

PATHOGENICITY OF ALTERNARIA ALTERNATA AND ITS ANTIBODY PRODUCTION IN EXPERIMENTAL ANIMALS¹

by

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Abstract

To determine the potential pathogenicity of *Alternaria alternata*, we exposed rabbits and guinea pigs to the organism by intradermal and intraperitoneal injection and by scarification. It caused superficial mycosis when inoculated by scarification. An antigenic extract of it that we prepared and used in ring precipitation tests demonstrated precipitating antibodies in the experimentally infected animals.

Introduction

Motta (1925) isolated *A. alternata* from the external auditory meatus of a man; Montpellier, Cabanci & Cattori (1929) isolated it from a subcutaneous 'nodule granulomateux' on the foot of an Algerian child; Henrici (1930) recovered it from superficial wounds and suggested that it may cause suppurative lesions in human beings. More recently it has been isolated from many different sources in man, including bile, cerebrospinal fluid, conjunctival sac, and skin (Ohashi, 1960; Ainsley & Smith, 1965; Botticher, 1966; Miller & Tindall, 1967). Delacretaz et al. (1970) reported two cases of Onchomycosis and one of acute skin infections from which he repeatedly isolated *A. tenuis*. In animals, Dovgich (1962) isolated the fungus from dogs with eczema.

We investigated the possible pathogenic role of *A. alternata* in rabbits and guinea pigs, and production of circulating antibodies after inoculating the organism.

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Materials and methods

Experimental animals

Thirty New Zealand white rabbits and 30 American short-haired, pigmented guinea pigs were divided into two major groups per species. Group I animals were controls. Group II animals were fasted 72 hours then inoculated. Groups I and II were subdivided into three groups. One control animal was used for each subgroup. Rabbits and guinea pigs of Group I were housed in separate rooms and received commercial rations¹ and water *ad libitum* during the experiment. Group II were fed and watered in the same manner.

Health of the animals was determined by checking general appearance, appetite, and rectal temperature. Samples were collected by gently combing hair from various areas of the body into sterile petri dishes. Hair samples were examined for dermatophytes by direct examination and by culturing. Each hair sample was inoculated into six tubes, 3 containing Mycobiotic agar² and 3 containing Sabouraud's Dextrose agar³ to detect fungi.

Inoculum

*Alternaria alternata*⁴ cultures grown on Potato Dextrose Agar⁵ (PDA) in Roux flasks were scraped from the surface of the medium and suspended in Phosphate Buffered Saline⁶ (PBS) pH 7.4. The culture suspension was homogenized in a tissue grinder⁷. Number of conidia and broken mycelia per milliliter were determined by direct count in a hemacytometer⁸. The suspension contained conidia and mycelia in a concentration of 8×10^4 particles/ml.

Viability of the organisms in the culture suspension was determined by standard plate-count techniques. In this test, serial dilutions from 10^0 to 10^{-6} of the culture suspension were made and plated on PDA. Volumes of 1 ml of each dilution were spread uniformly over the surface of the medium with a sterile glass rod. The plates were incubated 96 hrs. at 25 °C. Growth occurred in the first 5 dilutions.

Inoculation

All animals were inoculated with a culture suspension standardized to

¹ Purina Rabbit Chow and Purina Guinea Pig Chow, Ralston Purina Co., St. Louis, Mo.

² Bacto-Mycobiotic Agar (Dehydrated), Difco Laboratories, Detroit, Mich.

³ Bacto-Sabouraud Dextrose Agar (Dehydrated), Difco Laboratories, Detroit, Mich.

⁴ Cultures obtained from Pioneering Research Laboratories, U.S. Army Natick Laboratories, Natick, Mass.

⁵ Bacto-Potato Dextrose Agar (Dehydrated), Difco Laboratories, Detroit, Mich.

⁶ PBS- Na_2HPO_4 , 5.6 g; K_2HPO_4 , 2.7 g; NaCl, 4.1 g; and distilled water, 1000 ml.

⁷ Tissue Grinder (Homogenizers), Pyrex Brand, Fisher Scientific Company.

⁸ Bright Line, Improved Neubauer, A. O. Spencer.

Table 1. Details of inoculating animals with *A. alternata*

Species	Subgroups ¹ of Group I (normal)	Subgroups ¹ of Group II (stressed)	Dose 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	Intradermal
	C	C ₁	2.0	Intraperitoneal

¹ All controls were inoculated with sterile PBS. Dose and mode of inoculation were the same as those of their representative group.

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

8×10^4 fungal elements/ml. Three modes of inoculation were used (table 1).

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

Post-inoculation procedure

Clinical observations of the animals were on alternate days after inoculation. General physical condition was determined and inoculation sites were examined. Scrapings were collected aseptically from scarified areas and incubated on Sabouraud Dextrose Agar and incubated at 25 °C. Rectal temperatures were taken every fifth day.

Autopsy and culture

All animals were bled by intracardiac puncture two weeks before, and at 15-day intervals after, inoculation until they were euthanized. Aseptic precautions were taken during blood collecting and serum was separated and preserved at -25 °C.

Guinea pigs were necropsied 30 days post-inoculation; 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 into tubes of Mycobiotic agar. A swab of peritoneal fluid from each animal was streaked on Mycobiotic agar plates. Tubes and plates were incubated 7 days at 25 °C.

Antigen preparation

The antigen for serological tests was prepared according to the technique described by Thjotta et al. (1950) with following modifications.

Alternaria alternata was cultured in Roux flasks containing PDA media. After 7 days incubation, cultures were harvested with PBS and

pooled. Formalin was added to a final concentration of 0.5%, the suspension was stored at 4°C 24 hours and sodium bisulfite (meta) $\text{Na}_2\text{S}_2\text{O}_5$ was added to neutralize the formalin. Following sterility check on PDA media, the suspension was centrifuged and the pellet was lyophilized. The freeze-dried fungus was ground in a mortar to homogenous suspension with borate saline buffer¹⁰ and sonicated¹¹. The suspension was placed on a shaker for one hour, incubated at 4°C overnight, centrifuged for 30 minutes at 3,000 g, and the supernatant was used as an antigen to detect circulating antibodies in the sera of test animals after inoculating with *A. alternata*. The ring precipitation test was used.

Results

Direct examination for dermatophytes before inoculation was negative. *Alternaria* spp. were isolated from four hair samples of 180 obtained before inoculation.

In all scarified animals, inoculation sites were erythematous for four days after inoculation. In some the skin became dry and scaly; in others it was moist and crusted. Little hair growth was observed in scarified areas. In a few animals, lesions persisted two weeks or longer. In the uninoculated control group scarified areas healed within four days, and hair growth was normal and uniform. All scrapings from the scarified areas examined on alternate days (post-inoculation) revealed *A. alternata* organisms repeatedly for 2 weeks. Lesions of the rabbits were more inflammatory and more distinct longer than lesions of guinea pigs. On the second scarification of the rabbits their reaction was less severe and lesions healed in approximately 7 days with organisms being revealed for 5 days. With the intradermally-inoculated group of rabbits and guinea pigs, the inoculum was absorbed within one day. A red papule-like swelling developed and persisted two weeks. Skin at the injection site became dry and scaly and no hair grew. No organisms were recovered from the areas. Control animals had no observable reaction to the sterile inoculum.

The rabbits developed small abscesses after the second inoculation. When opened, the abscesses exuded a gelatinous pus. The pus was cultured on Mycobiotic agar and incubated at 24°C 7 days. No fungal growth was observed. All animals injected intraperitoneally remained normal throughout the experiment.

Necropsy of test animals revealed no systemic abnormalities. *A. alternata* was not recovered from tissues or peritoneal fluid.

Details on circulating antibodies detected against *A. alternata* in sera of test animals are presented in tables 2 and 3.

¹⁰ Borate Saline Buffer-Boric acid, 6.184 g; Sod. tetraborate (Borax), 9.536 g; Sod. chloride, 4.384 g; and distilled water, 1000 ml.

¹¹ Sonifier Cell Disruptor, Model W185; Heat Systems-Ultrasonics, Inc.

Table 2. Antibody response by rabbits to *alternaria alternata* inoculation 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	1/4	4/4	2/4
Scarification	A ₁	0/4	4/4	2/4
Intradermal	B	1/4	3/4	4/4
Intradermal	B ₁	0/4	4/4	4/4
Intraperitoneal	C	0/4	3/4	3/3*
Intraperitoneal	C ₁	0/4	3/4	4/4

Control animals of all groups were negative for circulating antibodies.

¹ Serum collected 15 days before inoculation.

² Serum collected 15 days after inoculation.

³ Serum collected 30 days after inoculation.

* One rabbit in group was accidentally killed.

Table III. Antibody response by guinea pigs to *alternaria alternata* inoculation 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 before inoculation.

² Serum collected 15 days after inoculation.

³ Serum collected 30 days after inoculation.

Discussion

A. alternata appears to be an 'opportunistic agent' as it produced superficial lesions on the skin when rabbits and guinea pigs were inoculated by scarification, which supports findings of Dvovich (1962). He reported characteristic eczema in his dogs inoculated with *A. tenuis* (*A. alternata*). Lesions produced in our experiments were eczematoid with peripheral spreading. Earlier work on the subject shows that hyphae of *Alternaria* spp. can disintegrate the cortex of hair, nails, and callus (English, 1965).

Our study suggests that the organism may produce superficial mycosis. Lesions were more prominent and prolonged in rabbits than in guinea pigs.

Injected intradermally, *A. alternata* produced reactions varying from red papule-like swellings to small abscesses. *A. alternata* was not patho-

genic when inoculated intraperitoneally, but antibodies to *A. alternata* were produced. There were no significant differences in regard to clinical symptoms and lesions between normal and fasted animals of either species.

Antigens obtained by cell disintegration are suitable for detecting antibodies to *A. alternata* for the study shows that the organisms can produce circulating antibodies in experimental animals. Guinea pig sera showed more constant and significant precipitation test results to *A. alternata* than did rabbit sera, indicating a better antigenic response. 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*.

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