The Effects of Steroids on the Virulence of *Cryptococcus neoformans*

ROBERT DUKE LONIAN, Douglass High, Oklahoma City

The administration and withdrawal of steroids is becoming more common in medical practice, often with great benefit to the patient. However, the many benefits of steroid treatment are accompanied by certain unfavorable side effects, one of the most serious being a reduction of the host's resistance. The enhancement of several bacterial and mycotic diseases, including cryptococcosis, due to prolonged steroid treatment is now recognized by many doctors.

INTRODUCTION

Cryptococcosis, also known as torulosis and European blastomycosis, is a mycosis of man whose etiologic agent is *Cryptococcus neoformans*, the most frequent cause of mycotic meningitis in man. Cryptococcosis effects people of all ages and has a world-wide distribution with the largest number of cases being reported in the United States and Australia.

Once considered a rare disease, cryptococcosis is being reported with a greater frequency—from 172 cases in 1952 to over 300 cases in 1960 in the United States (Littman & Zimmerman, 1960). This sudden increase in reported cases is believed to be the result of an increased interest in mycotic infections rather than a marked increase in the number of infections. There is good reason to believe that many cases of cryptococcosis go unrecognized and unreported because there is a great deal of discrepancy between its clinical and pathological diagnosis. Many cases occur as secondary infections and coexist with other diseases. Cryptococcosis effects all parts of the body, but cryptococcal meningitis, which is 98% fatal, is the most severe form (Bowman and Ritchey, 1954). Several treatments are used in treating cryptococcosis including X-ray therapy, surgery, and a large variety of chemicals. Amphoteracin-B seems to be one of the most effective treatments for disseminated and meningitic cryptococcosis, but it has several bad side effects which limit its usage (Rhoades and Muchmore, 1960).

C. neoformans is a spherical to oval, non-myceliated cell which varies in diameter from 2.5-8.0μ. The organism is a non-fermenter, reduces starch under suitable conditions, and is unique among pathogenic fungi in that it possesses a mucinous capsule which varies in thickness from very thin or undetectable to 7μ. Reproduction is by budding. In a culture, the cell develops a thick wall and contains few to several vacuoles. Macroscopically, *C. neoformans* forms a more or less dry cream to orange-tan, opaque, yeastlike, irregularly circular colony; however, shiny mucoid colonies often occur in cultures following passage through white Swiss mice by intracerebral injection.

C. neoformans is differentiated from other non-myceliated yeasts by its ability (1) to grow at 37°C, (2) to hydrolize urea, (3) to form wide capsules in mouse brain after intracerebral injection, and (4) by its ability to form capsules on *cryptococcus* capsule agar, and (5) by its virulence for white Swiss mice. It is differentiated from other species of Cryptococcus (1) by its ability to grow at 37°C; (2) by its ability to assimilate glucose, mannose, trehalose, xylose, galactose, maltose, and sucrose, all provided as separate carbon sources in synthetic media; (3) by its failure to assimilate lactose and potassium nitrate; and (4) by its virulence for mice (Benham, 1950-56; Emmons, 1952-60; Lonian, 1964).

Several studies involving the saprophytic occurrence of *C. neoformans* indicate that it is most prevalent in habitats containing pigeon droppings. The relationship between the pigeon and *C. neoformans,* however, is not of a pathogenic nature.

The *steroids* consist of an extensive group of colorless, and for the most part, saturated natural products possessing the tetracyclic carbon skeleton.

Except for slight variations due to the presence of nuclear substituents and the degree of saturation, the extensive types of compounds comprising this group are basically due to variations in side chains.

Prednisolone can be prepeared by microbiological dehydrogenation of cortisone and cortisol with *Corynebacterium simplex.* A 24-hour culture of the organism in a nutrient medium of 0.1% yeast extract buffered broth at pH 7 is inoculated with a solution of the substrate in methanol, and the mixture is shaken at 28°C for 3-34 hours. Extraction of the broth with chloroform and crystallization of the extracted material from acetone gives an excellent yield of prednisolone.

Steroids are now being used to treat a large variety of common diseases, including cancer and arthritis. In the treatment of cancer, approximately 80% of those treated experienced relief of pain with objective improvement in 15-30% of the cases. The mortality rate of 109 severe cases of ulcerative colitis (as compared with 205 cases treated during the procorticosteroid era) dropped from 38% to 10%, and approximately 75% of the patients gained significant or lasting benefits from steroids (Lischutz, 1950).

The adverse effects of steroid treatment also have attracted the attention of several doctors and researchers. The depression of antibody by cortisone during immunization in rabbits suggested that adrenal cortical hormone acts by either inhibiting the synthesis of antibody or accelerating its breakdown (Kass *et. al.,* 1955). Several other experimenters have reported a reduction in the number of circulating antibodies due to steroid treatment. Bjørneboe and Stoerk (1951) showed that antibody is not catabolized more rapidly in cortisone-treated animals and suggested that the resulting low antibody levels were due to an inhibition of synthesis. The effect of this reduction of resistance on the enhancement of human cryptococcosis is suggested by several, including Goldstein and Rambo (1962), who reported eight cases of cryptococcosis whose onset occurred during or immediately following steroid treatment. Additional data indicating that steroids encourage the enhancement of cryptococcosis has been collected from experiments involving animals.

It is the purpose of this report to (1) obtain additional information, using prednisolone, indicating the enhancement of cryptococcosis by steroids; (2) study the effects of steroids on the dissemination rate of cryptococcosis, from the central nervous system to the intraperitoneal cavity and visa versa; and (3) to assess the effect of steroid withdrawal, before and after involvement of the central nervous system on the prognosis of cryptococcal infection.

PROCEDURE

A group of 56 white Swiss mice was divided into equal groups, each of which was placed in one of seven metal cages labeled I-VII.

On January 22, 1965, water bottles containing a solution of 10mg of prednisolone per 50cc of distilled water were placed in cages VI and VII. This was continued for three weeks.

On February 12, 1965, a dense suspension of organisms from a five-day old culture of OKC4C (Lonian, 1964) was made in sterile physiological saline. The concentration of the organisms in this suspension was found using a Petroff-Hauser bacteria counter and additional saline was added to get a concentration of 1 X 10^6 cells per cc of saline.

A sterile 1cc graduated tuberculine syringe and a sterile 25 gauge, ¼" long hypodermic needle were used to make an intracerebral injection consisting of 0.05cc of the 1 X 10^6 cells per cc suspension into each mouse in cages II, IV, and VI. A 22 gauge, 1½" hypodermic needle was used with a 1cc syringe to give each mouse in cages III, V, and VII an intraperitoneal injection consisting of 0.15cc of the 1 X 10^6 cells per cc suspension.

The administration of prednisolone in cages II and III was discontinued at this point. Those mice in cage I were not injected but continued to get prednisolone. Daily checks of the cages were made to remove dead mice and to check the amount of water and food.

Beginning on February 19, 1965, two mice from each cage in which two mice had not died during the previous week were sacrified by ether inhalation. If one mouse had died during the week, only one mouse was sacrificed.

Autopsies were performed on those mice which died or were sacrificed. Brain cultures were made on agar plates containing Sabouraud's dextrose agar using portions of the brain and spinal fluid as an inoculant. The vicera (lungs, liver, and spleen) were aseptically ground in sterile tissue grinders and suspended in 1.5-2cc of sterile physiological saline. This suspension was used to inoculate agar plates containing Sabouraud's dextrose agar. Both brain and visceral cultures were incubated at 21°C.

Organisms from colonies which resembled those of *C. neoformans* were examined under oil immersion. Those inoculants which grew *C. neoformans* were presumed to be from mice killed by that organism.

RESULTS

An average of 7cc of water per day was consumed by each mouse. This amounted to approximately 10mg of prednisolone per week. A total of 48 mice were injected with *C. neoformans*, 24 intracerebrally (IC) and 24 intraperitoneally (IP). Out of these 48 mice, 43 had brain and visceral inoculants which grew *C. neoformans*, none had positive brain cultures and negative visceral cultures, and 43 had negative brain cultures and positive visceral cultures. The mice in cage IV had the shortest survival time. There was, however, little difference between the survival time of the mice in cages II and IV and in cages III and VII.

The mice in cages II and IV possessed bulging craniums at the time of autopsy, and the brains lacked in a firmness exhibited by the brains of those mice without bulging craniums. Except for VII-7-26, the mice in cages III, VI, and VII which died after March 5, 1965, also showed this effect. In several instances the lungs and liver were noted to be spotted, and in some of these cases the liver and lungs showed an uncommon deli-

cacy during autopsy. In all of these cases *C. neoformans* was grown from both the brain and visceral cultures.

The mice in cage I were infected with a gram negative bacterium which was isolated from the lungs on Sabouraud's dextrose agar plates incubated at 21°C. This infection was localized to cage I, and there was no evidence of infection in any other cage. All of the mice in cage I died.

DISCUSSION

The data obtained in this experiment indicates that prednisolone reduces the host resistance to cryptococcosis. Previous work done with steroids suggest that a more pronounced effect might have been noticed had the prednisolone been injected rather than administered orally (Thomas, 1952).

Consideration of the data from cages II, IV, and VI suggests that if the central nervous system is infected by *C. neoformans* during steroid treatment, little is accomplished in the way of prolonging the life of the host by withdrawing steroid treatment. Data from cages III, V, and VII, however, suggest that the withdrawal of steroid treatment might be of definite value to the host if cryptococcal infection is detected before the brain and/or its meninges are involved. This could be of special value in situations in which cryptococcosis is detected during steroid treatment for another disease. This is by no means meant to imply that steroid withdrawal should not be one of the first steps taken in such cases. It is intended to only further emphasize the importance of early diagnosis in such cases.

The isolation of *C. neoformans* from both the brain and viscera of mice IV-1-1, II-1-3, and II-2-3 demonstrates an unusually rapid rate of dissemination of the cryptococcal infection from the central nervous system to the intraperitoneal cavity. The fact that all of these mice received prednisolone suggest that the steroid treatment played a definite part in causing this rapid dissemination.

The *ante mortem* observation of a significant number of cranial bulges in those mice which received prednisolone and which died comparatively early strongly suggested a tremendously increased rate of reproduction of the cryptococci which were present in the central nervous system. Similar bulges, which appeared to be the result of an excessive number of *C. neoformans* organisms in the brain and/or its meninges, have been observed in experiments involving *C. neoformans* and white Swiss mice but which did not involve steroids (Lonian, 1964). The bulges observed in these previous experiments, however, were seen in mice who had been infected with *C. neoformans* for a significantly longer period of time.

The infection involving cage I is, from all indication, the result of some foreign pathogen which infected one or two of the mice then spread to the other inhabitants of the cage. From all indication, the organism infected only those mice in cage I. Since this cage was to be used as a control, the infection did not effect the information gained in this experiment in any adverse manner. It did however, suggest that the resistance of the mice was significantly decreased by the prednisolone, allowing infection to occur.

CONCLUSION

The data collected in this and similar experiments strongly indicates that prolonged steroid treatment definitely lowers the host resistance to cryptococcosis. This experiment also suggests that little is gained in prolonging the life of the host by discontinuing steroid treatment after *C. neoformans* has invaded the already highly susceptible central nervous system.

This, along with the suggested increase in the rate of dissemination due to steroid treatment, suggests that those who administer steroids should be more aware of the infections whose enhancement is more likely because of previous steroid treatments. If these administrators make their patients aware of the hazards caused by prolonged steroid therapy, it seems reasonable that the number of infections might be reduced since these informed patients could then attempt to avoid those places in their environment which have been shown to be likely to contain a large number of known pathogens. In cases in which infection occurred, an awareness of these diseases might result in an earlier diagnosis which, if made soon enough, might prevent the death of the patient.

BIBLIOGRAPHY

Benham, Rhoda W. 1950. Cryptococcosis and blastomycosis. Anals. of N. Y. Acad. Sci., 50.

Benham, Rhoda W. 1955. The genus *Cryptococcus:* The present status and criteria for the identification of species. Trans., N. Y. Acad. Sci. (series 2), 17.

Benham, Rhoda W. 1956. The genus *Cryptococcus*. Bacteriol. Rev., 20.

Bergman, R. 1961. Reproduction and pathogenicity of *Cryptococcus neoformans*. Sabouraudia, Malme, Sweden, 1.

Bjørneboe, M., E. E. Fischel, and H. C. Stoerck. 1951. The effect of cortisone and adrenocorticotropic hormone on the concentration of circulating antibody. J. Exper. Med., 93.

Bowman, H. E., and J. O. Ritchey. 1954. Cryptococcosis (torulosis) involving the brain, adrenal and prostate. J. Urol., 71.

Emmons, Chester W. 1952. Isolation of *Cryptococcus neoformans* from soil. J. of Bacteriol., 62.

Emmons, Chester W. 1955. Saprophytic sources of *Cryptococcus neoformans* associated with the pigeon. Amer. J. of Hyg., 62(3).

Emmons, Chester W. 1955. Saprophytic sources of *Cryptococcus neoformans* associated with the pigeon *(Columbalivia)*. Amer. J. Hyg., 62(3).

Emmons, Chester W. 1960. Prevalence of *Cryptococcus neoformans* in pigeon habitats. *Public Health Rep.*, Public Health Serv., U. S. Dept. of Health, Educ., and Welfare, 75.

Goldstein, E., and O. N. Rambo. 1962. Cryptococcal infection following steroid therapy. Ann. Int. Med., 56(1).

Kass, E. H., M. L. Kendrick, and M. Finland. 1955. Effects of cortisone, hydrocortisone, and corticotropin on production of antibodies in rabbits. J. Exper. Med., 102.

Lischutz, Alexander. 1950. *Steroid Hormones and Tumors*, Williams and Wilkins, Baltimore.

Lonian, Robert D. 1964. The isolation of *Cryptococcus neoformans* in Oklahoma City. Trans. Okla. J. Acad. Sci., 7.

Rhoades, Everett R., and Harold B. Muchmore. 1960. The effect of amphotericin on the respiration of *Cryptococcus*. Antibio. Annual, Antibiotica, Inc., New York.

(Editor's comment: Unfortunately, publication limitations made necessary the elimination of many citations in this bibliography. Only those cited in the paper were included).