Pathogenesis and Immunological Aspects of Experimental Histoplasmosis in Cynomolgus Monkeys (Macaca fascicularis)

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Studies were carried out to obtain basic information on the pathogenesis of experimental histoplasmosis in Cynomolgus monkeys (Macaca fascicularis) and to determine whether such infected primates can be used as a source of positive reference sera in serological tests for histoplasmosis. Ten monkeys were inoculated intra-peritoneally with approximately 9.1 × 10^9 viable Histoplasma capsulatum yeast-form cells or cell aggregates. At periodical intervals, their sera were tested for antibodies to H. capsulatum by the complement fixation (CF), immunodiffusion, and latex agglutination tests. Selected monkeys were also sacrificed at periodical intervals for cultural and pathological evaluation of their tissues. Infection with H. capsulatum elicited high-antibody responses, and the fungus disseminated to many organs. Initially, the infected monkeys developed CF titers as high as 1:256 to both histoplasmin and H. capsulatum yeast cell antigens. Subsequent challenges boosted the CF antibody titers to levels as high as 1:1,024. All of the monkeys developed M precipitins, and some also produced H precipitins. Latex agglutination titers as high as 1:1,024 were also demonstrated. Our findings show that Cynomolgus monkeys experimentally infected with H. capsulatum by the intraperitoneal route develop a mild form of histoplasmosis and that these animals can be used as a source of reference sera in serological tests for histoplasmosis.

Positive control sera are required for the proper performance of serological tests for histoplasmosis. Such sera now are obtained mainly from humans infected with histoplasmosis. Unfortunately, obtaining adequate amounts of this material is often difficult, and, as serological tests for this disease become more widely used, such difficulties will undoubtedly be compounded. These procurement problems emphasize the need to explore the possibility of using lower-animal sera as positive controls in serological tests for histoplasmosis.

The present investigation was carried out to study the immunological responses of Cynomolgus monkeys (Macaca fascicularis) to experimental infection with Histoplasma capsulatum and to determine whether sera from such experimentally infected monkeys can be used as positive controls in sero-diagnostic tests for histoplasmosis. We chose Cynomolgus monkeys because they are readily available, relatively inexpensive, and large enough to permit collection of adequate amounts of blood. Another purpose of this investigation was to study the pathogenesis of experimental histoplasmosis in this species of monkey. We felt that the basic information obtained from the pathogenesis study would add to our overall knowledge of this disease and would enable us to correlate serological responses with the stage of the disease process. It should be emphasized that the investigation was not carried out to develop an animal model for studying the disease as it occurs in man or in lower animals.

MATERIALS AND METHODS

Twelve adult Cynomolgus monkeys, five females and seven males, were used. They were kept in individual cages in one room and were fed laboratory chow supplemented with fresh fruit. At the outset of the study, the animals were examined and found to be in good physical condition. They were skin tested with tuberculin and with histoplasmin [Center for Disease Control (CDC) antigen lot H-42] and found to be negative to both antigens. In addition, the following data were obtained on the monkeys: body weights, complete blood counts and other hematological values including erythrocyte sedimentation rates, and also various blood chemistry values including results of several liver function tests. The animals also

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were bled and their sera were tested for antibodies to histoplasmin, *H. capsulatum* yeast cells, Blastomyces dermatitidis yeast cells, and coccidioidin by the CDC diagnostic complement fixation (CF) procedure (8). In addition, their sera were tested by the immunodiffusion (ID) technique for H and M precipitins (2) and by the latex agglutination (LA) procedure (3). Commercially prepared histoplasmin-sensitized latex particles (Colab Laboratories, Inc.) were used in the latter tests.

The yeast form of *H. capsulatum* (culture B923) was used to infect the monkeys. This strain was selected because it represents the most complete serotype of *H. capsulatum*. It contains factors 1, 2, 3, and 4 in its antigenic makeup (5). Monkeys were inoculated intraperitoneally with 0.5 ml of packed cells (packed by centrifugation for 15 min at 980 × g) from 5-day-old Brain Heart Infusion agar cultures grown at 37 C. The 0.5 ml of packed cells was suspended in a total volume of 3 ml of sterile saline solution. Plate counts showed that this inoculum contained approximately 9.1 × 10⁹ viable cells or cell aggregates. The intraperitoneal route was used because of ease of administration.

Initially, 10 of the 12 monkeys were inoculated. The two uninoculated monkeys served as controls. During the course of this study selected animals received two additional challenges with the standardized inoculum. These booster inoculations were given 6 and 9 months after the initial injection.

After inoculation the animals were observed daily for signs of clinical illness, and their body weights were recorded at weekly intervals. In addition, hematologic and blood chemistry determinations were made on all animals 1 week after inoculation and every 2 weeks thereafter.

All animals were skin tested with histoplasmin once a week for the first 3 weeks after the initial inoculation and then once more 2 months later. The noninfected control monkeys were also skin tested at the same time as the infected animals. Two dilutions of antigen were used, 1:10 and 1:100. Antigen (0.1 ml) was injected intradermally on the abdomen, and reactions were read after 24 to 48 hr. An area of induration of 5 mm or greater was considered a positive reaction.

All monkeys were bled every 2 weeks and their sera were tested for complement-fixing antibodies against histoplasmin, *H. capsulatum* yeast-form cells, *B. dermatitidis* yeast-form cells, and coccidioidin. In addition, their sera were examined for H and M precipitins by the ID technique and for agglutinins by the LA procedure. After these tests were performed, the sera were stored at −20 C and subsequently were retested to determine the effect of storage on the antibody stability of the infected monkey sera.

Once a week for the first 2 months after challenge, bone marrow and blood samples were collected from all animals and cultured for *H. capsulatum*. Bone marrow and blood samples were also collected at the same time from the uninoculated control monkeys and cultured for this fungus. The bone marrow specimens were obtained from the crest of the ilium. The medium used for isolation of *H. capsulatum* was yeast extract agar (7). The inoculated media were incubated at 25 C for 1 month before they were considered negative.

At periodical intervals selected monkeys were sacrificed and necropsied. One monkey was sacrificed at 10 days after the first inoculation, one at 21 days, and three at 3 months. In addition, two animals were sacrificed 10 days after the second inoculation. At necropsy, the animals were examined for the presence of gross lesions, and portions of their lungs, liver, spleen, kidneys, brain, thoracic and abdominal lymph nodes, and selected peripheral lymph nodes were cultured for *H. capsulatum*. The tissue specimens were inoculated on yeast extract agar and incubated at 25 C. Portions of all major organs were fixed in Formalin for histological examination.

**RESULTS**

With the exception of transient anorexia and inactivity during the first week after the initial inoculation, none of the animals showed any clinical signs of illness. Those receiving the second and third inoculations showed only transient anorexia during the first week after challenge. A large firm mass, apparently representing the tissue reaction to the inoculum, was palpable in the abdomen of all inoculated monkeys. These masses gradually decreased in size and could not be palpated after a period of approximately 1 month. Maintenance of body weight by all inoculated animals was comparable to that of the uninoculated controls.

With the exception of a marked increase in erythrocyte sedimentation rates (from an average of 1.2 to 29.2 mm/hr) during the first week after the initial inoculation, none of the infected monkeys showed any significant changes in hematological or blood chemistry values. The erythrocyte sedimentation rates declined rapidly after the first week, and by the end of 3 weeks most of the inoculated monkeys again had normal rates. After receipt of the second and third inoculation, the monkeys did not show increased erythrocyte sedimentation rates.

**Skin test results.** At the outset of the study, all monkeys were histoplasmin skin test negative. One week after administration of the *H. capsulatum* inoculum, 1 of the 10 monkeys converted from negative to positive. Two weeks after inoculation, all but one of the monkeys were skin test positive. The negative monkey had an M precipitin, CF antibodies to histoplasmin, and the yeast-form antigens of *H. capsulatum* and *B. dermatitidis* in its serum. In addition, *H. capsulatum* was recovered from the residual lesion in its abdominal cavity. Qualitatively similar skin test
results were obtained with the 1:10 and 1:100 dilutions of histoplasmin. However, we felt that the 1:10 dilution was preferable to the 1:100 dilution because, with positive reactions, areas of induration were more prominent and more easily read. None of the uninfected controls demonstrated a positive histoplasmin skin test.

**Culture results of bone marrow and blood.** One week after the initial inoculation, *H. capsulatum* was recovered from the bone marrow of 4 of the 10 monkeys. Two weeks after inoculation, the fungus was recovered from the bone marrow of three of the animals. Only one monkey was culturally positive during the third and fourth weeks. All bone marrow samples collected 1 month after challenge were negative for *H. capsulatum*. *H. capsulatum* was recovered from only one blood sample. This specimen was collected 2 weeks after the animal had been inoculated. The bone marrow culture from this monkey was also positive at that time.

*H. capsulatum* was not isolated from the bone marrow or blood from any of the monkeys after the second and third inoculations. Neither was the fungus recovered from the blood or bone marrow samples of any of the un inoculated control animals.

**Necropsy results.** In all instances, gross lesions were confined to the site of inoculation in the abdominal cavity. Occasional whitish foci were present in the liver. The two monkeys that were sacrificed at 10 and 21 days after inoculation had a localized peritonitis involving the serosa of the intestines, urinary bladder, mesentery, and omentum. In the females, the serosa of the uterus was often involved. This inflammatory process accounted for the firm abdominal mass previously detected by palpation. The three monkeys that were sacrificed 3 months after the initial inoculation showed localized fibrous adhesions involving the abdominal visera. In addition, one of these animals had a 2- by 3-cm mass in the mesentery which was closely adhered to the colon. This mass was well encapsulated and exuded a yellowish-white purulent material when sectioned. Occasional yellowish-white foci were noted in the liver of two of the three animals. Gross lesions were not observed in any other organs. The two monkeys that were sacrificed 10 days after receiving the second inoculation showed lesions in the abdominal cavity similar to those noted in the animals that were sacrificed 10 and 21 days after the first injection.

Histological examination of grossly involved tissues revealed a similar pattern in all animals, with differences limited primarily to degree of cellular reaction. Tissues at the site of inoculation showed an extensive cellular infiltrate which consisted primarily of lymphocytes, plasma cells, and large mononuclear histiocytic-type cells. Multinucleated giant cells were numerous in all sections. Areas of intense polymorphonuclear cell infiltration with varying degrees of necrosis were noted in tissue sections from all animals, except two of the three that were sacrificed 3 months after the initial challenge. Definite *H. capsulatum* cells were demonstrable by conventional staining procedures in tissue sections of all animals except these latter two, which showed occasional questionable organisms.

Other histological observations included foci of mononuclear cells in the liver, kidneys, and lungs. Lymphoid tissues often appeared hypercellular, with an increased population of plasma cells.

**Results of culture of tissue.** Cultural studies of the various organs of the two monkeys that were sacrificed 10 and 21 days after the first inoculation showed that *H. capsulatum* was widely disseminated throughout the reticulo-endothelial system. The fungus was recovered from such diverse sites as the liver, spleen, lymph nodes in the abdominal and thoracic cavities, and an axillary lymph node. In addition, fungus was recovered from the localized lesion in the abdominal cavity. In contrast, *H. capsulatum* was isolated only from the residual lesion in the abdominal cavity of one of the three animals that were sacrificed 3 months after the first inoculation. This culture-positive monkey was skin test negative. The other organs cultured from these three animals were negative.

Cultural studies of the two animals that were sacrificed 10 days after receipt of the second inoculation indicated that the infection apparently had been contained at the site of the injection. *H. capsulatum* was recovered only from the localized lesions in the abdominal cavities.

**Serological results: CF tests.** Two weeks after receipt of the first inoculation the monkeys developed relatively high levels of complement-fixing antibodies to histoplasmin and to the yeast-form antigens of *H. capsulatum* and *B. dermatitidis*. The titers ranged from 1:32 to 1:256 with the respective antigens, depending upon the individual monkey. The serum from one of the monkeys at this time also had a CF titer of 1:16 with coccidioidin. The CF titers with histoplasmin and with the *H. capsulatum* and *B. dermatitidis* yeast cell antigens fluctuated to some extent, but remained essentially at these levels for approximately 3 months. Then, they gradually declined, and by the end of 6 months the titers ranged from 1:8 to 1:16 with these antigens. At various times during the first 3 months after the initial inoculation, sera from all of the surviving monkeys also showed CF titers with coccidioidin.
These titers varied from 1:8 to 1:32. Sera collected during the third to the sixth month after inoculation were negative with coccidioidin.

After the second injection of *H. capsulatum*, the CF titers with the antigens histoplasmin, *H. capsulatum* yeast cells, and *B. dermatitidis* yeast cells rose rapidly, reaching their peak at the second week after receipt of the inoculum. In most cases, the titers with these three antigens exceeded those noted earlier. In some cases these titers rose as high as 1:1,024 and 1:2,048. The titers with coccidioidin also were elevated and ranged from 1:8 to 1:128. The CF titers declined after the second week, and by the end of 4 months ranged from 1:8 to 1:32 with the three antigens, depending upon the individual monkey. A similar pattern of CF titer change was noted in the sera collected from monkeys that received the third inoculation. It is noteworthy that in most cases the height of the CF titers obtained after the third inoculation exceeded the maximum titers noted after the second injection. The titers gradually declined, and 6 months after the third injection they ranged from 1:8 to 1:32, depending upon the individual monkey. Figure 1 graphically illustrates the heights of the CF titers and their movement in one of the infected monkeys that had received all three inoculations. This animal was selected because its CF responses were typical of the group.

Fig. 1. Effect of histoplasmin skin tests and repeated challenge with viable cells of Histoplasma capsulatum on complement fixation titers to fungal antigens in a Cynomolgus monkey infected with *H. capsulatum*.

That the intradermal injection of histoplasmin in skin test-positive people may stimulate CF antibodies to *H. capsulatum* antigens (1, 6) has been established. Therefore, it is of interest to note the findings of the present study in this regard. As previously pointed out, 3 weeks after the initial inoculation with *H. capsulatum*, four of the monkeys had converted from negative to positive by the histoplasmin skin test. Serum samples collected from these positive animals 1 week after skin testing showed a twofold rise in the histoplasmin CF titer in three cases and a fourfold rise in one case. In contrast, two of the sera showed no change in CF titer to *H. capsulatum* yeast-form antigen; one showed a twofold rise and one a twofold decline. The skin test-negative monkeys did not show any apparent stimulation in their CF titers to histoplasmin or to the *H. capsulatum* yeast-form antigen after the intradermal injection of histoplasmin. In three of these skin test-negative monkeys the histoplasmin CF titers remained the same, and, in the other three, titers showed a twofold decline. Similarly, in four of these skin test-negative monkeys the CF titers to *H. capsulatum* yeast-form antigen remained the same, and in two of them these titers showed a twofold decline. Two months after the fungus was administered, all but one monkey was skin test positive to histoplasmin. In nearly all cases, sera collected 2 weeks after this skin test showed a twofold or a fourfold rise in histoplasmin CF titers. At this time, the sera from four of these skin test-positive monkeys showed a twofold rise in CF titer to *H. capsulatum* yeast-form antigen, one showed a fourfold rise, and four showed no change. These findings suggest that the intradermal injections of histoplasmin may have, in part, stimulated increases in histoplasmin CF titers in the skin test-positive monkeys. In only a few cases, however, was such an apparent stimulation noted in CF titers to *H. capsulatum* yeast-form antigen.

In this study, sera from two of the monkeys...
were frequently anti-complementary (AC). Consequently, these animals would have been of limited value as sources of positive control sera for CF tests for histoplasmosis. However, sera from four of the monkeys were never AC, and only occasional samples from the remainder were AC.

**Immunodiffusion tests.** None of the monkeys had M or H precipitins in their sera prior to challenge. Two weeks after the initial inoculation, one of the monkeys had M precipitins in its serum. One month after inoculation six of the monkeys had these antibodies. After 2.5 months sera from all of the inoculated animals had M precipitins. Once they appeared, these precipitins persisted until the study was terminated.

In contrast, only two monkeys developed H precipitins. Furthermore, the H precipitins appeared later than the M precipitins, and they persisted for relatively brief periods of time. These H precipitins were first detectable 2.5 months after infection and in each case persisted for less than 1 month.

None of the uninoculated control monkeys developed either M or H precipitins.

**LA tests.** Two weeks after the initial inoculation, sera from five of the monkeys reacted in the LA test, with titers ranging from 1:16 to 1:64. After 1 month, the sera from all inoculated monkeys reacted in the LA test. At this time the titers ranged from 1:16 to 1:256. As time passed, LA titers increased, and at the end of 2 months they ranged from 1:64 to 1:1,024. The titers remained at a high level for 1 or 2 additional months and then gradually declined. By the end of 6 months, sera from nearly all surviving inoculated monkeys were negative. Two weeks after the second inoculation, titers rose to levels of 1:64 to 1:512. Titers of sera from all but one monkey remained at high levels (1:32 to 1:256) for 4 months, at which time the monkeys received the third inoculation. The LA titer of the serum from one monkey declined rapidly and was negative 4 months after the second inoculation. After the third inoculation, the LA titer of this particular animal rose rapidly to 1:64 but after one month it was negative. The LA titers of the other monkeys did not rise after the administration of the third inoculation. They gradually declined to a level of 1:32 and remained at this level until the study was terminated.

**Effect of storage on stability of sera.** After samples were collected and tested, surplus sera were stored at -20 C. After storage for 1 year, five sera that did not originally show AC activity were removed from the freezer and allowed to thaw. These sera were tested by the CF procedures for antibodies to histoplasmin, *H. capsulatum* yeast cells, *B. dermatitidis* yeast cells, and coccidioidin, and by the ID procedure for H and M precipitins. Then they were stored at 4 C. These samples were subsequently retested by the CF and ID procedures nine different times over a 5-month period. The samples tested after removal from the freezer and also 2 weeks later had approximately the same CF titers they had at the time of collection. With continued storage at 4 C, all but one of the samples showed AC activity. Precipitin activity was not affected by these conditions of storage.

The effect of alternate freezing and thawing on the stability of the sera was also investigated. The aforementioned five sera were also used in this study. After storage for 1 year at -20 C, the sera were tested by the CF and ID procedures. The sera were then stored at -20 C for 1 week, at which time they were removed from the freezer, tested, and then refrozen. This sequence of alternate thawing, testing, and refreezing was carried out on a weekly basis for 9 weeks. During the first month, the CF antibody levels were not affected by the alternate freezing and thawing. Two of five sera showed AC activity after 1 month, whereas two showed a two- to fourfold decline in CF titers. Precipitin activity was unaffected by these conditions of storage.

Some of the infected monkey sera have been used in parallel with human histoplasmosis case sera as positive controls in routine diagnostic CF tests. Comparable results were obtained with the two types of sera. The findings of these preliminary studies suggest that the infected monkey sera may be used as effective control specimens.

**DISCUSSION**

In an earlier study Hill and Marcus (4) found that Cynomolgus monkeys were highly resistant to challenge with the yeast form of *H. capsulatum* by either the intratracheal or intracardial route. They inoculated six monkeys intratracheally with a suspension of the fungus in gastric mucin. The animals showed no clinical signs of disease during a 6-month period after challenge and did not develop the delayed type of cutaneous hypersensitivity to histoplasmin. Furthermore, demonstrable antibodies were not produced, and *H. capsulatum* was not recovered from spleen, liver, or lung tissue at necropsy. Intracardial inoculation of the fungus in five monkeys also failed to cause overt symptoms. However, all of the animals developed demonstrable CF antibodies in their sera, and three became histoplasmin skin test positive. *H. capsulatum* could not be recovered from various organs at autopsy. The results of the present study agree with those of Hill and Marcus that Cynomolgus monkeys have a high degree of innate resistance to challenge with the yeast form.
of *H. capsulatum*. Our findings, however, indicate that this species of monkey can be infected by the intraperitoneal injection of the yeast form of *H. capsulatum*. The recoveries of *H. capsulatum* from bone marrow, major organs, and lymph nodes as well as from local lesions at the site of inoculation, the marked increase in erythrocyte sedimentation rate after inoculation of the fungus, the serological responses, the development of skin sensitivity to histoplasmin, and the pathological findings clearly indicate that infection indeed was established. However, the monkeys were able to withstand repeated challenges with large numbers of *H. capsulatum* yeast cells. They developed only a transient, mild form of the disease. This innate resistance to challenge along with their serological responses to infection make Cynomolgus monkeys suitable animals for the production of reference serum.

The sequence in the appearance of the various antibodies is noteworthy. Two weeks after the initial inoculation, the sera from all of the infected monkeys had relatively high CF titers to histoplasmin and also to the yeast-form *H. capsulatum* and *B. dermatitidis* antigens. At this time the titers ranged from 1:32 to 1:256 with the different antigens, depending upon the individual monkey, but the sera from only five monkeys were positive by the LA test, and only one had demonstrable M precipitins. At the end of 1 month, the sera from all inoculated monkeys were positive by the LA test, but only six had demonstrable M precipitins. By the 10th week after inoculation, all sera had demonstrable M precipitins. Both CF antibodies and latex agglutinins remained at high levels for 3 to 4 months and then gradually declined. By the end of 6 months, most of the sera from the infected animals were negative by these two tests. Although M precipitins appeared later than the CF antibodies and latex agglutinins, once they were demonstrable, they persisted throughout the study. In contrast, only two monkeys developed H precipitins, and these antibodies were first detected 2.5 months after inoculation. In addition to their relatively late appearance, they persisted for less than 1 month. As was the case with the latex agglutinins, by the second week after the initial inoculation, approximately half of the monkeys had become histoplasmin skin test positive. At the end of 2 months, all but one of the inoculated monkeys were skin test positive.

The results of this study indicate that Cynomolgus monkeys infected with the yeast form of *H. capsulatum* by the intraperitoneal route may be useful as a source of positive control sera for the serodiagnosis of histoplasmosis. Such monkeys develop relatively high CF titers to histoplasmin and to yeast-form *H. capsulatum* and *B. dermatitidis* antigens and also develop high LA titers. These titers persist at high levels for several months, during which time the animals can be repeatedly bled for reference sera. After these titers have declined to low levels, the monkeys can be given a booster inoculation which induces rapid rise in antibody levels, often exceeding those noted after the initial infection. Because all of the infected monkeys develop M precipitins that persist for long periods of time, they can be repeatedly bled for sera that contain this antibody. In this study only two monkeys developed demonstrable H precipitins in their sera, and these antibodies persisted for less than 1 month. Therefore, Cynomolgus monkeys infected by the intraperitoneal route might not be the reliable sources of reference sera with H precipitins.

Our preliminary study indicated that monkey sera could be stored for at least 1 year at −20 °C without any deleterious effect on CF antibody levels or on precipitin activity. Whether the sera can be stored for considerably longer periods of time at this temperature or whether other conditions of storage such as lyophilization might be preferable remains to be determined. It is important that, although the infected monkey sera withstood storage at −20 °C for a relatively long period of time, alternate thawing and refreezing resulted in antibody loss and in AC activity. Therefore, we recommend that such sera be stored in small quantities so that they can be used shortly after thawing. However, sera that must be refrozen should be retested for antibody potency and AC activity prior to use.

Although our findings indicate that Cynomolgus monkeys can serve as a source of reference sera, we recognize that other animals, particularly large domestic animals, may be better for this purpose. This possibility remains to be investigated.

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LITERATURE CITED