Determination of total serum antibody

This test was based on the quantitative precipitin test developed by Heidelberger and Kendal (cited by Hudson and Hay, 1976). Increasing quantities of antigen (ranging from 5ug/tube to 60ug/tube) were added to 0.45ml of Phosphate buffered saline (pH 6.8) in serological tubes. 0.1ml of serum samples from the primary and secondary challenges were respectively added to each tube and thoroughly mixed. The mixtures were incubated at 37°C for one hour and at 4°C for 8 days.

At the end of the incubation periods, the mixtures were spined at 3,000 rpm for 5 minutes and the supernatants discarded. The precipitates were washed twice in cold phosphate buffered saline by centrifugation. The final precipitates were redissolved in 3mls of 0.1 N sodium hydroxide. The extinctions were read at 250nm (SP6 - 200 spectrophotometer; Pye Unicam. Ltd., England). Serum antibodies (ug/ml were obtained using the formular:

$$\text{Serum ab} = \frac{\text{OD} \times \text{extinction coefficient}}{\text{sample volume}}$$ (Hudson and Hay, 1976).

where: ab = antibody concentration

OD = Optical Density.

The test was carried out with the different antigen preparations.

CHAPTER FOUR

RESULTS

A total of 2,808 fungal colonies were obtained from 102 plates exposed to the air in the year round survey for fungal aeroallergens in Zaria atmosphere; 229 colonies were obtained from a 12 weeks survey of an irrigation farm site and 796 colonies were obtained from the homes of asthmatic and non-asthmatic individuals during the same period of 12 weeks. Thus, 3,833 colonies were isolated during the period of this survey. The average number of colonies sampled per plate was about 9, and the average per week was about 60. The highest count of 350 colonies was obtained in January, while the least count of 93 colonies occured in April. Other high counts were obtained in February, May, November and December with total colony counts of 266, 334, 297 and 275 respectively (Appendix 1).

More than 38 genera of fungi were identified from the air sampling of the open field. As there were no statistically significant differences between the colony counts at the high and low altitudes ($P = 0.05$), they were merged and treated as total count for the individual month (Appendix 1). More than 21 fungal genera were identified from the

irrigation farm site (Table 1) and more than 18 genera from both the homes of asthmatic and non-asthmatic individuals (Table 2).

The most common genus isolated from the air spora was Fusarium spp (14% of total). This was closely followed by Curvularia spp (12.71% of total), and Saccharomyces spp (10.58% of total). The least encountered genera were: Cunninghamela, Dreschslera, Pestalotia, Spegazzinia, and Syncephalastrum (all accounting for 0.2% of total colonies isolated) (Appendix 1). Fusarium spp, Saccharomyces spp. Curvularia spp. Aspergillus spp and Penicillium spp dominated the dry season isolates accounting for 17.39%, 11.28%, 10.71%, 9.63% and 7.66% of total dry season isolates respectively (Table 1). On the other hand, Curvularia spp. Gliocladium spp, Saccharomyces spp, Fusarium spp, Aspergillus spp and Penicillium spp. dominated the wet season isolates accounting for 15.79%, 14.29%, 12.41%, 18.08%, 6.2% and 6.58%, respectively of the wet season isolates (Table 1). The dry season isolates of Fusarium, and Aspergillus occured at a significantly higher level ($P = 0.05$) than the wet season isolates. Generally, the dry season fungal isolates occured at a significantly higher level ($P = 0.05$) than the wet season isolates.

Gliocladium spp, Curvularia spp, Penicillium spp, Rhodotorula spp and Saccharomyces spp dominated the irrigation farm-site isolates accounting for 22.7%, 16.16%, 10.92%, 7.42% and 6.99% of total farm site isolates respectively, (Table 1). Aspergillus spp and Penicillium spp dominated the indoors fungal isolates together accounting for about 41% of the total indoors isolates (Table 2). There were no significant differences (P = 0.05) between the total isolates in the homes of asthmatic and non-asthmatic patients.

The total fungal particle counts (direct microscopic count on grease-coated glass slides) was 4,114.08 with the highest counts occuring during the harmattan periods (between October and February, inclusive). 615.57 particles were counted in October, while the counts for the months of November and December were 431.24 and 465.17 respectively. The least count was obtained in April, and this amounted to 120.46 particles (Appendix 4). The mean percentage monthly distribution of 7 most abundant genera is shown in Figure 1.

10 genera of the Agaricales and 5 genera of the Polyporales were collected during this investigation. They were commonly encountered during the raining season between the months of June and September.

Some members of the Polyporales flourished even beyond October (Table 3).

The physical reactions of experimental animals to selected fungal antigens are shown in Table 4. All animals given oral challenge with fungal antigens showed no observable responses, while those given nasal challenges expressed some forms of irritation. 5 of the 6 animals given intravenous inoculation died within a week after the second challenge. One died within 3 hours of second challenge with mixed antigens. All the antigens used elicited some forms of physical responses in the animals.

The mean differential blood cells count on 5 blood samples from each animal are shown in Table 5. It can be seen from the table that the antigens used and the route of inoculation did not produce any change from normal differential count, and were therefore reported as the mean of total differential blood cells count for all the routes.

After the secondary challenge with antigen, there was a need to increase the quantity of Curvularia antigen by 37.15% to be able to achieve a 50% basophil degranulation in vitro. The corresponding rise in the quantity of Rhizopus antigen needed was

38.86%. 35.83% increase was needed with the mixed antigen preparation (Table 6).

Serum antibodies were demonstrated against antigens that were intravenously incoulated, but not with oral and nasal inoculations. The mean serum antibody levels generated by the different types of antigen preparations (as determined by optical density measurements) are shown in Table 7. Curvularia antigen caused about 120% rise in serum antibody, after a second inoculation. The corresponding values were 33% and 50% for Rhizopus and mixed (Curvularia + Rhizopus) antigen preparations respectively (Table 7).

Regression analysis using the spearman's rank correlation co-efficient (R = - 1 to +1), showed that majority of the fungi isolated during this investigation had positive correlation with the mean of annual asthma attendance at the Ahmadu Bello University Teaching Hospital (Asthma Clinic) based on five-years record considered (Table 8). Similarly, the occurrences of some of the fungal isolates in the air correlates significantly (P = 0.05) with those of the others; thus, for Helminthosporium and Fusarium, R = 0.632; for Aureobasidium and Fusarium, R = 0.680; for Saccharomyces and Curvularia,

R = 0.758. Others were: Sclerotium and Mycelial sterilia R = 0.953; Syncephalastrum and Mycelial sterilia, R = 0.942; Torula and Cladosporium, R = 0.918; Geotricum and Cladosporium, R = 0.944; Arthrographis and Epicoccum, R = 0.924; Spegazzinia and Candida, R = 0.951; Syncephalastrum and Sclerotium, R = 0.946; Cunninghamela and Monilia, R = 1.000 etc (Appendix 5).

TABLE 1: PERCENTAGE VIABLE COUNT OF AIRBORNE FUNGI (8 Plateau/month) COLLECTED BETWEEN OCTOBER 1989 AND SEPTEMBER 1990

ORGANISMS	DRY SEASON % OF TOTAL	WET SEASON % OF TOTAL	DRY & WET SEASON % OF TOTAL	IRRIGATION FARM SITE % OF TOTAL
Fusarium	17.39	8.08	13.99	3.06
Curvularia	10.71	15.79	12.71	16.16
Saccharomyces	11.28	12.41	10.58	6.99
Gliocladium	5.95	14.29	9.15	22.7
Aspergillus	9.63	6.20	8.41	6.11
Penicillium	7.65	6.58	8.08	10.92
Helminthosporium	6.63	4.79	5.98	1.31
Mycelial sterilia	5.21	2.16	4.1	3.06
Rhodotorula	2.72	5.83	3.92	7.42
Aureobasidium	3.29	3.10	3.24	5.24
Cladosporium	3.06	2.73	2.96	-
Cephalosporium	1.76	2.54	2.07	0.44
Alternaria	1.13	3.76	1.89	0.87
Epicoccum	2.15	1.41	1.89	0.87
Candida	1.81	1.79	1.82	5.24
Trichoderma	2.10	1.03	1.71	0.44
Mucor	0.28	3.67	1.57	2.18
Rhizopus	1.13	2.07	1.5	3.06
Nigrospora	0.91	0.47	0.61	-
Humicola	0.96	0.28	0.75	0.87
Others*	4.25	1.03	2.83	3.49
Total	100	100	100	100

(LSD = (P=0.05)

*Phoma, Torula, Stemphylium, Geotricum, Arthrographis, Papularis, Bipolaris, Sclerotium, Blastomyces, Monilia, Stylopage, Chaetomium, Cunninghamela, Dreschslera, Pestalotia, Spegazzinia, Syncephalastrum. A few unidentified.

TABLE 2: FUNGI VIABLE COUNT OF AIR SAMPLING IN THE HOMES OF ASTHMA AND NON-ASTHMA PATIENTS - A TWELVE WEEK SURVEY

ORGANISMS	ASTHMA PATIENT % OF TOTAL	NON-ASTHMA PATIENT % OF TOTAL
Aspergillus	17.9	17.81
Penicillium	21.94	19.18
Saccharomyces	13.71	8.90
Rhizopus	9.36	19.86
Curvularia	6.77	5.48
Mycelia sterilia*	6.13	5.48
Mucor	4.35	10.96
Rhodotorula	5.81	0
Helminthosporium	2.58	8.90
Fusarium	4.35	0
Gliocladium	2.90	0
Candida	0.81	2.74
Aureobasidium	0.97	0
Monilia	0.97	0
Alternaria	0.65	0
Neurospora	0.48	0
Epicoccum	0.32	0
Unidentified	0	0.69
Total	100	100

LSD = (P=0.05)

* Non-sporulating species.

TABLE 3: AGARICALES AND POLYPORALES COLLECTED DURING THE MONTH OF JUNE-SEPTEMBER, 1990.

ORDER	ORGANISM (Species)	SOURCES OF ORGANISM	DURATION OF FLOURISH	FREQUENCY OF COLLECTION
Agaricales	Agaricus bisporus	Open field	June through July	Frequent
	Agaricus campestris	"	July through August	"
	Amanita muscaria	"	" " "	"
	Amanita muscaris	Open field, under larger trees.	"	"
	Coprinus congregatus	Around dead stumps	"	Infrequent
	Coprinus disseminatus	" " "	"	Frequent
	Coprinus niveus	On animal dungs	June through September	"
	Leucoagaricus bresadolae.	On refuse dungs	July	Infrequent
	Pleurotus spp	On dead stumps	June through September	"
	Psathyrella spp	Open field, usually under the shade of taller trees.	June through July	"
Polyporales	Ganoderma lucidum	On large, lateral roots extending from main roots.	July on	Very Frequent
	Lenzites betulina	On dead woods	" "	" "
	Polyporus squamosus	On tree trunks, usually in thickly shaded areas.	" "	" "
	Polyporus varius	On stumps	" "	Infrequent
	Trametes versicolor	On dead woods	June on	Very frequent

KEY: Very frequent = found in more than 20 locations
Frequent = found in 10 to 20 locations
Infrequent = found in less than 10 locations

TABLE 4: PHYSICAL REACTIONS OF RABBITS AFTER THE FIRST AND SECOND INOCULATIONS WITH SELECTED ANTIGENS

	FIRST INOCULATION							SECOND INOCULATION						
	Curvularia			Rhizopus			Mixed Antigen				Curvularia		Rhizopus Mixed Antigen	
TIME	Nasal	Oral	I.V.	Nasal	Oral	I.V.	I.V.	Nasal	Oral	I.V.	Nasal	Oral	I.V.	I.V.
0-10min	Scratching of nose & mouth (a)	-(a)	-(a)	Scratching of nose & mouth (a)	-(a)	-(a)	-	Scratching of nose & mouth (a)	-	*+(a)	Scratching of nose & mouth (a)	-	**(b) *+(b)	(*+(b) (**(b)
1-3hrs.	-	-	*(a)	-	-	*(a)	*(a)	-	-	*(a)	-	-	*	-(b) ***(b)
After 6hrs	-	--	-	-	-	-	-	-	-	-	-	-	-	-
After 12hrs	-	-	-	-	-	-	-	-	-	-	-	-	-	-
After 6days	-	-	-	-	-	-	-	-	-	***(b)	***(a)	-	***(b)	***(b)

KEY

1. * Animals assumed resting posture
2. *+ Animals assumed resting posture; with elevated breathing rate
3. ** Animals exhibited violent reactions to the fungal antigens; characterized by complete loss of consciousness, elevated breathing rate, stretching and rolling on the floor, inability to stand on feet etc.
4. *** Animals died within a week after the second inoculation.
5. (a) All of the animals inoculated
6. (b) One of the animals inoculated
7. I.V. Intravenous.

LSD = (P = 0.05).

TABLE 5: MEAN DIFFERENTIAL BLOOD CELL COUNTS ON FIVE BLOOD SAMPLES COLLECTED FROM TEST AND CONTROL ANIMALS (ALL ROUTES OF INOCULATION*)

		CELL TYPES AND MEAN NUMBER COUNTED					
Nos of Animals Used	Sources of Antigen.	Baso-phil	Eosino-phil	Band Cells	Neutro-phil	Lympho-cytes	Mono cyte
6	Curvularia	0	0.44	0.80	41.49	57.72	0.49
6	Rhizopus	0	0.91	0.13	41.43	57.07	0.37
2	Mixed (Curvularia + Rhizopus)	0	0.4	0.3	36.6	56.7	1
7	Controls	0	0.59	0.19	42.31	55.96	0.93

* Route of inoculation did not influence the cell counts.

LSD = (P=0.05)

TABLE 6: MEAN % BASOPHIL DEGRANULATION IN RELATION TO TYPES AND CONCENTRATIONS OF ANTIGENS IN INTRAVENOUSLY INOCULATED ANIMALS (AS DETERMINED IN VITRO)

	FIRST INOCULATION				SECOND INOCULATION		
ANTIGEN CONCENTRATION (Ug)	CURVULARIA	RHIZOPUS	MIXED (RHIZOPUS + CURVULARIA)	ANTIGEN CONCENTRATION (Ug)	CURVULARIA	RHIZOPUS	MIXED(RHIZOPUS + CURVULARIA
	% DEGRA.[1]	% DEGRA.	% DEGRA.		% DEGRA.	% DEGRA.	% DEGRA.
0	0	0	0	*50	0.39	1.64	2.11
15	2.23	5.95	7.7	65	1.53	6.21	5.78
30	3.23	19.5	16.8	80	7.37	19.8	14.57
45	11.8	28.7	25.2	95	38.98	31.04	27.89
60	33.8	45.9	39.9	110	45.2	46.9	42.71
75	59.4	63.6	50.7	125	61.66	64.5	54.27
90	77.4	74.5	54.0	140	67.63	68.4	62.31
50% Degra.	63.8ug	59ug	73.9ug	50% Degra.	101.51ug	96.51ug	115.16ug
Degra Unit	1.6	1.7	1.36	Degra Unit	0.99	1.03	0.87

*NOTE: Preliminary experiments had shown that no appreciable degranulations were obtained with less than 50ug of antigens after the second inoculation. 50ug was consequently used as the starting concentration.

LSD = (P = 0.05)

1. DEGRA = DEGRANULATION

TABLE 7: MEAN SERUM ANTIBODY LEVELS (mg/ml) TO CHALLENGES WITH VARIOUS FUNGAL ANTIGENS (INTRAVENOUS ROUTE)

NO. OF ANIMALS USED	SOURCES OF ANTIGEN	MEAN SERUM ANTIBODY LEVELS (mg/ml)		
		1st INOCULATION	2nd INOCULATION	% Ab Rise
2	Curvularia	2.75×10^{-3}	6×10^{-3}	120.67
2	Rhizopus	2.65×10^{-3}	3.85×10^{-3}	33.80
2	Mixed (Rhizopus + Curvularia)	5.7×10^{-3}	8.6×10^{-3}	50.65

LSD = (P=0.05)

Ab = Antibody

TABLE 8: Spearman's Rank correlation of the fungal genera isolated with a 5-years asthma attendance at the Ahmadu Bello University Teaching Hospital (Asthma Clinic)

	Fungal genera	Spearman's correlation Coefficient.
1	Fusarium	0.225
2	Curvularia	0.185
3	Saccharomyces	0.134
4	Gliocladium	0.056
5	Aspergillus	-0.461
6	Penicillium	-0.165
7	Helminthosporium	0.260
8	Mycelial sterilia	-0.155
9	Rhodotorula	0.082
10	Aureobasidium	0.258
11	Cladosporium	-0.315
12	Cephalosporium	0.345
13	Alternaria	0.073
14	Epicoccum	0.262
15	Candida	0.234
16	Trichoderma	0.270
17	Rhizopus	0.282
18	Mucor	0.365
19	Humicola	0.119
20	Nigrospora	-0.168
21	Phoma	-0.179
22	Torula	0.204
23	Stemphylium	-0.281
24	Geotricum	0.273
25	Arthrographis	0.126
26	Papularia	-0.231
27	Bipolaris	0.037
28	Sclerotium	0.194
29	Blastomyces	-0.077
30	Monilia	-0.231

TABLE 8 CONT.

	Fungal genera	Spearman's correlation Coefficient.
31	Stylopage	0.126
32	Chaetomium	0.394
33	Cunninghamela	-0.231
34	Dreschslera	0.037
35	Pestalotia	-0.498
36	Spegazzinia	-0.052
37	Syncephalasrum	0.216
38	Unidentified	-0.517

LSD = (P = 0.05)

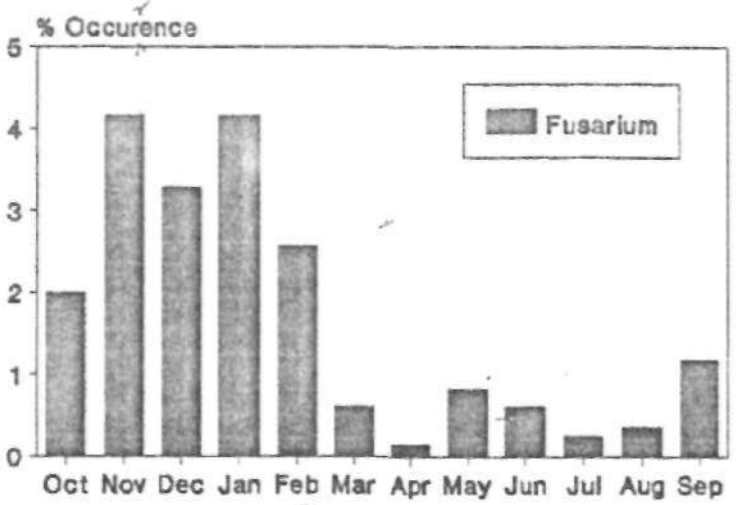
NOTE (APPENDIX 5)

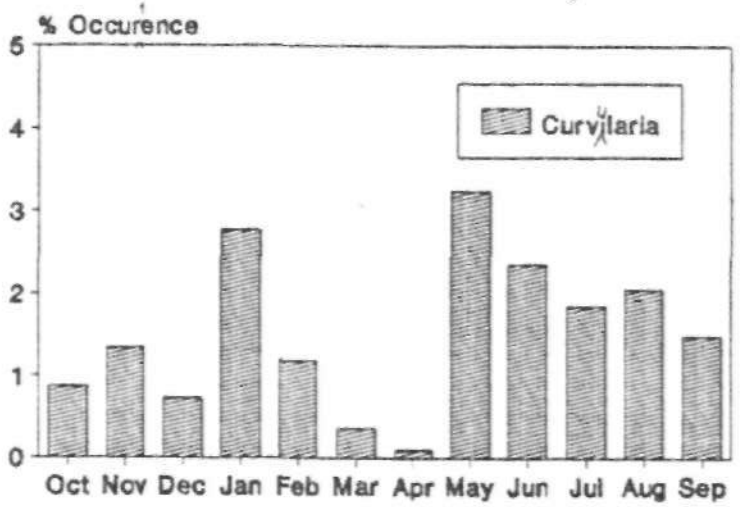
V1 = Mean of 5-year asthma attendance at the Ahmadu Bello University Teaching Hospital (Asthma Clinic).

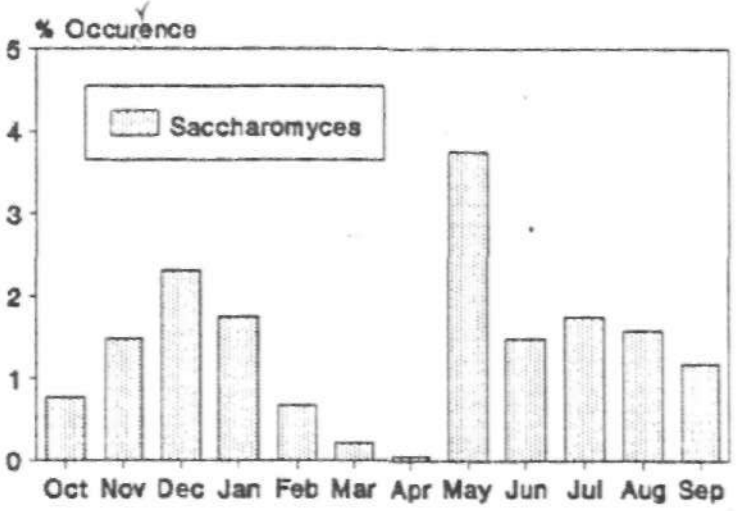
V2-V39 = Fungal isolates from the atmosphere in descending order of numerical occurences.

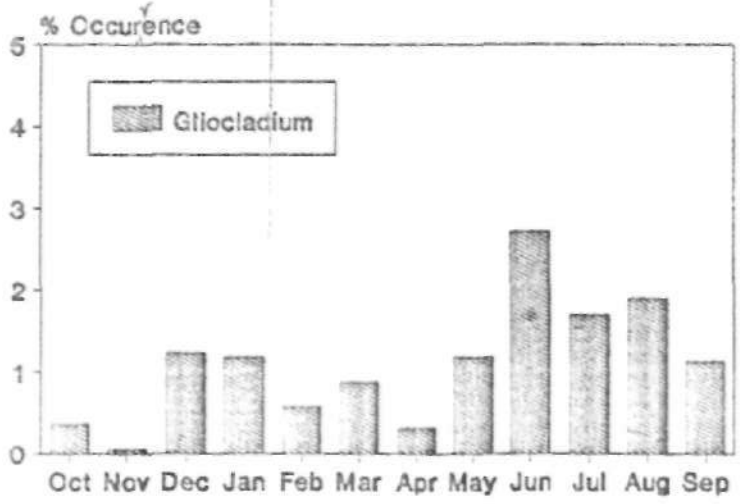
Figure 1

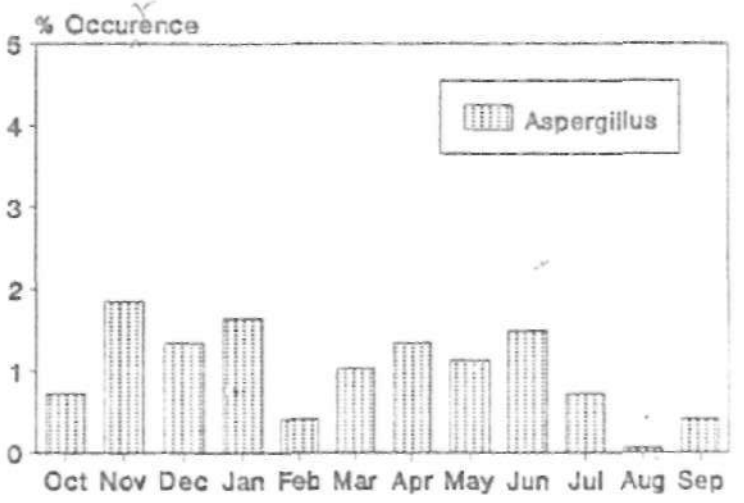
Percent Monthly Occurence
of the Seven Most Abundant Fungi

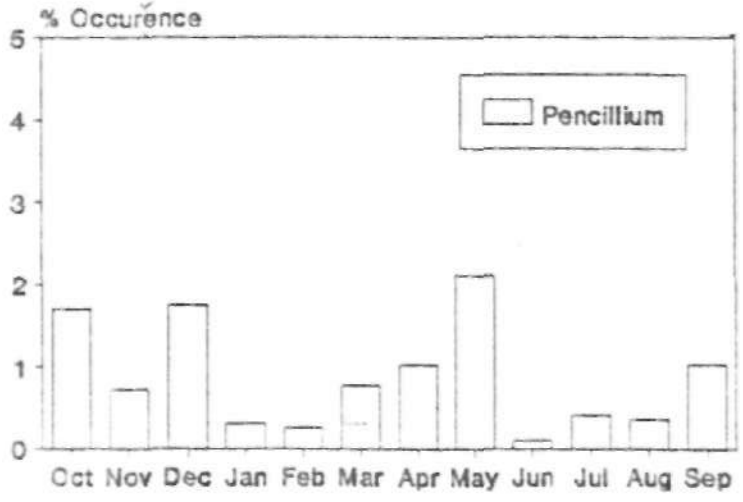







% Occurence
Gliocladium
0
1
2
3
4
5
Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug Sep


% Occurence
Aspergillus
0
1
2
3
4
5
Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug Sep


% Occurence
Pencillium
0
1
2
3
4
5
Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug Sep

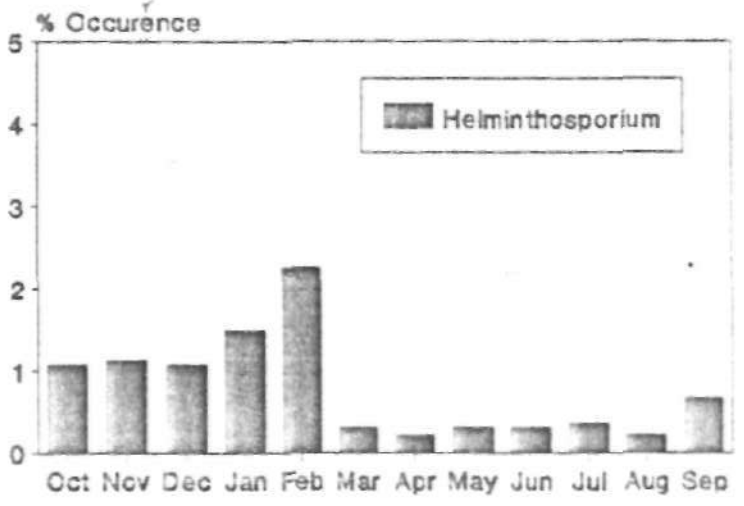

% Occurence
Helminthosporium
0
1
2
3
4
5
Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug Sep

Figure 2

Mean Monthly Values of Some Meteorological Data (1989-1990)

Figure 2a: Windrun

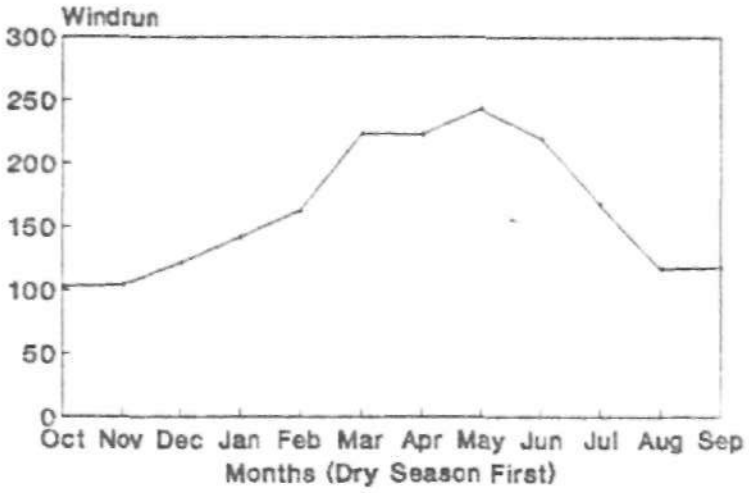


Figure 2b: Maximum-Minimum Temperatures

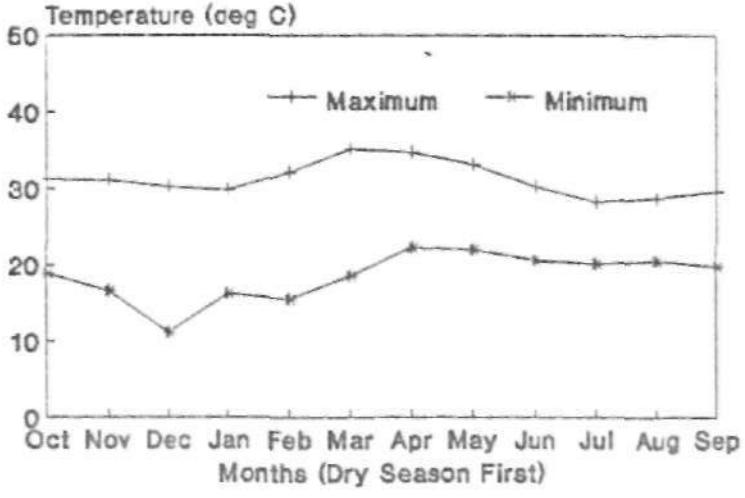


Figure 2c: Relative Humidity

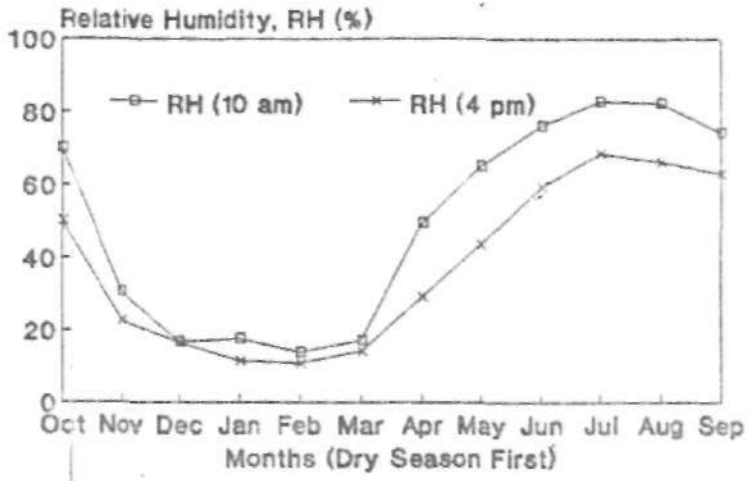
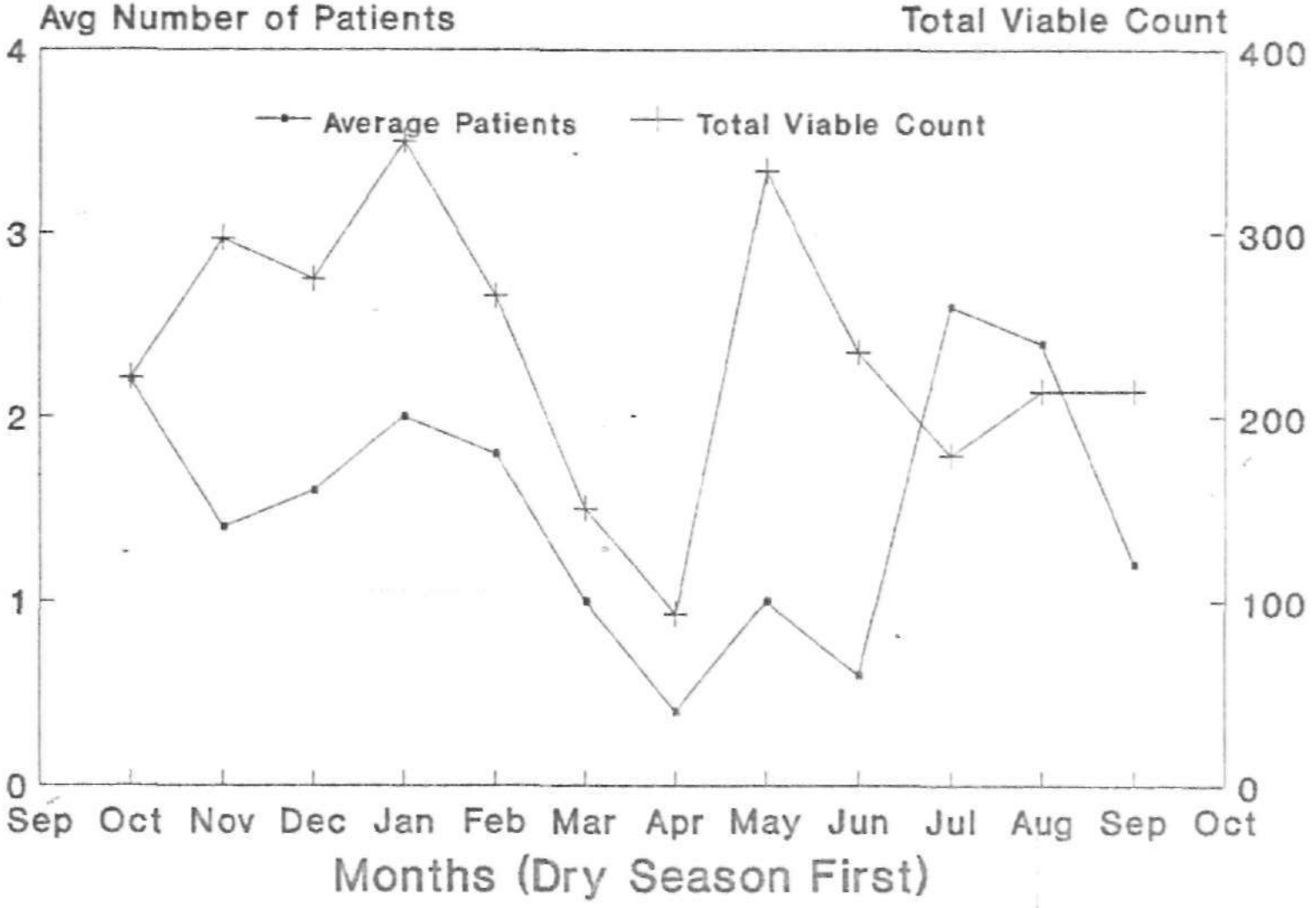


Figure 3 Yearly Mean Asthma Patients and Viable Fungal Colony Count
ABUTH Asthma Clinic, 1984-1988



CHAPTER FIVE

DISCUSSION

This study reveals that a high level of various genera of fungi are present in the Zaria atmosphere during the greater part of the year. Airborne fungi have a global distribution (Southworth, 1974), and with the exception of Spondylocladium and Cryptococcus, all the fungus genera classified as universal dominants (Morrow and Meyer, 1964) were isolated in this survey viz: Alternaria, Helminthosporium, Aspergillus, Penicillium, Phoma, Trichoderma, Fusarium, Curvularia, and Rhodotorula.

This study which was for a longer duration than that done by Lawande and Onyemelukwe (1984) confirms Fusarium as the most frequently isolated genus during the harmattan months in Zaria atmosphere. This however contrasted with the results of Dransfield (1966) who isolated more Cladosporium than any other genus from the aerospora of an agricultural field in Zaria. Curvularia which was the next most common fungus in this survey was similarly reported by Dransfield (1966) who found it to have taken a leading distribution in the tropics just as Alternaria in the temperate zones. These findings confirms the earlier observation by Meyer, et al. (1983) that the annual distribution of

the dominant, universal moulds are fairly constant. Curvularia appeared to be more evenly distributed all the year round compared to Fusarium which dominated the harmattan months of October to February (Appendix 1)

The peak periods of fungal isolates were October to February and May/June (Figure 1). These months comprise the dry season in Zaria. Dust carrying particles are generally more airborned at this period unlike during the wet season when rain continually wash off particles from the atmosphere. The peak periods of aerial fungal presence corresponds to the peaks of asthma clinic attendance at the Ahmadu Bello University Teaching Hospital (Figure 3).

Although the level of fungal air spora fell between June and September (Figure 3); which is the raining season in Zaria, asthma attendance at the Ahmadu Bello University Teaching Hospital (Asthma Clinic) increased during the same period. The rise in asthma figure may be explained based on two possibilities:

(a) The annual flourish of Agaricales and Polyporales which was found to be between June and September (Table 3) correspond to this period.

(b) More trees and grasses are probably shedding pollen into the atmosphere at this period. There are no informations on the peak periods of trees and

grasses pollens in Zaria atmosphere, however, preliminary observations suggested the periods between June and September. Fungal particles occur over a rather extended period of the year (Appendix 1).

The general correlations between the levels of fungal particles and the mean annual asthma clinic attendance (Table 8) suggests the need to consider their roles when any determination of the causes of asthma in Zaria area is being made. Lehrer et al. (1982) have also demonstrated a relationship between high atmospheric fungal spores concentration and asthma admission at a local hospital. Lawande and Onyemelukwe (1984) also observed increased asthma admission in the Ahmadu Bello University Teaching Hospital during the harmattan months. Although it has been shown that pollens can cause asthma (Larsen, 1981), and that extracts from Agarics and Polypores gave positive skin tests in asthma patients (Gregory and Hirst, 1952), their roles in asthma attendance at the Ahmadu Bello University Teaching Hospital (Asthma Clinic) has not been considered. It is therefore necessary to consider other parameters to be able to make categorical statements on the increase in Asthma Clinic attendance between the months of June and September (Figure 3).

The following 8 genera which accounted for the majority of fungi isolated in this work, viz:

Epicoccum, Candida, Fusarium, Cladosporium, Curvularia, Alternaria, Helminthosporium and Stemphylium were also reported by Champman and Sharon (1984) as the fungal aeroallergens most frequently causing positive skin test reactions.

Of the various meteorological parameteres studied, only windrun appears to influence the fungal particle recovery (Figure 2), and this probably accounts for the observed disparity between the total viable colony counts on the farm site (where windrun was more stable due to vegetation) and those isolated from the air (Table 1-dry season). It should also be observed that the longest peak of fungal particles in the atmosphere (Figure 1) corresponds with the dry months (Table 1). Amin and Bockhari (1979) have observed that there is a definite correlation between dryness of the air and the incidence of fungal spores.

In a 24-month survey of seasonal fungus prevalence inside and outside dwellings, Beaumont (1984) observed that the total numbers of indoor Penicillium was significantly higher than outdoor catch, and that the concentration of indoor Aspergillus was more than double the outdoors concentration. This was also observed by Calvo et al. (1982), who found that the Penicillium-Aspergillus groups were the dominant fungal spores (90%) present in the homes of asthmatics.

They also observed that the same group dominates the homes of healthy persons (92.5%). In the present study, Aspergillus and Penicillium were the most commonly isolated genera in the homes of asthmatic and normal persons - accounting for more than 41% of the total indoors isolates (Table 2). Sneller and Roby (1979), were of the opinion that indoor spora is influenced by outdoor spora, and this might be the case in this study. One possible explanation for the more abundance of fungi in the indoor air is that garbage thrown around houses could support rapid proliferation of the organisms. Similarly, the humidity and temperature of the enclosed air in the homes could support the growth of the organisms.

It is generally accepted that exposure to high concentrations of mould spores, reactivity to more than one mould and antigenic cross reactivity between spores of different fungal genera are important factors leading to exacerbation of allergic symptoms - in the mould-sensitive patient; and that in almost all instances, there is greater percentage correlation of positive skin test with indoor colony forming units compared with outdoor colony forming units (Roby and Sneller, 1979). Housewives and children formed the bulk of asthma patients that visited the Ahmadu Bello University Teaching Hospital (Asthma Clinic) annually.

It is noteworthy that women and children spend a lot of their time indoors and are thereby more exposed to the indoor mould spores. It is significant that farmers reporting at the Clinic are, much fewer; this further suggests the role of the indoor spora in asthma attacks. Farmers spend most of their time outdoors and are therefore less exposed. Three of the 5 patients whose homes were sampled are housewives and two of them had at least one child who also suffered from asthma attack. The nature of the household chores of the Nigerian woman also exposes her to more fungal flora: women engage in threshing of grains and gathering of fire woods from the bush during the harmattan; these in addition to prolonged exposure to indoor flora expose them to allergens more than their male counterparts.

The results of animal inoculation (Table 4) using Curvularia Lunata and Rhizopus nigricans showed that the route of inoculation is very important in the induction of respiratory allergy. Bellanti (1978) has made similar observations. Allergenicity as determined by basophil degranulations (Table 6) shows that intravenous route of inoculation is perhaps more important in provoking allergy than oral and nasal routes. There were also evidences of allergenicity through the nasal route (Table 4); no similar observations were made for the oral route of inoculation.

The more positive correlation seen with intravenous route of inoculation indicates that the route is more effective in bringing about basophil degranulation although this is not the route of natural sensitization. Penetration of nasal linings by diffusible antigenic determinants may account for the sensitization of basophil cells when the animals were given nasal inoculation; since it is known that mast cells (tissue basophils) do not migrate back into circulation (Bellanti, 1978). The nature of antigen and the route of inoculation did not affect the differential blood cell count (Table 5).

Table 6 shows that 37.15% increase in the quantity of Curvularia antigen was needed to cause 50% basophil degranulation after a second animal inoculation, while that needed for Rhizopus antigen was 38.86%. This result agrees with the observation that more antigens (sometimes up to 50% increase) is needed to cause 50% basophil degranulation after the second inoculation than after the first inoculation (Benveniste, 1977). It has been observed that there may be complete blockage of basophil degranulation after more than two animal inoculations or in hyper-immunized animals. Such blockage was thought to be due to the production of blocking antibodies after multiple inoculation (Maunsell, 1968). Associated changes in both immunoglobulin E and Immunoglobulin G antibodies

may be related to clinical improbement, and a possible explanation for this association could be the suppression of reagin synthesis by blocking antibodies (Pepys, 1975).

It might be possible to classify fungal allergenicity on the basis of their basophil degranulation capacity. It has been shown that basophils and mastocytes contain effector molecules of anaphylaxis (Benveniste, 1977; Benveniste, 1978) and that these cells have the capacity to bind to immunoglobulin E chains of reaction between the immunoglobulin E and the stimulating antigen lead to the degranulation of the cells and the consequent release of effector molecules of allergic reactions. If the level of serum IgE rises therefore, more basophil cells are covered by the immunoglobulin and when degranulated will cause the release of more effector molecules. The possibility of classifying fungal allergenicity on the basis of their basophil degranulation has not been experimented, but if found useful, may be of great improvement on the present diagnostic procedures in selecting the most probable aetiologic agents of allergic reactions. This is because basophil degranulation test is an indirect way of quantitating the serum level of Immunoglobulin E (Benveniste, 1977) which has been shewn to be responsible for type I, anaphylatic reactions (Ishizaka and

Ishizaka, 1968). Fungal antigens capable of causing more basophil degranulation will by extension, be those that will favour the production of more mediators of allergy. Another advantage of basophil degranulation test is that it is carried out *in vitro*. This eliminates the danger of sensitizing the patients.

When compared to *Curvularia*, *Rhizopus* appears to be more effective in generating IgE as evidenced by the basophil degranulation unit (Table 6). However, in nature, *Curvularia* causes positive skin reaction in more individuals than *Rhizopus* (Champman and Sharon, 1984). This may be due to the relatively more abundance of *Curvularia* in nature than *Rhizopus* (Appendix 1) so that more people are sensitized to *Curvularia* than *Rhizopus*. Similarly, *Curvularia* is shown (Table 7) to be more immunogenic than *Rhizopus*; and it may favour the production of Immunoglobulin G rather than Immunoglobulin E. As much as 120% rise in serum antibody was noticed with *Curvularia* antigen, and 33.8% with *Rhizopus* antigen when administered intravenously (Table 7). This is typical of immunological secondary antibody response (Bellanti, 1978) and it suggests an increased synthesis of blocking antibodies since allergy is manifestation of Immunoglobulin E activity while serologic reactivity is manifestation of IgG and IgM activity (Nazer, *et al*., 1981).

One of the serologic reactivities in allergy manifestations is the blocking of immunoglobulin E activities (Pepys, 1975).

Although much of this discussion has been limited to the dominant fungal isolates, only the development of specific IgE and inhalation provocation studies or other sensitive studies will reveal the true importance of the rare moulds as aeroallergens. It has been shown that atopic individuals are allergic to wide varieties of particles including fungal particles (Benveniste, 1978); it has also been shown that the levels of reactivities to these particles by atopic individuals depend more on the frequency of sensitization, rather than the concentration of the allergens at any given time (Frazier, 1980). Therefore, the rare moulds should be given adequate considerations during allergological studies. Moreover, it has been suggested that any mould or yeast encountered in significant proportions in a fungal aerospora survey can be considered as a potential aeroallergen (Leventine and Hordwitz, 1978).

The use of 50% degranulation level as the basis for basophil degranulation test may not be ideal in light of the fact that as little as 15ug of antigen could cause 2.23% degranulation (Curvularia) 5.95% degranulation (Rhizopus) and 7.7% degranulation (mixed: Curvularia + Rhizopus)(Table 6). It should also be

observed that the quantities of antigen required to cause 50% basophil degranulation were too large (Table 6). Since large concentrations of allergens are not pre-requisite for allergenicity, but rather the frequency of sensitization (Frazier, 1980) couple with the fact that atopic individuals react to relatively minute quantities of allergens (Benveniste, 1978), it may be necessary to use a low percentage degranulation as the basis for comparing the allergenicity of different fungi. It should also be noted that whereas fungal particles are present over longer periods in the atmosphere, they are not always present in very large quantities (Lopez, et al., 1976; Lehrer et al., 1986), therefore allergologic tests using fungal particles may reflect natural situations better if smaller quantities of allergen are employed.

RECOMMENDATIONS

In concluding this report, the following suggestions on fungal aeroallergens in Zaria may be considered important:

There is a need for continuous monitoring of the atmosphere on a national basis; available evidences showed that this is not being done at the moment. As rightly observed by Lawande and Onyemelukwe (1984) in an allergological investigation, it is necessary to carry out further allergological tests on patients by using various *in vitro* and *in vivo* tests. This work attempted an *in vitro* comparative allergenicity test, which if improved may be a useful replacement of the methods presently in use.

Since it has been shown that indoors, dominant genera correlate better with positive skin tests (Roby and Sneller, 1979) it will be informative to carry out more wide-spread investigations on the indoor fungal spora in different parts of Nigeria. Better informations on the correlation between peak values in spore concentration and their ability to produce an attack of asthma is also desirable.

It is estimated that there are more than 10 million Nigerians who suffer from one form of allergic attack or the other. Therefore, any efforts to describe, understand and monitor Nigerian allergens can form the

basis of a public enlightenment program supported by appropriate therapeutic procedures.

Public education on the potentials of the various allergens, the times and seasons of the year when the allergen are common in the atmosphere can assist victims in avoiding undue exposure to such allergens.

Sensitivity of an individual to a particular allergen can only be ascertained after specific in vivo test. These tests should be incorporated into our health policies as a means of quick, reference diagnosis.

Laboratory workers who show positive allergic test to any fungus should not be allowed to work with such fungus, but if they must work, they should undergo some forms of hyposensitization therapy before such work.

Finally, informations on aeroallergens should be incorporated into our Agricultural Extension Programmes as a wal of educating our farmers in the rural areas on the need to seek medical assistance. Same education is very essential to housewives.

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APPENDIX 1: VIABLE COUNT OF AIRBORNE FUNGI (8 PLATES/MONTH) COLLECTED BETWEEN OCTOBER 1989 AND SEPTEMBER, 1990

		1989			1990										
	FUNGI	OCT.	NOV.	DEC.	JAN.	FEB.	MAR.	APR.	MAY	JUNE	JULY	AUG.	SEPT.	TOTAL	% TOTAL
1	Fusarium	39	81	64	81	50	12	3	16	12	5	7	23	393	13.99
2	Curvularia	17	26	14	51	23	7	2	63	46	36	40	29	357	12.71
3	Saccharomyces	15	29	45	34	13	4	1	73	29	34	31	23	297	10.58
4	Gliocladium	7	1	24	23	11	17	6	23	53	33	37	22	257	9.15
5	Aspergillus	14	36	26	32	8	20	26	22	29	14	1	8	236	8.41
6	Penicillium	33	14	34	6	5	15	20	41	2	8	7	20	225	8.08
7	Helmintho-sporium	21	22	21	14	44	6	4	6	6	7	4	13	168	5.98
8	Mycelia sterilia	5	16	5	8	22	17	2	22	6	5	3	4	115	4.1
9	Rhodotorula	0	0	5	3	22	3	5	10	16	4	28	14	110	3.92
10	Aureobasi-dium	11	6	17	18	6	1	2	8	5	0	8	9	91	3.24
11	Cladosporium	14	15	15	5	1	11	12	5	3	2	2	8	83	2.96
12	Cephalos-porium	7	3	2	14	2	7	0	3	6	9	1	4	58	2.07
13	Alternaria	10	1	0	2	7	0	3	7	10	3	9	8	53	1.89
14	Epicoccum	5	3	0	9	15	5	0	6	1	2	3	4	53	1.89
15	Candida	9	11	3	11	0	2	2	3	1	2	2	5	51	1.82
16	Trichoderma	2	6	0	22	4	5	0	0	2	4	1	2	48	1.71
17	Mucor	4	3	1	0	0	0	0	1	4	4	17	10	44	1.57
18	Rhizopus	3	10	4	1	1	0	0	4	3	5	6	5	42	1.5
19	Humicola	0	0	0	2	11	3	1	0	0	0	2	1	20	0.71
20	Nigrospora	0	0	0	3	1	8	1	3	0	0	3	2	21	0.75
21	Phoma	0	5	0	0	1	0	0	10	1	2	0	0	19	0.68
22	Torula	0	0	0	4	10	0	0	0	0	0	0	0	14	0.5
23	Stemphylium	0	5	0	0	0	1	1	0	1	0	0	0	8	0.29
24	Geotricum	5	2	0	0	0	0	0	0	0	0	0	0	7	0.25
25	Arthrogra-phis	0	0	0	0	6	0	0	0	0	0	0	0	6	0.21

APPENDIX 1 Cont.

		O	N	D	J	F	M	A	M	J	J	A	S		
26	Papularia	0	0	0	0	0	0	0	6	0	0	0	0	6	0.21
27	Bipolaris	0	0	4	0	0	0	0	0	0	0	0	0	4	0.14
28	Sclerotium	0	1	0	3	0	0	0	0	0	0	0	0	4	0.14
29	Blastomyces	0	0	0	0	1	1	0	0	0	0	0	0	2	0.07
30	Monilia	0	0	0	0	2	0	0	0	0	0	0	0	2	0.07
31	Stylopage	0	0	0	0	2	0	0	0	0	0	0	0	2	0.07
32	Chaetomium	0	0	0	0	0	0	0	0	0	0	2	0	2	0.07
33	Cunninghamela.	0	0	0	0	0	1	0	0	0	0	0	0	1	0.04
34	Dreschsler	0	0	1	0	0	0	0	0	0	0	0	0	1	0.04
35	Pestalotia	0	0	0	0	0	0	1	0	0	0	0	0	1	0.04
36	Spegazzinia	0	1	0	0	0	0	0	0	0	0	0	0	1	0.04
37	Syncephalastrum	0	0	0	0	0	0	0	0	0	0	0	0	1	0.04
38	Unidentified	0	0	0	1	0	2	1	2	0	0	0	0	5	0.18
	TOTAL	221	297	275	350	266	150	93	334	235	179	214	214	2808	100
	% of TOTAL	7.82	10.5	9.72	12.38	9.41	5.3	3.29	1.81	8.31	6.33	7.57	7.57	100	

APPENDIX 2: MEAN MONTHLY VALUES OF SOME METEOROLOGICAL DATA TAKEN DURING THE SAMPLING PERIOD

	MONTHS	TEMPERATURE MAX.°C	TEMPERATURE MIN.°C	REL.HUMIDITY 10 A.M	REL.HUMIDITY 4 P.M	SUN-SHINE	WIND-RUN
1	OCTOBER '89	31.2	18.9	70.1	50.2	8.9	103.1
2	NOVEMBER '89	31.1	16.6	30.7	22.8	9.7	104.2
3	DECEMBER "	30.2	11.2	16.8	16.7	9.4	121.6
4	JANUARY '90	29.8	16.25	17.6	11.4	9.1	142
5	FEBRUARY "	32.1	15.5	13.8	10.8	9.4	162.8
6	MARCH "	35.13	18.55	17.18	14.2	9.4	223.4
7	APRIL "	34.7	22.25	49.55	29.23	7.9	223
8	MAY "	33.15	22.08	65.1	43.6	8	243
9	JUNE "	30.2	20.6	76.1	59.5	7.5	219.5
10	JULY "	28.2	20.1	82.9	68.6	6.6	167.8
11	AUGUST "	28.7	20.5	82.4	66.3	5.6	117.1
12	SEPTEMBER "	29.6	19.8	74.4	63.1	7.8	118.1

APPENDIX 3: REPORTED CASES OF ALLERGIC ASTHMA AHMADU BELLO UNIVERSITY TEACHING HOSPITAL (1984-1988) (USED WITH PERMISSION)

		1984	1985	1986	1987	1988	TOTAL	AVERAGE
	MONTHS/ YEARS.	NUMBER OF CASES						
1	JANUARY	3	2	2	3	0	10	2
2	FEBRUARY	2	3	2	1	1	9	1.8
3	MARCH	1	0	1	1	2	5	1
4	APRIL	0	1	0	0	1	2	0.4
5	MAY	2	0	1	2	0	5	1
6	JUNE	0	1	1	1	0	3	0.6
7	JULY	3	3	3	3	1	13	2.6
8	AUGUST	3	3	4	1	1	12	2.4
9	SEPTEMBER	0	0	4	0	2	6	1.2
10	OCTOBER	5	2	3	1	0	11	2.2
11	NOVEMBER	2	3	2	0	0	7	1.4
12	DECEMBER	4	1	3	0	0	8	1.6
	TOTAL	25	19	26	13	8	91	
	AVERAGE	2.08	1.58	2.17	1.08	0.67		

APPENDIX 4: TOTAL (VIABLE AND NON-VIABLE) FUNGAL PARTICLE COUNTS PER MONTH (DIRECT MICROSCOPY)

MONTH	TOTAL PARTICLE COUNT	% OF TOTAL
OCTOBER	532.41	25.9
NOVEMBER	431.24	20.91
DECEMBER	465.17	22.57
JANUARY	580.22	28.25
FEBRUARY	411.04	19.94
MARCH	277.54	13.45
APRIL	120.46	5.85
MAY	386	18.82
JUNE	336.8	16.64
JULY	267.7	13.55
AUGUST	295.5	14.42
SEPTEMBER	413.95	20.04
TOTAL	4,114.08	100

APPENDIX 5: CORRELATION MATRIX

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MTB > PRINT V1-V39
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ROW	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12	V13
1	2.0	81	54	34	23	32	6	14	8	3	18	5	14
2	1.8	50	23	13	11	8	5	44	22	22	6	1	2
3	1.0	12	7	4	17	20	15	6	17	3	1	11	7
4	0.4	3	2	1	6	26	20	4	2	5	2	12	0
5	1.0	16	63	73	23	22	41	6	22	10	8	5	3
6	0.6	12	46	29	53	29	2	6	6	16	5	3	6
7	2.6	5	36	34	33	14	8	7	5	4	0	2	9
8	2.4	7	40	31	37	1	7	4	3	28	8	0	1
9	1.2	23	29	23	22	8	20	13	4	14	9	8	4
10	2.2	39	17	15	7	14	33	21	5	0	11	14	7
11	1.4	81	26	29	1	36	14	22	16	0	6	15	3
12	1.6	64	14	45	24	26	34	21	5	5	17	5	2

ROW	V14	V15	V16	V17	V18	V19	V20	V21	V22	V23	V24	V25	V26
1	2	9	11	22	1	0	2	3	0	4	0	0	0
2	7	15	0	4	1	0	11	1	1	10	0	0	6
3	0	5	2	5	0	0	3	8	0	0	1	0	0
4	3	0	2	0	0	0	1	1	0	0	1	0	0
5	7	6	3	0	4	1	0	3	10	0	0	0	0
6	10	1	1	2	3	4	0	0	1	0	1	0	0
7	3	2	2	4	5	4	0	0	2	0	0	0	0
8	9	3	2	1	6	17	2	3	0	0	0	0	0
9	8	4	5	2	5	10	1	2	0	0	0	0	0
10	10	5	9	2	3	4	0	0	0	0	0	5	0
11	1	3	11	6	10	3	0	0	5	0	5	2	0
12	0	0	3	0	4	1	0	0	0	0	0	0	0

ROW	V27	V28	V29	V30	V31	V32	V33	V34	V35	V36	V37	V38	V39
1	0	0	3	0	0	0	0	0	0	0	0	1	0
2	0	0	0	1	0	2	0	0	0	0	0	0	0
3	0	0	0	1	2	0	0	1	0	0	0	0	2
4	0	0	0	0	0	0	0	0	0	1	0	0	1
5	6	0	0	0	0	0	0	0	0	0	0	0	2
6	0	0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	8	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0	0	0
11	0	0	1	0	0	0	0	0	0	0	1	0	0
12	0	4	0	0	0	0	0	0	1	0	0	0	0

MTB > CORR V1-V39

	V1	V2	V3	V4	V5	V6	V7	V8
V2	0.225							
V3	0.185	0.045						
V4	0.134	0.137	0.758					
V5	0.056	-0.411	0.546	0.356				
V6	-0.461	0.478	0.053	0.153	-0.148			
V7	-0.165	0.021	-0.110	0.411	-0.360	0.072		
V8	0.260	0.632	-0.213	-0.155	-0.461	-0.098	-0.048	0.425
V9	-0.155	0.233	0.187	0.223	-0.312	0.114	0.097	0.428
V10	0.082	-0.342	0.286	0.060	0.049	-0.647	-0.380	0.078
V11	0.258	0.689	0.265	0.392	-0.006	0.177	0.295	0.278
V12	-0.315	0.194	-0.530	-0.394	-0.712	0.383	0.379	-0.049
V13	0.345	0.254	0.361	0.040	0.202	0.256	-0.279	-0.113
V14	0.073	-0.381	0.359	0.063	0.360	-0.537	-0.036	0.006
V15	0.262	0.345	0.211	-0.065	-0.269	-0.264	-0.216	0.671
V16	0.234	0.702	0.135	0.072	-0.430	0.447	0.132	0.130
V17	0.270	0.581	0.332	-0.023	-0.056	0.375	-0.423	0.111
V18	0.282	0.237	0.252	0.414	0.020	0.032	0.050	0.008
V19	0.365	-0.330	0.202	0.056	0.398	-0.635	-0.199	-0.288
V20	0.119	0.132	-0.159	-0.366	-0.215	-0.394	-0.398	0.687
V21	-0.168	-0.239	-0.031	-0.147	-0.010	-0.137	-0.035	-0.332
V22	-0.179	0.015	0.538	0.715	-0.086	0.254	0.416	-0.083
V23	0.204	0.375	0.048	-0.188	-0.201	-0.189	-0.387	0.775
V24	-0.281	0.366	-0.179	-0.136	-0.389	0.574	-0.146	0.076
V25	0.273	0.262	-0.230	-0.188	-0.646	0.024	0.344	0.263
V26	0.126	0.185	-0.113	-0.237	-0.223	-0.341	-0.296	0.808
V27	-0.231	-0.179	0.559	0.739	0.034	0.068	0.585	-0.215
V28	0.037	0.334	-0.265	0.283	0.055	0.185	0.414	0.189
V29	0.194	0.672	0.377	0.109	-0.109	0.507	-0.289	0.070
V30	-0.077	-0.028	-0.368	-0.461	-0.236	-0.246	-0.257	0.439
V31	-0.231	-0.222	-0.382	-0.384	-0.095	0.010	-0.051	-0.215
V32	0.126	0.185	-0.113	-0.237	-0.223	-0.341	-0.296	0.808
V33	0.394	-0.276	0.172	0.056	0.334	-0.546	-0.247	-0.269
V34	-0.231	-0.222	-0.382	-0.384	-0.095	0.010	-0.051	-0.215
V35	0.037	0.334	-0.265	0.283	0.055	0.185	0.414	-0.189
V36	-0.498	-0.318	-0.466	-0.433	-0.330	0.185	0.071	-0.269
V37	-0.052	0.516	-0.063	0.023	-0.437	0.477	-0.075	0.215
V38	0.216	0.516	0.408	0.104	0.034	0.361	-0.271	00.000
V39	-0.517	-0.408	-0.041	0.101	-0.164	0.124	0.415	-0.412

MTB > CORR V1-V39

	V9	V10	V11	V12	V13	V14	V15	V16
V2								
V3								
V4								
V5								
V6								
V7								
V8								
V9								
V10	0.021							
V11	-0.156	-0.071						
V12	-0.028	-0.676	-0.086					
V13	-0.067	-0.428	0.234	-0.066				
V14	-0.141	0.599	-0.021	-0.256	-0.153			
V15	0.647	0.262	0.155	-0.268	0.215	0.155		
V16	-0.059	-0.579	0.507	0.259	0.462	-0.187	0.959	
V17	0.077	-0.280	0.412	-0.040	0.791	-0.331	0.429	0.599
V18	-0.036	0.021	0.036	0.118	-0.215	0.049	-0.310	0.354
V19	-0.457	0.607	-0.002	-0.214	-0.241	0.527	-0.237	-0.073
V20	0.505	0.481	-0.101	-0.356	-0.144	0.075	0.826	-0.345
V21	0.342	0.041	-0.159	0.061	0.181	-0.250	0.223	-0.132
V22	0.626	-0.094	-0.122	0.010	-0.157	0.023	0.079	0.071
V23	0.476	0.347	0.137	-0.408	0.063	0.062	0.886	-0.120
V24	0.240	-0.348	-0.253	0.585	-0.170	-0.359	-0.215	0.424
V25	-0.081	-0.430	0.148	0.630	0.109	0.264	0.001	0.579
V26	0.523	0.448	-0.088	-0.377	-0.225	0.162	0.788	-0.344
V27	0.523	0.029	0.023	-0.122	-0.145	0.162	0.118	-0.101
V28	-0.196	-0.145	0.522	-0.122	-0.225	-0.405	-0.329	-0.101
V29	0.023	-0.314	0.535	0.048	0.662	-0.343	0.299	0.711
V30	0.620	0.173	-0.336	-0.087	-0.039	-0.180	0.616	-0.390
V31	0.312	-0.215	-0.365	0.260	0.172	-0.405	0.043	-0.182
V32	0.523	0.448	-0.088	-0.377	-0.225	0.162	0.788	-0.344
V33	-0.277	0.657	0.023	-0.313	-0.304	0.324	-0.105	-0.182
V34	0.312	-0.215	-0.365	0.260	0.172	-0.405	0.043	-0.182
V35	-0.193	-0.145	0.522	-0.122	-0.225	-0.405	-0.329	-0.101
V36	-0.319	-0.145	-0.310	0.324	-0.383	-0.162	-0.329	-0.182
V37	0.270	-0.320	-0.088	0.515	-0.145	-0.324	-0.105	0.547
V38	-0.067	-0.215	0.578	-0.122	0.727	-0.243	0.341	0.547
V39	0.492	-0.188	-0.362	0.218	-0.120	-0.236	-0.002	-0.273

MTB > CORR V1-V39

	V17	V18	V19	V20	V21	V22	V23	V24
V2								
V3								
V4								
V5								
V6								
V7								
V8								
V9								
V10								
V11								
V12								
V13								
V14								
V15								
V16								
V17								
V18	-0.176							
V19	-0.263	0.095						
V20	0.131	-0.409	-0.168					
V21	0.213	-0.398	-0.023	0.187				
V22	-0.160	0.391	-0.192	-0.199	-0.229			
V23	0.362	-0.368	-0.304	0.918	-0.032	-0.121		
V24	0.053	0.550	-0.128	-0.190	-0.135	0.279	-0.196	
V25	-0.060	0.221	0.004	-0.226	-0.316	-0.022	-0.164	0.224
V26	00.000	-0.274	-0.226	0.944	-0.101	-0.061	0.924	-0.146
V27	-0.209	0.055	-0.165	-0.169	0.168	0.875	-0.122	-0.146
V28	-0.209	0.055	-0.165	-0.169	-0.235	-0.165	-0.122	-0.146
V29	0.953	-0.036	-0.234	-0.022	0.088	-0.045	0.250	0.166
V30	0.039	-0.487	-0.335	0.800	0.549	-0.167	0.595	-0.054
V31	0.052	-0.383	-0.226	0.135	0.841	-0.165	-0.122	0.073
V32	00.000	-0.274	-0.226	0.944	-0.101	-0.061	0.924	-0.146
V33	-0.157	0.274	0.823	0.034	0.168	-0.165	-0.122	-0.146
V34	0.052	-0.383	-0.226	0.135	0.841	-0.165	-0.122	0.073
V35	-0.209	0.055	-0.165	-0.169	-0.235	-0.165	-0.122	-0.146
V36	-0.209	-0.383	-0.226	-0.067	-0.101	-0.165	-0.122	0.073
V37	0.105	0.712	-0.041	-0.169	-0.235	0.355	-0.122	0.951
V38	0.942	-0.274	-0.226	0.034	0.168	-0.165	0.296	-0.146
V39	-0.191	-0.379	-0.367	-0.049	0.698	0.457	-0.222	-0.027

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MTB > CORR V1-V39
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	V25	V26	V27	V28	V29	V30	V31
V2							
V3							
V4							
V5							
V6							
V7							
V8							
V9							
V10							
V11							
V12							
V13							
V14							
V15							
V16							
V17							
V18							
V19							
V20							
V21							
V22							
V23							
V24							
V25							
V26	-0.122						
V27	-0.122	-0.091					
V28	-0.122	-0.091	-0.091				
V29	-0.023	-0.118	-0.118	-0.118			
V30	-0.181	0.674	-0.135	-0.135	-0.175		
V31	-0.122	-0.091	-0.091	-0.091	-0.118	0.674	
V32	-0.122	10.000	-0.091	-0.091	-0.118	0.674	-0.091
V33	-0.122	-0.091	-0.091	-0.091	-0.118	-0.135	-0.091
V34	-0.122	-0.091	-0.091	-0.091	-0.118	0.674	10.000
V35	-0.122	-0.091	-0.091	10.000	-0.118	-0.135	-0.091
V36	-0.122	-0.091	-0.091	-0.091	-0.118	-0.135	-0.091
V37	0.296	-0.091	-0.091	-0.091	0.237	-0.135	-0.091
V38	-0.122	-0.091	-0.091	-0.091	0.964	-0.135	-0.091
V39	-0.222	-0.165	0.629	-0.165	-0.215	0.344	0.629

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MTB > CORR V1-V39
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	V32	V33	V34	V35	V36	V37	V38
V2							
V3							
V4							
V5							
V6							
V7							
V8							
V9							
V10							
V11							
V12							
V13							
V14							
V15							
V16							
V17							
V18							
V19							
V20							
V21							
V22							
V23							
V24							
V25							
V26							
V27							
V28							
V29							
V30							
V31							
V32							
V33	-0.091						
V34	-0.091	-0.091					
V35	-0.091	-0.091	-0.091				
V36	-0.091	-0.091	-0.091	-0.091			
V37	-0.091	-0.091	-0.091	-0.091	-0.091		
V38	-0.091	-0.091	-0.091	-0.091	-0.091	-0.091	
V39	-0.165	-0.165	0.629	-0.165	0.232	-0.165	-0.165