EXPERIMENTAL CRYPTOCOCCAL-INDUCED MYOCARDITIS

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Rabbits and Wistar rats developed myocarditis after intracardiac inoculation with Cryptococcus neoformans. Myocardial lesions were observed on the 1st, 2nd, 3rd and 4th weeks after this inoculation in all animals. The cardiac lesions consisted of focal necrosis with mononuclear inflammatory cells infiltration in the myocardium. Cryptococcus neoformans itself was found by PAS stain and indirect immunofluorescence stain by the 2nd week after the inoculation. Maximal cardiac lesions were observed in the 2nd week and thereafter, the lesions showed progressive scarring. In the 9th week, there were fibrotic lesions and we were not able to demonstrate cryptococcal antigens in these lesions. Fungemia and antibody for Cryptococcus neoformans were not found over the entire period. Cryptococcal meningeal lesions were observed in all animals. In the animals given an intracardiac administration of saline, Pseudomonas aeruginosa, and Serratia marcescens, there were no myocardial lesions. The relationship of this experimental fungal myocarditis and fungal endocarditis in human is discussed.

 FUNGUS is a pathogen of increasing significance in man. Cryptococcus neoformans is the common cause of fungal infection in patients with underlying diseases. Infection by Cryptococcus neoformans is known to cause acute, subacute or chronic pulmonary, systemic or meningeal lesions1. In recent years, cryptococcal infections have been recognized as a common cause of nosocomial infections. Involvement of the heart by Cryptococcus neoformans is being recognized more often than in the past. Although cryptococcal endocarditis on prosthetic heart valves has received the most attention2,3 direct involvement of myocardium has been little discussed. Our experiments revealed, however, that the existence of cryptococcal myocarditis in human must be taken into consideration. This paper describes a method for inducing cryptococcal myocarditis and the histological features of the cardiac lesions.

MATERIALS AND METHODS

Animals

Strain 22 inbred Wistar male rats weighing 200 to 250 gm and 16 male white New Zealand rabbits weighing 2 to 2.5 kg were used.

Three groups were studied: a) group 1 (2 rabbits and 4 rats) was used as sham-inoculated control (same inoculation minus microorganism), b) group 2 (4 rabbits and 6 rats) was used as Pseudomonas aeruginosa and Serratia marcescens inoculated animals, c) group 3 (10 rabbits and 12 rats) was used as Cryptococcus neoformans inoculated animals. The method employed for establishing myocarditis depends upon inoculating with about 0.1 ml saline containing approx-

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imately $10^5$ colony forming units per ml of desired microorganisms into the myocardium and intracardiac cavity through a 26-gauge needle, percutaneously under thiopental anesthesia.

Briefly: when pulsatile movements of the needle synchronous with the cardiac cycle was felt, the saline suspension of desired organism was introduced through a 26-gauge needle attached to a 2 ml syringe.

The strain of Pseudomonas aeruginosa (11D–1042) and the strain of Serratia marcescens (11D–620) were obtained from the Institute of Medical Science, the University of Tokyo. Cryptococcus neoformans was obtained from a patient with cryptococcal meningitis in Kyoto University Hospital. Cryptococcus neoformans was identified by India ink preparation, the ability to grow at 37°C, a characteristic assimilation pattern, a pathogenicity toward experimental animals and the ability to utilize creatinine as a sole carbon source.

**Antiserum**

Antiserum was produced in rabbits by inoculating heat-killed organisms intravenously. The antigen was standardized so that 1 ml contained $10^6$ organisms. Ten consecutive daily inoculations of 3 ml each were given. The rabbits were test-bled 7 days following the last inoculation and the titration of antiserum was carried out in immunofluorescence tests on smears of the organism. If the titer of the serum was 1:160 or above, the rabbits were exsanguinated. The serum was removed from the clot, merthiolate added, and the material refrigerated until use.

**Microbiologic methods**

After the injection of each organism, the animals were kill at intervals by intravenous pentobarbitone, at a predetermined time. Examination of the heart and other organs was carried out under sterile conditions. All chambers of the heart were carefully examined for lesions and all lesions were smeared and studied by Gram stain. About one third of heart, kidney, liver, spleen, lung and brain were rapidly removed with aseptic precautions and were homogenized in trypticase soy broth. A loopful of homogenate was spread on blood agar and Sabouraud's dextrose agar, and added to thyoglycollate broth. The thyoglycollate broth was incubated at 36°C for 7 days. After which subcultures onto blood agar and Sabouraud's dextrose agar were carried out. Each organisms was identified according to

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standard procedures. All rabbits and rats were subjected to blood and urine (suprapubic aspirations) cultures at days 1, 2, 3, and 7, and then weekly after the intracardiac inoculation. Blood in the rabbits was taken from a lateral ear vein when the rabbits were alive and from cardiac puncture at the completion of an experiment. The blood in the rats was taken from the tail when the rats were alive and cardiac puncture at the completion of an experiment. The blood and urine cultures were treated likewise.

For immunofluorescence examination, part of tissue specimens was prepared and the methods of tissue fixation and fluorescent staining for immunoglobulines have been described previously. Fluorescent staining for each organism was done by an indirect immunofluorescent technique, using rabbit antiserum against each organism followed by FITC-labelled goat antirabbit IgG (Behring-Werke). By immunoelectrophoresis, this conjugate revealed a single, strong precipitation line corresponding to rabbit IgG. The molar ratio was 1.8. Nonspecific fluorescence in the section was checked in the following ways. (a) In the direct procedure, reaction of antiserum fluorescence was specially blocked by an unconjugated sample of the same antiserum. (b) In the indirect procedure (i) the sections were covered only with the conjugated goat antirabbit IgG, and (ii) the sections were first covered with normal rabbit serum for 30 min. After rinsing, the conjugated goat antirabbit IgG was used. No fluorescence was noted when methods (i) and (ii) were used. The remaining pieces of tissue were fixed in formal saline and prepared for conventional light microscopy.

RESULTS

Bacteriological and morphological studies of group 1 were carried out 15 - 30 days later and those of group 2 were carried out 15 - 60 days later. There were no spontaneous deaths. In group 1, there were no lesions, either grossly and microscopically observed in the examined organs. There were also no bacteremia and no significant bacteriuria. In group 2, the only lesion consisted of pyelonephritis in 2 of 4 rabbits and in 2 of 6 rats. The other examined organs in group 2 showed no evidence of infectious lesions. Low numbers of inoculated bacteriuria with $10^4 - 10^5$ colony-forming units per ml. were observed up to 60 days after the challenges in the animals with pyelonephritis. Thus, it was apparent that myocarditis did not
result from the intracardiac inoculation of Pseudomonas aeruginosa and Serratia marcescens in numbers sufficient to produce pyelonephritis in animals. Fungal and morphological studies of group 3 were carried out 1 week – 9 weeks later. Nine rabbits and eight rats were sacrificed on the following weeks: 1, 2, 3, 4, and 9. Histologic evidence of myocardial damage was observed on the 1st post-inoculation week. Maximal cardiac damage was observed in specimens taken in the 2nd week (Fig. 1). Thereafter, lesions showed progressive scarring. Histologic evidence of myocarditis was observed in 7 in 7 rabbits and 6 in 6 rats (100 per cent) sacrificed in the first, second, third, and fourth weeks. One of 10 rabbits and 2 of 12 rats died within 2 – 3 hours after the intracardiac inoculation. In these particular animals, there were no infectious lesions in the examined organs. Nine weeks after inoculation, 2 rabbits and 2 rats were sacrificed. In these animals, there were occasional fibrotic lesions without cell infiltration or with sparse cell infiltrations (Fig. 2). Sections of the brain revealed several foci of necrosis and meningeal lesions due to Cryptococcus neoformans 2nd week after intracardiac inoculation and persisted to the end of the experiment in all animals of group 3 (Fig. 3). Sections of the liver, kidneys, lungs and spleen revealed no histologic alterations.

**Immunofluorescence findings**

Cryptococcal antigens were demonstrated by an indirect immunofluorescent antibody method in the foci of the necrosis. Cryptococcus neoformans itself was observed the 2nd week after inoculation (fig. 4). From the 3rd to the 4th week, Cryptococcus neoformans itself was not observed, but specific green fluorescence for Cryptococcus neoformans was present in the foci of damaged area of the myocardium (Fig. 5). In the animals sacrificed in the 9th week, cryptococcal antigens were not present in the fibrotic lesions, but IgG was diffusely present in these lesions (Fig. 6).

**Microbiological findings**

No fungemia and no inoculated fungus in the urine were found throughout. Cultures of Cryptococcus neoformans in the homogenized heart were positive up to the 2nd week, and in the homogenized brain, were positive to the end of this experiment (the 9th week) in the group 3. Cultures of inoculated bacteria in the homoge-
nized kidney in the group 2 were positive up to the 1st week. The cultures of other homogenized organs were all negative in the group 2 and

3. In the control animals (the group 1), cultures of organisms in the homogenized brain, heart, lungs, liver, spleen and kidneys were all negative.
DISCUSSION

Certain strains of Coxsackie virus induce experimental myocarditis and cases of myocarditis in humans caused by virus have been described in numerous reports. To our knowledge, cryptococcal myocarditis, however, has not been documented. Development of experimental cryptococcal myocarditis in rats and rabbits in the present study suggests that Cryptococcus neoformans may also cause myocarditis in humans.

Cryptococcal infection is usually an opportunistic infection. Primary metabolic disorders such as diabetes mellitus and Cushing’s syndrome, alterations of host metabolism in the course of such diseases as chronic pulmonary and hepatic disease and renal failure, hematologic disorders including leukemia and lymphoma, and the use of steroid or broad spectrum antibacterial agents are known to predispose the patients to fungal infection. During the past three decades, the reported incidence of fungal endocarditis has increased simultaneously with the increased use of broad-spectrum antibiotics, corticosteroids, and cytotoxic and immunosuppressive drugs, prolonged intravenous therapy, development of cardiac surgery and introduction of foreign intracardiac materials, emergence of patient populations in which host defenses have been altered by immunosuppressive therapy. Endothelial damage may also result from mechanical trauma due to intracardiac catheters, pacemakers, or surgery. This type of injury, not necessarily associated with subendocardial changes, is the mechanism by which the most reliable experimental models produce nonbacterial thrombotic endocarditis. Diagnostic and therapeutic catheterization of the heart is now common, and use of such catheters may lead to nonbacterial thrombotic endocarditis. The current increase in these treatments or procedures has also contributed to the increased incidence of fungal endocarditis. Foreign intracardiac materials, such as sutures and pacemakers, have also provided a nidus for fungal endocarditis. Our experimental cryptococcal myocarditis suggest that such patients run an increased risk of developing cryptococcal myocarditis.

Cryptococcus neoformans is present in remarkable abundance in old accumulation of pigeon excretion and less frequently in other avian excreta and in soil. Susceptible persons have frequent chances of exposure to Cryptococcus neoformans and myocarditis is frequent in patients with infective endocarditis. Myocarditis was
observed at necropsy in 88 – 100 percent of 187 patients with infective endocarditis. Myocarditis was also considered to be a major cause of congestive heart failure in patients with active infective endocarditis. One of the most common causes of death is congestive heart failure in patients with active infective endocarditis. Fungal endocarditis is a disease in which the diagnosis has been difficult to confirm because of fungal growth characteristics, difficulty in blood stream isolation, and the response to treatment is poor. Blood cultures were positive in only 50 percent of proven cases of fungal endocarditis. Even after the active myocarditis subsided, there was a subsequent fibrosis and although there was no existence of cryptococcal antigens in our experimental myocarditis, the presence of cryptococcal foci was found in the brain. Such may explain why some cases of fungal infections have been reported as cures and an infection was manifested 1 – 2 years later. We did not detect Cryptococcus neoformans in blood cultures and antibody for Cryptococcus neoformans by means of immunofluorescence “sandwich” method over the entire period, despite the presence of active myocarditis and meningoencephalitis. Thus, detection of the case of cryptococcal myocarditis may be difficult.

Patients who have predisposing factors of cryptococcal infections, or who have already demonstrated the cryptococcal lesions, myocardial involvement with Cryptococcus neoformans should be considered when a patient has major intraventricular or A – V conduction defect, new QRS changes or an arrhythmia. Cryptococcal myocarditis probably occurs more often than is generally known.

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Experience with two patients previously reported.

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