PATHOGENICITY OF *ALTERNARIA ALTERNATA* AND ITS ANTIBODY PRODUCTION IN EXPERIMENTAL ANIMALS

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INTRODUCTION

Alternaria alternata[1] (A. tenuis) is a universal fungus and usually lives as a saprophyte. The fungus is well known as one of the ubiquitous plant pathogens.

A number of instances have been reported in which the fungus A. alternata was isolated from man. As early as 1925, A. tenuis was isolated by Motta from the external auditory meatus, while Montpellier, Cabanci and Cattori (1929) isolated the organism from a subcutaneous "nodule granulomateux" on the foot of an Algerian child.

Hopkins et al. (1930) reported a case of asthma due to A. tenuis. The same authors later reported a case of eczema which was extremely sensitive to contact with Alternaria. Thus the allergenicity of this fungus for man is now firmly confirmed.

Henrici (1930) suggested that Alternaria may cause suppurative lesions in humans, but the evidence for this is inconclusive. Ohue (1930) had isolated the fungus from the urine and the spinal fluid of a patient with meningitis. Borsook (1933) isolated the organism from a skin infection on the hand of a woman by a splinter.

Ohashi (1960) described "alternariasis" as a rare disease and has experienced 37 cases in Japan. He isolated the organism first from the urine of an 11-year-old boy with the complaint of pollakisuria, hematuria and urodynia, and later from the cerebrospinal fluid, the gall and the sputum

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[1] Simmons, E. G. (1967) presented evidence based on the international rules of nomenclature that the valid name for Alternata tenuis (Nees) is Alternaria alternata.

of other patients with hematuria, meningitis, cholecystopathy and other conditions.

English (1965) had shown the ability of the organism to disintegrate in vitro all the forms of keratinized materials such as the cortex of hair, human nails and callus. From this it is evident that the organism could lead a pathogenic role in man and animals under certain circumstances. Ainsley and Smith (1965) isolated the organism from the conjunctival sac in healthy and diseased eyes.

Botticher (1966) raised doubt about the nonpathogenic nature of Alternaria spp. She has isolated Alternaria spp. on 121 occasions from 2,381 specimens of suspected superficial mycosis of the skin, hair and nails. At the Duke University Medical Center, Alternaria sp. was cultured on four separate occasions from cutaneous lesion on the lower calf of a woman with discoid lupus erythematosus and leukopenia (Miller and Tindall, 1967).

Delacretaz et al. (1970) reported two cases of onychosis and one case of acute skin infection on the back of the hand from which he isolated repeatedly A. tenuis, and discussed the possible pathogenic role of this fungi.

The frequent isolation of the organism from hair samples and skin scrapings of horses and dogs received at the Veterinary Mycology Laboratory, College of Veterinary Medicine, K.S.U., gave suspicion that A. alternata could be a pathogen. It is a known fact that under certain conditions of actual mutation or modification of the host, various microorganisms may pass from simple parasitism to pathogenisis. It is thought that the fungus A. alternata may be one of those opportunistic agents.

This study was undertaken to investigate the possible pathogenicity of A. alternata. Two species of experimental animals were used. The experiment was conducted under normal conditions and also by altering the resistance of the animals by starvation. Three different modes of inoculation were adopted in the experiment. Further, the production of antibodies by A. alternata was investigated.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Thirty New Zealand white rabbits and thirty American short-haired, pigmented guinea pigs were used. Each species was divided into two major groups. Group I consisted of animals held under normal conditions, and Group II of animals that were fasted. Each of the two major groups of each species were further divided into three subgroups: A, B, C (normal) and A_1, B_1, and C_1 (fasted). A control animal was used for each subgroup. All animals were marked for identification and the two species were housed in separate rooms. All animals received commercial rations[2] and water ad libitum during the experiment.

Health of the animals was determined by checking general appearance, appetite and rectal temperatures. Hair samples from each animal were collected by combing gently on different parts of the body into separate sterile petri dishes. Sterile precautions were taken in combing between animals. The hair samples thus collected were examined for dermatophytes by direct examination (clearing with 10% NaOH and checking for spores) and culturing

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[2]Purina Rabbit Chow and Purina Guinea Pig Chow, Ralston Purina Co., St. Louis, Mo.

on agar media. Each of the hair samples was inoculated into six tubes, three tubes containing Mycobiotic agar[3] media and three tubes containing Sabouraud's Dextrose agar[4] media to determine the presence of fungi on the body of animals.

INOCULUM

Cultures of A. alternata[5] grown on Potato Dextrose Agar[6] (PDA) in Roux flasks were scraped and suspended in Phosphate Buffered Saline[7] (PBS) pH 7.4. The culture suspension was ground in a tissue grinder.[8] The number of conidia and broken mycelia per milliliter were determined by direct count in a hemocytometer,[9] using the following formula.

$$\text{No. of organisms counted} \times \text{Vol. cor. factor} \times \text{Dil. cor. factor} = \text{Number of conidia and mycelia per milliliter.}$$

It was determined that a concentration of 8×10^4 conidia and mycelia per milliliter was present in the suspension.

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[3]Bacto-Mycobiotic Agar (Dehydrated), Difco Laboratories, Detroit, Mich.

[4]Bacto-Sabouraud Dextrose Agar (Dehydrated), Difco Laboratories, Detroit, Mich.

[5]Cultures obtained from Pioneering Research Laboratories, U. S. Army Natick Laboratories, Natick, Mass.

[6]Bacto-Potato Dextrose Agar (Dehydrated), Difco Laboratories, Detroit, Mich.

[7]PBS--Na_2HPO_4, 5.6 g; K_2HPO_4, 2.7 g; NaCl, 4.1 g; and distilled water, 1000 ml.

[8]Tissue Grinder (Homogenizers), Pyrex Brand, Fisher Scientific Company.

[9]Bright Line, Improved Neubeuer, A. O. Spencer.

The ground culture suspension was placed in a spectrophotometer.[10] A forty-five percent transmittance was recorded using a wave length of 590 mu.

The concentration of conidia and mycelia was finally verified and confirmed by establishing the viable count of the culture suspension, using standard plate count technique. In this test serial dilutions from 10^{0} to 10^{-6} of the culture suspension and PDA plates were used. Volumes of 1 ml. of each solution were spread uniformly over the surface of the media with a sterile glass rod. The plates were incubated for 96 hrs. at 25°C. The plates of 10^{0}, 10^{-1} and 10^{-2} dilutions revealed the growth continuous and fully covered over the entire media. 10^{-3}, 10^{-4} and 10^{-5} dilution plates gave 90, 10 and 1 colonies respectively, while 10^{-6} dilution plates were negative.

INOCULATION

Both species of animals were inoculated with the culture suspension standardized to 8×10^{4}. Three modes of inoculation were used. The Group II animals were fasted for 72 hours to alter their resistance immediately before inoculation. The subgroups A and A_1 were inoculated by scarification, B and B_1 were inoculated intradermally and C and C_1 intraperitoneally. The groups, dose and modes of inoculation are shown in Table I.

The rabbits were reinoculated with the same concentration of the organisms and in the same manner 45 days after the first inoculation.

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[10] Coleman Junior II Spectrophotometer, Model 6/20, The Perkin-Elmer Corporation, Maywood, Illinois.

TABLE I

INOCULATION OF ANIMALS WITH A. ALTERNATA

Species	Subgroups[1] of Group I (normal)	Subgroups[1] of Group II (fasted)	Dose in ml.	Mode of inoculation
Rabbits	A	A_1	1.0	Scarification
	B	B_1	1.0[2]	Intradermal
	C	C_1	4.0	Intraperitoneal
Guinea pigs	A	A_1	0.5	Scarification
	B	B_1	0.5[2]	Intradermal
	C	C_1	2.0	Intraperitoneal

[1]All the controls were inoculated with sterile PBS. The dose and mode of inoculation used were the same as that of their representative group.

[2]Total dose used on two sides, and on each side two sites.

POST INOCULATION PROCEDURE

After inoculation, clinical observations of the animals were taken on alternate days. General physical condition and lesions at the site of injection were examined. Scrapings from the site of scarified areas were collected aseptically and inoculated on Sabouraud Dextrose Agar media and incubated at 25°C. Rectal temperatures of all animals were taken every fifth day.

After the second inoculation of the rabbits the same post inoculation procedures were repeated.

AUTOPSY AND CULTURE

One of the phases of the studies was to investigate the circulating antibody production following the inoculation with the organism. The animals

were bled by intracardiac puncture two weeks prior to inoculation and subsequently at 15 day intervals after inoculation until they were euthenized. Blood was collected using aseptic precautions and serum was separated and preserved at -25°C.

All guinea pigs including controls were euthenized using Euthenol[11] 30 days after inoculation and necropsied. Similar procedure was carried out in rabbits 60 days after the first inoculation and 15 days after the second inoculation. Sections of liver, spleen and lungs were collected aseptically from each animal and inoculated separately in tubes consisting of Mycobiotic agar media. A swab of peritoneal fluid from each animal was also streaked on Mycobiotic agar plates. The tubes and plates were incubated for 7 days at 25°C.

ANTIGEN PREPARATION

The antigen was prepared according to the technique described by Thjotta et al. (1950) with some modification. The procedure adopted essentially consisted of preparing soluble antigens suitable for serological tests as well as for immunization purposes.

The Alternaria alternata were cultured in Roux flasks containing PDA media. After 7 days of incubation the growth of the cultures was harvested with PBS and pooled in a sterile flask. To this suspension of organisms formalin was added to a final concentration of 0.5% and refrigerated. After 24 hours an equal amount of sod. bisulfite (meta) $Na_2S_2O_5$ was added to neutralize the formalin and again refrigerated for 24 hours. Sterility was checked by using PDA media (Appendix 4). The culture suspension was then

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[11] Barb-Euthol, Nitrogenized, Hoover-Lockhart Laboratories, U.S.A.

centrifugalized and the centrifugate lyophalized. This freeze-dried fungus was ground in a mortar until a fine homogeneous mass was obtained. This homogeneous mass was further sonified[12] and the resulting mass suspended in Borate Saline Buffer.[13] The material was placed on a shaker for one hour and then held in a refrigerator (4°C) overnight. The next day it was centrifuged for 30 minutes at 4,000 rpm. The sediment was discarded and the clear opalizing supernatant liquid collected in a sterile bottle. This crude antigen was used to evaluate the presence of circulating antibodies in the test animals following inoculation with A. alternata. A precipitate ring test was conducted on all the sera collected from the test and control animals.

RESULTS

On determining the health standards of test animals, the general appearance and the appetite of all animals were satisfactory. The rectal temperatures recorded for each animal (Appendix 2) were considered within normal range limits for the respective species. Direct examination for dermatophytes prior to inoculation were negative. On examination of the tubes inoculated with hair samples, containing Mycobiotic agar media and Sabouraud Dextrose agar media, after 7 days of incubation 17 tubes out of 180 tubes of Mycobiotic agar and 53 tubes out of 180 tubes of Sabouraud Dextrose agar media showed growth (Appendix 1). On microscopic examination of these growths in Lactophenol cotton blue wet mounts, four samples revealed

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[12]Sonifier Cell Disruptor, Model W185; Heat Systems--Ultrasonics, Inc., N. Y.

[13]Borate Saline Buffer--Boric acid, 6.184 g; Sod. tetraborate (Borax), 9.536 g; Sod. chloride, 4.384 g; and distilled water, 1000 ml.

Alternaria spp. and the rest were common saprophytic fungi and bacteria.

For one day following the inoculation all the test animals became lethargic and anorexic.

In the scarified animals the sites of scarification were erythematous for approximately 4 days after inoculation. In some animals the skin became dry and scaly, while in others it was moist and crusted. These areas showed retarded hair growth and new hair grew in patches. In a few animals the scarified lesions took more than two weeks to heal. In the control group the scarified lesions healed within 4 days and hair growth was normal and uniform over the area. Scrapings from the scarified areas (post-inoculation) revealed A. alternata organisms repeatedly for 2 weeks. It was observed that the rabbits were more susceptible to the organisms than guinea pigs.

On the second inoculation of the rabbits the dermal reaction was less severe and lesions healed in approximately 7 days.

In the intradermally inoculated group of animals the inoculum was absorbed within one day. A red papule-like swelling developed and persisted for two weeks. Hair grew in the adjoining area except at the site of injection. At this site the skin became dry and scaly. In control animals the inoculum swelling had absorbed completely within 2 days and did not show any reaction. In these animals the site of injection became indistinguishable from the adjoining area.

The rabbits upon second inoculation developed small abscesses. When these were opened a gelatinous pus exuded. This material was cultured on Mycobiotic agar and incubated at 24°C for 7 days. No fungal growth was observed.

The intraperitoneally injected animals did not show any abnormalities either after first or second inoculation.

On necropsy of the test animals no systemic abnormalities were found. The cultures from tissues and peritoneal fluid did not reveal the presence of A. alternata (Appendix 5).

The determination of the circulating antibodies against A. alternata in the sera of the test animals is given in Tables II and III.

TABLE II

ANTIBODY RESPONSE TO ALTERNARIA ALTERNATA INOCULATION IN RABBITS AS DETERMINED BY THE PRECIPITATION RING TEST

Mode of Inoculation	Subgroup of Animals	Number of Animals Positive			
		Serum I[1]	Serum II[2]	Serum III[3]	Serum IV[4]
Scarification	A	1/4	4/4	2/4	0/4
Scarification	A_1	0/4	4/4	2/4	0/4
Intradermal	B	1/4	3/4	4/4	2/4
Intradermal	B_1	0/4	4/4	4/4	0/4
Intraperitoneal	C	0/4	3/4	3/3[#]	1/3
Intraperitoneal	C_1	0/4	3/4	4/4	3/4

Control animals of all groups were negative for circulating antibodies.

[1]Serum collected 15 days prior to inoculation.

[2]Serum collected 15 days after inoculation.

[3]Serum collected 30 days after inoculation.

[4]Serum collected 60 days after initial inoculation (15 days after the second inoculation).

[#]A rabbit of this group died due to an accident.

TABLE III

ANTIBODY RESPONSE TO ALTERNARIA ALTERNATA INOCULATION IN GUINEA PIGS AS DETERMINED BY THE PRECIPITATION RING TEST

Mode of Inoculation	Subgroup of Animals	Number of Animals Positive		
		Serum I[1]	Serum II[2]	Serum III[3]
Scarification	A	0/4	3/4	4/4
Scarification	A_1	0/4	4/4	4/4
Intradermal	B	0/4	4/4	4/4
Intradermal	B_1	0/4	4/4	4/4
Intraperitoneal	C	0/4	4/4	4/4
Intraperitoneal	C_1	0/4	4/4	4/4

Control animals of all groups were negative for circulating antibodies.

[1]Serum collected 15 days prior to inoculation.

[2]Serum collected 15 days after the inoculation.

[3]Serum collected 30 days after the inoculation.

DISCUSSION

In standardizing the inoculum the concentration of the suspension with the fungal elements was determined in a hemacytometer. This study gave the number of conidia and mycelial segments present in a milliliter of the suspension. To know the viable fungal elements present in the suspension and also to verify their number a viable count of the inoculum was conducted. The figures derived from these two different techniques in standardizing the dose were closely correlated.

The rabbits were reinoculated 45 days after the initial inoculation in order to observe instances of infection due to a second exposure to the organisms and a possible heightening of the degree of the observable reactions. In guinea pigs as considerable mortality had been experienced during the third bleeding due to the multiple punctures of the heart reinoculation was not carried out.

The results show that the A. alternata are "opportune agents" and the organisms produce superficial lesions on the skin when inoculated by scarification on the back of the experimental animals. This study supports the findings of Dovgich (1962) that an artificial inoculation of A. tenuis (A. alternata) infected 3 or 5 dogs, in which characteristic eczema symptoms were noted on the back of the dogs. The results of the present studies reveal that the lesions produced were eczematoid with peripheral spreading. The hair grew on healed areas in patched. A perusal of the earlier work on the subject shows that the Alternaria spp. are capable of disintegrating in vitro the cortex of hair, nails and callus by means of their boring hyphae (English, 1965). In view of this study it is possible that the organism may be capable of producing superficial mycosis. The lesions were more prominent and prolonged in rabbits than in guinea pigs.

The organisms when injected intradermally produced reactions varying from red papule-like swellings to small abscesses. It appears that the organisms have a tendency to cause suppurative lesions intradermally. When inoculated intraperitoneally the organisms appear not to be pathogenic, but the antibodies against A. alternata were produced. However, the ubiquitous character of the organism makes it difficult for critical interpretation of the possible systemic pathogenicity of the fungus although Ohue (1930),

Ohashi (1960) and others reported previously isolation of this fungus from body fluids.

There were no significant differences in regard to the clinical symptoms and lesions between the normal and fasted animals of both species.

The antigens obtained by cell disintegration were found to be suitable for detecting antibodies against A. alternata in the sera of the test animals. No natural antibodies reacting with the antigens prepared from this fungus were detected in the control animals. However the preinoculation sera of two test rabbits reacted with the antigen. This may be due to a nonspecific reaction or these rabbits may have already been exposed to the fungus A. alternata. The second inoculation of the rabbits produced less reaction which is evidence of the immunizing capabilities of the organism. By this study it is clear that the organisms are capable of producing circulating antibodies in experimental animals. It is observed that the guinea pigs were better in antigenic response as the ring formation was more consistently significant in the precipitation test. The lesions produced in the guinea pigs however were not as severe as in rabbits.

The cause of disappearance of antibodies after the reinoculation of organisms in rabbits is not understood. However, it may be due to collecting the serum during Lag phase of the antibody production. The few doubtful reactions recorded at second and third bleeding tests could not be considered of significance. In the present study the number of subjects studied and the strain of the organisms used suggest further experiments be conducted to better understand the pathogenic role of A. alternata.

SUMMARY

Experimental studies to determine the potential pathogenicity of Alternaria alternata in rabbits and guinea pigs have been conducted. The animals were inoculated with the organisms by the three different modes of inoculation: scarification, intradermal and intraperitoneal. The results show that A. alternata causes superficial mycosis when inoculated by scarification. The ubiquitous character of the organism makes it difficult to interpret the possible pathogenic role played by the organism when inoculated intradermally or intraperitoneally. The antigens of A. alternata have been prepared to detect the circulating antibodies against the same organism. A precipitate ring test was conducted against the sera collected from all the animals and was found to be positive in all the test animals.

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APPENDIX

APPENDIX I

EXAMINATION OF HAIR SAMPLES

In ascertaining the health standards of rabbits and guinea pigs an external examination for the presence of fungal organisms prior to inoculation with A. alternata was conducted. Pre-inoculation hair samples were collected by gently combing the hair on different parts of the body into separate sterile petri dishes. Care was taken to include the basal portion of the hairs. Sterile precautions were taken in combing between animals. The hair samples were examined first for dermatophytes by Direct Examination method. All the samples were found negative for spores of dermatophytes. Hair samples then were cultured on agar media. Each sample was inoculated on six agar slants, three containing Mycobiotic agar media and the other three with Sabouraud's Dextrose agar media. The slants were incubated at room temperatures and examined for growth after 7 days. Seventeen samples out of 180 tubes of Mycobiotic agar media and fifty-three samples out of 180 tubes of Sabouraud's Dextrose agar media showed positive growth while the remaining were negative. The positive growth slants were examined by making Lactophenol Cotton Blue Tease-mounts. The fungal isolates found on the positive slants are listed below:

ISOLATES ON MYCOBIOTIC AGAR MEDIA

ORGANISMS ISOLATED	GUINEA PIGS	RABBITS
Alternaria spp.	-	4
Cladosporium spp.	2	2
Helminthosporium spp.	-	1
Penicilium spp.	1	1
Scopulariopsis spp.	5	1

ISOLATED ON SABOURAUD'S DEXTROSE AGAR MEDIA

ORGANISMS ISOLATED	GUINEA PIGS	RABBITS
Alternaria spp.	-	3
Aspergillosis spp.	4	9
Cladosporium spp.	-	5
Penicilium spp.	-	1
Mucor spp.	-	2
Scopulariopsis spp.	3	2
Bacterial organisms	15	9

APPENDIX 2

The rectal temperatures of rabbits and guinea pigs were taken at 5 day intervals. The readings obtained were considered within the normal range limits for the respective species.

RECTAL TEMPERATURES OF RABBITS

Rabbit Number	Number of Readings							
	I	II	III	IV	V	VI	VII	VIII
1	102	102.4	102	102	102	102	103.8	103
2	101.8	102	102	103	102.2	102	103	103
3	102	102.6	102	102	103	103	103	103
4	102	103.2	103.2	102	102.4	102	102.8	102
5	103	104	104	102	102	102	102.8	102.4
6	103	103.4	103	103	103	103	103.8	103
7	102	102.8	102.8	103	102.8	102.8	103.4	102.8
8	102	102.8	102.8	102.2	102.2	102.2	102.8	102.6
9	102	103	102	102	102	102	103	103
10	102	102.8	102.8	103	102.8	102.6	103	103
11	101.8	102	101.8	102.8	101.8	102	103	103
12	102.4	102.8	102.8	102.8	102.4	102.8	103	103
13	102.4	102.8	102.4	102.4	102	102	102.8	102.4
14	102.4	102.8	102.4	102.4	102.4	102	102.8	102.8
15	103	103.2	103	103	102	102	102.8	103
16	103	103	103	103	103	102.8	103	103
17	102.8	103	103	102	102	102	102.8	103
18	103	103.6	103.6	d*	d	d	d	d

RECTAL TEMPERATURES OF RABBITS (cont'd.)

Rabbit Number	Number of Readings							
	I	II	III	IV	V	VI	VII	VIII
19	103	103.4	103	102.4	102.4	102.4	103.2	103
20	102.4	102.8	102.4	102.4	102.8	102.4	102.8	102.8
21	103	103	103	102.4	102.4	102.8	103.2	103
22	102	102.6	102.6	102.8	102.8	102.6	103	102.6
23	102	103	102.4	102.8	102.8	102.8	103.2	103
24	102.6	102.8	102.8	103	103	103	103.2	103
A	102.6	102.8	102.6	102.8	102.8	102.8	103	103
B	103	103	103	103	103	103	103	103
C	102.4	103	102.8	102.8	102.8	102.8	102.8	102.4
A_1	102.6	102.8	102.6	102.6	102.6	102.6	103	102.8
B_1	102.8	103	103.4	102.8	102.8	102.6	103	102.8
C_1	102.8	103	102.8	102.8	102.6	102.6	102.8	102.6

I Temperature taken pre-inoculation.

II through VI Temperature after first inoculation.

VII and VIII Temperature after second inoculation.

d* - died due to an accident.

RECTAL TEMPERATURES OF GUINEA PIGS

Guinea Pig Number	I	II	III	IV	V	VI	VII
1	101	102	102	101.4	101.2	101	101
2	101	103	102	100.8	101	101	101
3	101	102	102	100.6	101	101	101
4	101	102.6	102	101.4	100.8	101	101
5	100	101.8	102	101	101	101	100
6	100.4	103	102.6	101.4	100.8	100.8	100.6
7	100.4	101.8	100.2	100.2	100.6	100.6	100.6
8	100.6	103	102.2	100.8	100.8	100.8	100.6
9	101	102.2	102	101.6	101.6	101.2	101
10	100.4	103	102.4	101	101	101	100.8
11	100.2	103	102.6	101	101	101	100.8
12	101	102.8	101.8	101	100.8	100.8	100.8
13	100	102.8	102	102	102	101	101
14	101	103	101.4	101	101	101	101
15	100	102.4	102.4	101.2	101	101	100.8
16	101	102.2	102	100.8	100.8	100.8	100.8
17	101	102.4	102.8	101.4	101	101	101
18	101	102	102	101.2	101.2	101	101
A	101	101.4	101.2	101.2	101.6	101	101
B	101	102.4	102	101	101	101	101
C	101	102.4	101	100	100.8	101	101
A_1	101	102.4	102	101.4	101	101	101
B_1	101	102.4	102	101.4	101	101	101
C_1	101	102	102	101	101	101	101

I Temperature taken pre-inoculation.

II through VII Temperature taken after the inoculation.

APPENDIX 3

VIABILITY OF THE A. ALTERNATA IN PHOSPHATE BUFFER SALINE

Initially, the viability of the A. alternata in Phosphate Buffer Saline (PBS) pH 7.4, was tested. Three slants of A. alternata cultures were harvested with PBS and pooled in a sterile flask. From this suspension, kept at room temperature, an inoculum was taken at 30 minute intervals streaked on a Sabouraud's Dextrose agar media slants until 5 hours, total time had elapsed. All the tubes were incubated at 25°C. The tubes showed luxuriant growth of A. alternata after four days of inoculation, indicating that the organisms would be viable in PBS solution.

APPENDIX 4

ANTIGEN PREPARATION

STERILITY TEST

In the process of antigen preparation the fungal elements were killed by adding formalin to the suspension of A. alternata. To know the killing effect of formalin on the fungus A. alternata a portion of culture suspension was divided into twelve test tubes of 10 ml each; to these ten tubes 0.06, 0.12, 0.18, 0.25 and 0.5 ml of formaldehyde (40%) was added in duplicates. Thus the final concentrations of formalin in these tubes were .25%; .5%; 1%; 1.5% and 2%. Two other tubes of culture suspension in PBS were kept as controls. The viability of the organisms keeping all the tubes at room temperature was tested by standard plate count technique. One ml of the suspension solution from each of the tubes was spread uniformly over the surface of Potato Dextrose agar media in petri dishes with a sterile glass rod every 24 hrs interval. The plates were incubated at 25°C. The readings of the plates after 96 hrs of incubation is as shown below:

Culture Suspension Age	Growth in Formalin Dilutions					Growth in Control Dilution[1]
	.25%	.5%	1%	1.5%	2%	
0 hrs	-	-	-	-	-	+ (43 colonies)
24 hrs	-	-	-	-	-	+ (44 colonies)
48 hrs	-	-	-	-	-	+ (41 colonies)
72 hrs	-	-	-	-	-	+ (42 colonies)
96 hrs	-	-	-	-	-	+ (42 colonies)

[1]Control suspension was diluted to 10^{-2} and plated.

APPENDIX 5

AUTOPSY AND CULTURE

The animals were bled by intracardiac puncture 2 weeks prior to inoculation with A. alternata organisms, and subsequently at 15 day intervals after inoculation. The site of puncture was cleansed with alcohol. Rabbits were bled using disposable syringes with monoject needles 19 g x 1 1/2", while guinea pigs were bled using disposable syringes with 20 g x 1 1/2" needles. Blood was collected aseptically and serum was separated and preserved at -25°C. The guinea pigs were euthenized using "Euthenol" 30 days after inoculation with organisms, and necropsied. The rabbits were euthenized 60 days after the first inoculation (which was 15 days after their second inoculation).

Sections of liver, spleen, and lungs were collected aseptically from each animal and inoculated separately on Mycobiotic agar slants. Also a swab of peritoneal fluid from each animal was streaked on a Mycobiotic agar plate. The tubes and plates were incubated at 25°C for one week. The cultures from tissues and peritoneal fluids did not reveal the presence of A. alternata, but the following fungal isolates were observed.

Animal Number	Tissue	Organism Found
Rabbit 2	Lung	Aspergillus spp.
Rabbit 3	Lung	Aspergillus spp.
Rabbit 4	Lung	Aspergillus spp.
Rabbit 8	Lung	Aspergillus spp.
Rabbit 12	Lung	Aspergillus spp.

Animal Number	Tissue	Organism Found
Rabbit 14	Spleen	*Aspergillus* spp.
Rabbit 16	Lung	*Cladosporium* spp.
Rabbit 19	Lung	*Cladosporium* spp.
Rabbit 21	Lung	*Cladosporium* spp.
Rabbit 25	Lung	*Aspergillus* spp.
Rabbit 27	Lung	*Aspergillus* spp.
Guinea pig 8	Spleen	*Penicilium* spp.
Guinea pig 12	Spleen	*Penicilium* spp.

PATHOGENICITY OF *ALTERNARIA ALTERNATA* AND ITS ANTIBODY PRODUCTION IN EXPERIMENTAL ANIMALS

by

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Information is lacking in the literature regarding the pathogenicity of Alternaria alternata (A. tenuis) in man and animals. As early as 1925, Motta had isolated A. tenuis from the external auditory meatus, while Perazzi (1925) isolated the fungus from female genitals. Montpellier, Cabanci and Cattori (1929) had also isolated the organism from a subcutaneous "nodule granulomateux" on the foot of an Algerian child. Hopkins et al. (1930) confirmed A. tenuis as a cause of asthma and its allergenicity in man. Ohue (1930) isolated the fungus from the urine and spinal fluid of a patient with meningitis. Henrici (1930) suggested that A. tenuis may cause suppurative lesions, but the evidence was inconclusive. Since then this fungus has been isolated in man from many different sources, including bile, cerebrospinal fluid, the conjunctival sac and skin (Ohashi, 1960; Ainsley and Smith, 1965; Botticher, 1966; and Miller and Tindall, 1967) and in animals, from dogs with eczema (Dovgich, 1962).

This fungus has also been isolated frequently from equine and canine specimens received at the Mycology Laboratory, College of Veterinary Medicine, Kansas State University.

As Alternaria is a common contaminant in laboratories, it has generally been regarded as insignificant when cultured from clinical materials. Also, it is not justifiable from the mere findings of A. alternata in human and animal specimens to conclude that the fungus appears as a parasite on man and animals. However, it is questionable whether the fungus A. alternata is always nonpathogenic to man and animals.

Because of the frequent isolation of the fungus from clinical cases in man and animals, it was deemed important to investigate the parasitic nature

of A. alternata. With this in mind, experiments were conducted for the following purposes: first, to determine the potential pathogenicity of A. alternata in rabbits and guinea pigs; and second, to detect possible circulating antibodies against A. alternata.

Experiments were conducted on 30 New Zealand white rabbits and 30 American short-haired pigmented guinea pigs. The results are discussed and the conclusion is drawn that the fungus is an opportunistic agent. A. alternata did produce superficial mycoses and also had the tendency to cause suppurative lesions intradermally. However, the ubiquitous nature of the fungus makes it difficult for critical interpretation regarding its pathogenicity. The antigens prepared by disintegrating the cells by means of grinding and sonic vibration were found to be suitable for detecting circulating antibodies in the rabbits and guinea pigs. The antibody response was positive against A. alternata in these experimental animals.