Experimental aspergillus endocarditis in rabbits

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Aspergillus endocarditis in man usually occurs on prosthetic cardiac valves and gives rise to large vegetations which embolize easily producing peripheral organ infarction and infection. Blood cultures are usually sterile and the disease is difficult to cure with antimicrobial agents. Aspergillus endocarditis was studied in rabbits to determine the course, degree of fungemia, and response to treatment with amphotericin B (A), 5 flucytosine (5 FC) or A + 5 FC. Polyethylene tubing was introduced into the left ventricle through the carotid artery and 24 hours later animals were inoculated with 10^4 to 10^7 spores of a strain of *Aspergillus fumigatus*. Large occlusive vegetations developed on the aortic valves. Spontaneous mortality reached 67 per cent after 3 days. Despite large aggregates of mycelia seen beneath a layer of amorphous material on microscopic sections, vegetations contained only 10^3 to 10^5 colony forming units (CFU) of aspergilli per gram, suggesting the aspergilli in tissues were clumped. Disseminated infection involving kidney, lung, liver, spleen, and brain occurred. Animals without intracardiac tubing which received the same inoculum of spores did not develop endocarditis, but showed evidence of disseminated infection. Blood after 24 hours of infection grew aspergilli only when large volumes were cultured and then only a small fraction of the total volume of blood obtained for culture yielded aspergilli, suggesting that the aspergilli in blood were clumped. Sterile vegetations in the absence of an intracardiac catheter were resistant to infection with aspergilli, but once established, infection with aspergilli persisted on vegetations despite removal of the catheter. Treatment of infected animals with A (1 mg. per kilogram), 5 FC (25 or 50 mg. per kilogram) or A + 5 FC daily intraperitoneally, significantly lowered the number of CFU per gram of vegetation.

Aspergillus endocarditis, although rare, has increased in incidence, mainly in association with insertion of prosthetic cardiac valves.[1, 2] Characteristically, large vegetations are produced and blood cultures are usually sterile. Aspergillus endocarditis is usually fatal and valve replacement plus antifungal therapy has been necessary to achieve the few reported cures. Experimental endocarditis in the rabbit was studied to determine the predisposing factors, the course, degree of fungemia, and response to chemotherapeutic agents.

Materials and methods

Micro-organism. The organism used in all experiments was a clinical isolate of *Aspergillus fumigatus* (Ryan strain), kindly supplied by Dr. Irving Abrahams. The spores were scraped from the

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surface of stock cultures grown for one week at room temperature on Sabouraud's dextrose agar slants (Difco), diluted as necessary in saline solution, and counted in a hemocytometer chamber. The spore suspension was adjusted to give a final inoculum of 10^4, 10^5, or 10^7 spores per milliliter.

Production of endocarditis. White female New Zealand rabbits weighing 2 kilograms (West Jersey Biological Farm, Wenonah, N. J.), were anesthetized with 60 mg. of sodium pentobarbital intravenously. Polyethylene tubing (Intramedic, PE 90/S36, Clay Adams, Parsippany, N. J.) was passed through the right carotid artery into the left ventricle and secured as previously described.[3] Groups of rabbits were inoculated with 10^4 to 10^7 spores, in a 1 ml. volume, into a marginal ear vein 24 hours after insertion of the polyethylene tubing. Rabbits which died spontaneously were discarded. The remaining rabbits were killed by intravenous injection of sodium pentobarbital 6 hours to 10 days after injection of the test organism. Vegetations and tissue samples from lung, liver, kidney, spleen, and brain were excised, weighed, and homogenized in sterile saline with a Teflon tissue grinder (Tri-R Instruments, Inc., Rockville Center, N. Y.). Blood was obtained from various sites in the arterial and venous system for culture.

Microbial enumeration. Serial 10-fold dilutions of the inocula or homogenized tissue suspension were made in sterile saline. The number of colony forming units (CFU) of aspergilli per milliliter of blood or saline or per gram of tissue was determined by plating 1.0 and 0.1 ml. of each inoculum, blood or homogenized tissue and 0.1 ml. of each serial dilution onto Sabouraud's dextrose agar plates. The Sabouraud's dextrose agar plates were incubated at room temperature for one week.

Growth in serum. To determine the antifungal activity of rabbit serum, 1 ml. samples of sera were obtained from normal rabbits and from rabbits 5 days after infection. Undiluted serum was inoculated with 0.1 ml. of a spore suspension containing 10^2 spores of *A. fumigatus*. The tubes were inspected daily for turbidity; if the tubes were clear, 0.1 ml. aliquots were cultured on Sabouraud's dextrose agar plates.

Treatment of aspergillus endocarditis. In vitro studies were performed to determine the minimal inhibitory concentration of flucytosine (5 FC), amphotericin B, and combinations of these drugs for the strain of *A.fumigatus*.[1] Groups of rabbits with the intracardiac catheters remaining in place were treated starting 6 hours after receiving 10^7 spores intravenously. The treatment groups were rabbits receiving either amphoterocin B (1 mg. per kilogram per day), 5 FC (25 or 50 mg. per kilogram per day) intraperitoneally, or both 5 FC and amphoterocin B. The animals were killed at 3 to 5 days after initiation of therapy and the vegetations, blood, and tissues cultured. Serum levels of 5 FC were determined by an agar diffusion technique using *Saccharomyces cerevisiae*, ATCC 9763, as the assay organism.[4]

Results

Rabbits without intracardiac catheters: Two rabbits without intracardiac catheters were given 10^7 aspergillus spores intravenously and were autopsied six hours later. Each 1 ml. aliquot of 5 ml. samples of blood from the right atrium, abdominal vena cava, and aorta contained 2 to 20 CFU of aspergilli per milliliter. No evidence of endocarditis was present grossly.

Of 9 rabbits without intracardiac catheters, given 10^7 spores intravenously, 6 died within 7 days. At autopsy, examination of the heart failed to reveal gross evidence of endocarditis and the aortic valves were sterile ($< \log_{10}$ 2.0 CFU of aspergilli per gram). One milliliter samples of blood from the aorta, vena cava, and right atrium were also sterile ($<$ 1 CFU of aspergilli per milliliter); but brain, liver, kidney, and spleen contained mean $\log_{10}$ 2.5, 4.4, 3.7, and 4.0 CFU of aspergilli per gram, respectively, on culture.

Rabbits with intracardiac catheters. Of 9 catheterized rabbits which were given 10^4 spores intravenously and were autopsied 2 days later, only one had infective endocarditis. Of 4 catheterized rabbits which were given 10^5 spores intravenously and were autopsied 2 days later, 2 had infective endocarditis. In the 3 infected rabbits the vegetations contained approximately $\log_{10}$ 2.0 CFU of aspergilli per gram.

As shown in Table I, 34 of 37 catheterized rabbits which received 10^7 spores intravenously and were autopsied 6 hours to 10 days later had infected vegetations on culture of the aortic valve. Another rabbit had a sterile culture of the vegetations which,

Fig. 1. Aortic valve vegetations (indicated by arrows) in the heart of a rabbit which had an intracardiac catheter for 5 days after infection with 10^7 spores of *A. fumigatus*. (The catheter has been removed to fully expose the vegetations.)

Table I. Aspergillus endocarditis in catheterized rabbits receiving 10^7 spores intravenously

Duration of infection (days)	*No. Infected/ total No. rabbits*	*Mean $\log_{10}$ CFU/gram of vegetation in infected rabbits*	*No. With aspergilli in 1 ml. of right atrial blood/total No. infected rabbits*
0.25	7/7	3.4	7/7*
1	3/4	3.0	2/3†
2	4/4	4.9	1/4‡
4	9/9	4.6	0/9
6, 7	10/11	4.2	0/7
9, 10	1/2	3.6	0/2
	34/37		

*Seven rabbits had 12 to 70 CFU of aspergilli per milliliter of right atrial blood.
†One rabbit had 1 CFU per milliliter and 1 rabbit 7 CFU per milliliter.
‡One rabbit had 1 CFU of aspergilli per milliliter of right atrial blood.

however, revealed hyphae on histologic sections. After 2 days of infection, the aortic vegetations were large and appeared to completely fill the aortic orifice (Fig. 1). Sections stained with periodic acid-Schiff (PAS) confirmed aspergilli in aortic valve vegetations (Fig. 2). The aspergilli appeared to be distributed in concentric layers, separated by thrombotic material. The mean $\log_{10}$ CFU of aspergilli per gram of vegetation were 3.4 at 6 hours, 3.0 at 1 day, 4.9 at 2 days, 4.6 at 4 days, and 4.2 at 1 week (Table I). One milliliter samples of right atrial blood contained 12 to 70 CFU of aspergilli per milliliter at 6 hours. Despite infective endocarditis, 1 ml. samples of right atrial blood after 24 hours were usually sterile (< 1 CFU per milliliter).

In another group of 10 rabbits with aspergillus endocarditis, 34 of 37 one milliliter samples of blood from the marginal ear vein daily from the second to the thirteenth day of

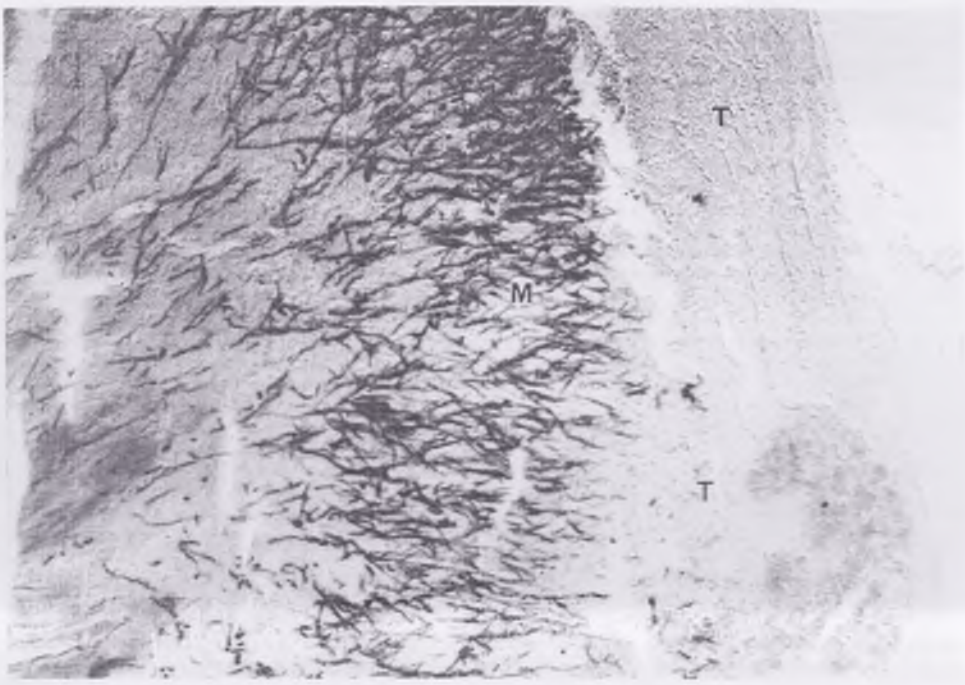

Fig. 2. Microscopic section of an aortic valve vegetation, 5 days after infection with 10^7 spores of *A. fumigatus* (PAS stain), showing a ring of mycelia (M) surrounded by thrombotic material (T).

Table II. Distribution of colony forming units (CFU) of aspergilli in cultures of aortic blood from rabbits with aspergillus endocarditis

Rabbit No.	*Total volume cultured (ml.)*	*Volume sterile (ml.)*	*CFU of aspergilli per total volume (ml.) containing aspergilli*
1	14	14	0
2	39	38	2/1
3	41	29	109/12
4	31	24	39/7
5	36	31	22/5
	161	136	172/25

infection were sterile. To determine if the sterility of blood cultures was due to a very low degree of fungemia, 5 rabbits with intracardiac catheters were killed 4 to 5 days after infection with 10^7 spores and large volumes of aortic blood (14 to 41 ml. per rabbit) were cultured in 1 ml. aliquots. As shown in Table II, in rabbit 1, all 14 one milliliter aliquots were sterile; in rabbit 2, 38 of 39 ml. were sterile; in rabbit 3, 29 of 41 ml.; in rabbit 4, 24 of 31 ml.; and in rabbit 5, 31 of 36 ml. were sterile. Approximately 85 per cent of the total volume cultured was sterile and 15 per cent, or 25 ml., contained a total of 172 CFU (2 to 35 CFU in each 1 ml. aliquot).

Of 8 rabbits with intracardiac catheters infected with 10^7 spores, all died within 7 days. Peripheral organs were examined in catheterized rabbits 4 to 5 days after infection with 10^7 spores. Grossly visible lesions were noted commonly in the kidneys and livers,

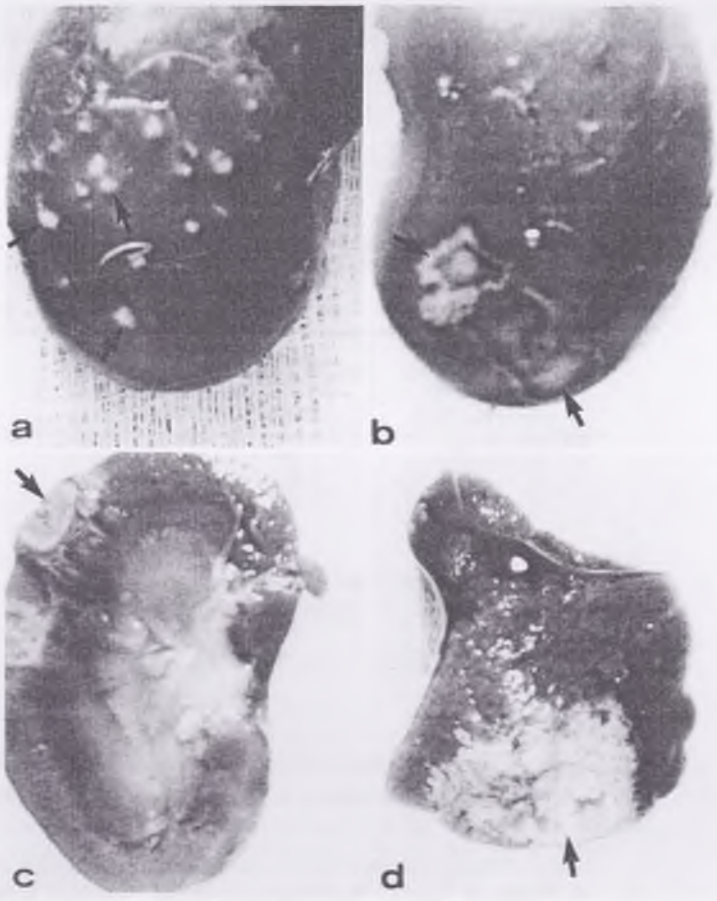

Fig. 3. Multiple infarctions (indicated by arrows) of the kidneys (A,B,C) and liver (D) of rabbits with intracardiac catheters 5 days after infection with 10^7 spores of *A. fumigatus*.

rarely in spleens, and never in the lungs or brains. The gross lesions in the liver or kidneys were usually multiple pinhead-sized abscesses or occasional infarcts (Fig. 3). There were mean $\log_{10}$ 2.5 to 3.9 CFU of aspergilli per gram of kidney, liver, spleen, brain, and lung.

Twenty-four hours after intracardiac catheterization sterile macroscopic vegetations are regularly produced. To evaluate whether the presence of these sterile vegetations alone is sufficient to reliably produce aspergillus endocarditis, catheters were removed from a group of 5 rabbits, 24 hours after intracardiac catheterization, and the rabbits were immediately injected with 10^7 aspergilli. At autopsy 4 days later, none of the 5 rabbits had macroscopic vegetations; 3 had sterile valves ($< \log_{10}$ 2.0 CFU per gram); 1 rabbit had $\log_{10}$ 2.0 CFU per gram; and another rabbit had $\log_{10}$ 4.5 CFU per gram of valve.

To evaluate whether the continuous presence of the intracardiac catheter is required for the perpetuation of aspergillus endocarditis, the intracardiac catheters were removed from a group of 5 rabbits 6 hours after infection with 10^7 spores. At autopsy 5 days later, all these rabbits had macroscopic vegetations (mean weight, 40 mg.). However, these vegetations were significantly smaller than vegetations from continuously catheterized animals after 5 days of infection (mean weight, 133 mg.), $P < 0.05$. The number of

Table III. Treatment of aspergillus endocarditis in rabbits

Intraperitoneal therapy	*No. Rabbits*	*Per cent survival 3 days*	*Vegetation mean ± S.E. Log_{10} CFU/Gm.*
None	9	78	4.87 ± 0.16
Amphotericin (1 mg./Kg./day)	20	65	3.76 ± 0.28
Flucytosine (25 mg./Kg./day)	8	88	3.47 ± 0.57
Amphotericin (1 mg./Kg./day) + flucytosine (25 mg./Kg./day)	8	88	4.16 ± 0.38
Flucytosine (50 mg./Kg./day)	15	20	2.64 ± 0.40
Amphotericin (1 mg./Kg./day) + flucytosine (50 mg./Kg./day)	14	43	2.97 ± 0.40

aspergilli per gram of vegetation was similar to the number in continuously catheterized rabbits; ie., 4 rabbits had $\log_{10}$ 4.6 to 5.3 CFU per gram of vegetation and 1 rabbit had $< \log_{10}$ 2.0 CFU per gram.

Growth of aspergilli in serum. Using an inoculum of 10^2 spores, both serum from 2 uninfected rabbits and serum from 2 infected rabbits contained 10^2 CFU of aspergilli per milliliter at 24 hours and were turbid at 48 hours.

Treatment of aspergillus endocarditis. The minimal inhibitory concentration of amphotericin B for the strain of *A.fumigatus* was 12.5 μg per milliliter and of 5 FC, 15.6 μg per milliliter, in Sabouraud's dextrose broth. When combined, concentrations of amphotericin B and 5 FC which inhibited growth were less than ½ the minimal inhibitory concentration of either agent alone. The mean serum level of 5 FC in 4 rabbits 1 hour after administration of 25 mg. per kilogram intraperitoneally was 13.4 μg per milliliter; 1 hour after 50 mg. per kilogram it was 42.6 μg per milliliter.

Results of therapy are shown in Table III. The 3-day survival was not significantly different among any of the treatment regimens ($P > 0.05$, by chi-square test) except the 3-day survival of the group treated with 5 FC, 50 mg. per kilogram per day was significantly lower when compared to the untreated group ($P < 0.05$), the amphotericin group ($P < 0.05$) and the 5 FC, 25 mg. per kilogram per day, or the amphotericin plus 5 FC, 25 mg. per kilogram per day groups ($P < 0.01$). However, the titers of vegetations were significantly lower in rabbits treated with amphotericin B, 1 mg. per kilogram per day ($P < 0.01$), 5 FC, 25 mg. per kilogram per day ($P < 0.05$), 5 FC, 50 mg. per kilogram per day ($P < 0.0l$), or amphotericin B plus 5 FC, 50 mg. per kilogram per day ($P < 0.0l$) by t-test for unpaired observations; but the vegetation titers in the untreated rabbits and in rabbits treated with amphotericin or 5 FC, 25 mg. per kilogram per day, were not significantly different from titers in rabbits treated with amphotericin plus 5 FC, 25 mg. per kilogram per day ($P > 0.05$).

Discussion

Aspergillus endocarditis only developed in rabbits if an intracardiac foreign body was present. Rabbits without intracardiac catheters fail to develop endocarditis after intravenous injection of a large inocula of spores, but did develop fatal disseminated aspergillosis. Sterile vegetations which the intracardiac catheter induced were relatively resistant to infection with aspergilli and spontaneously healed as judged by the absence of macroscopic vegetations if the catheter was removed immediately prior to infection. In contrast,

infection of sterile vegetations in the absence of the catheter has been reported to be more readily produced with *Streptococcus viridans* in this rabbit model.[5] In rabbits in which the catheter was removed 6 hours after initiation of infection with aspergilli, the vegetations tended to be smaller than vegetations in continuously catheterized rabbits but contained as many aspergilli per gram of vegetation. These findings are in accord with endocarditis due to *S. viridans*[6] and *Staphylococcus aureus*[7] reported in this same rabbit model. Therefore, the intravascular catheter apparently is required for the initiation, but not necessarily for the maintenance of aspergillus endocarditis.

Infected vegetations were large and occlusive, but contained 10^5 CFU of aspergilli per gram of vegetation, despite large numbers of mycelia in tangled masses in histologic sections. Similar histology of vegetations was observed in candida endocarditis[8]; but $> 10^5$ CFU of candida were cultured from these vegetations. This suggests that either the majority of aspergilli seen on microscopy were dead, or that, more likely, the tangled masses of mycelia seen on microscopy gave rise to fewer CFU due to clumping.

In addition, blood cultures were usually sterile in aspergillus endocarditis in rabbits as has been reported in man with aspergillus endocarditis[1, 2] and in rabbits with candida endocarditis.[8] This is unlike the regular occurrence of positive blood cultures in man and rabbits with *S. viridans*[9] and *S. aureus*[10] endocarditis (10^2 to 10^4 organisms per milliliter of blood). When large volumes of aortic blood were obtained in rabbits with aspergillus endocarditis, low numbers of aspergilli per milliliter of blood were found. This is probably a consequence of the low number of CFU of aspergilli per gram of vegetation. Apparently, the aspergilli in blood also are clumped, being distributed in a small portion of the total volume obtained for culture. Perhaps blood cultures in aspergillus endocarditis in man would be more frequently positive if larger volumes of blood were obtained.

The high mortality of aspergillus endocarditis can in part be attributed to concomitant disseminated aspergillosis which was itself rapidly fatal. However, toxin production may also explain the mortality seen in both aspergillus endocarditis and disseminated aspergillosis, since candida endocarditis has been reported to produce as much disseminated infection but nevertheless is not fatal.[8]

The 3-day survival of rabbits treated with 5 FC, 50 mg. per kilogram per day was significantly lower than 3-day survival in all other treatment groups except in those rabbits treated with amphotericin plus 5 FC, 50 mg. per kilogram per day. The decreased survival is probably the result of 5 FC toxicity at this dosage. The fact that rabbits treated with the combination of amphotericin plus 5 FC, 50 mg. per kilogram per day, were spared significantly increased mortality can probably be attributed to the beneficial effect of this combination of antifungal agents. The number of CFU of aspergilli in vegetations was significantly lowered by treatment except in the group treated with amphotericin plus 5 FC, 25 mg. per kilogram per day. This latter effect may have been due to antagonism of the combination at this dose of 5 FC. More prolonged courses of therapy are needed to evaluate efficacy of these agents fully. In addition, it is possible that more dramatic reduction in microbial counts would have been found in treated rabbits if the intracardiac catheters had been removed before therapy had been initiated, since in man usually the intracardiac prosthesis must be removed to achieve favorable response to antifungal chemotherapy.

This animal model of aspergillus endocarditis closely resembles aspergillus endocarditis in man: Intracardiac foreign bodies are required for production of endocarditis; vegetations are occlusive; infection is disseminated; and blood cultures are usually sterile.[1, 2]

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