Serological Response of Rhesus Monkeys to *Histoplasma*, *Blastomyces*, and *Coccidioides* Antigens

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Fifteen adult rhesus monkeys were inoculated with nonviable *Histoplasma capsulatum*, *Blastomyces dermatitidis*, or *Coccidioides immitis*. Antibody assays were made periodically during a 2-year period by use of a complement-fixation (CF) test employing four antigens and a latex-agglutination test. Selected sera were also studied in an immunodiffusion test and a coccidioidin-precipitin test. The serological patterns obtained with the anti-*Histoplasma*, anti-*Blastomyces*, and anti-*Coccidioides* monkey sera were comparable to those of sera from patients with diseases caused by the respective organisms. Rhesus monkeys should provide a good laboratory model for additional studies, including the influence of multiple antigenic stimuli on serologic response and patterns. Monkeys could also be used for the production of antisera required for studies to improve the specificity of the currently available CF antigens.

In the past 15 years, the early recognition and diagnosis of histoplasmosis, coccidioidomycosis, and blastomycosis has been facilitated by the increased use of serological tests. Frequently, these tests provide the initial clue, or serve to substantiate a clinical impression, leading to the subsequent confirmation of the disease. The fluctuation of titers may also provide valuable prognostic information.

Unfortunately, the antigens currently available for use in the most commonly employed serological tests, the complement-fixation (CF) and latex-agglutination (LA) tests, are not specific and therefore require careful interpretation to be of maximal value. The multiplicity of cross-reactions and problems of interpretation clearly indicate a need for further attempts to improve the specificity of antigens currently available. However, as a prerequisite to such a study, the investigator must have access to large quantities and numbers of human sera with a reliable history of past and present infections. As an alternative, antisera can be produced in laboratory animals. For these reasons, the rhesus monkey was investigated as a potential laboratory model for the production of antisera comparable to that produced during the course of human disease.

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MATERIALS AND METHODS

Preparation of antigens. The strains of *Histoplasma capsulatum* (G-92), *Blastomyces dermatitidis* (A-22, A-26, A-31), and *Coccidioides immitis* (C-25) used for the preparation of antigens were stock strains isolated from human clinical material.

The *H. capsulatum* and *B. dermatitidis* antigens were prepared in an identical manner, with the exception that the *Blastomyces* antigen was prepared from equal proportions of three strains, whereas the *Histoplasma* antigen was prepared from a single strain. Nine Kolle flasks containing Brain Heart Infusion (Difco)-glucose (BHIG) agar with 200 units of penicillin and 250 mg of streptomycin per ml were inoculated with the parasitic form (yeast form) of the organism. The flasks were fitted with Vaspar-treated cotton plugs and were incubated at 37 C for 7 days. The organisms were washed from the agar surface with 0.85% NaCl containing 10% Formalin. Incubation of the pooled cells was continued, with constant rotation, for an additional 6 days. The yeast cells were washed four times and suspended in 60 ml of distilled water containing Merthiolate in a final concentration of 1:10,000.

Coccidioides antigen was prepared by harvesting the submerged growth of the saprophytic form (mycelial form) of *C. immitis* from BHIG broth. Four 1-liter flasks each containing 250 ml of medium were inoculated with *C. immitis* and were incubated with constant rotation at 37 C for 2 weeks. Formalin was then added to a final concentration of 10%, and the incubation was continued for 6 days. The mycelial mass was recovered by filtration, washed twice with 0.85% NaCl, homogenized in a blender, centrifuged, and suspended in 65 ml of distilled water containing Merthiolate (1:10,000).

The protein nitrogen content of each antigen was determined by use of a coulometric method (2).

Immediately prior to inoculation, a water-in-oil emulsion of each antigen was prepared by adding equal volumes of antigen to Incomplete Freund Adjuvant (Difco) and then briefly homogenizing the mixture with a Branson sonifier.

Monkeys. Fifteen adult rhesus (*Macaca mulatta*) monkeys, 12 males and 3 females, were used for the production of antifungal sera. Two months prior to the initiation of this study, eight of these animals had been exposed to an intranasal inoculation of viable *Neisseria meningitidis*. There was no apparent reaction, and after a period of observation these animals were considered comparable to the seven monkeys which had not received previous manipulation.

All animals were tested for hypersensitivity to tuberculin (PPD), histoplasmin, blastomycin, and coccidioidin by dropping a 1:10 dilution of the antigen into the eye. Examinations for reactions were conducted at 24, 48, and 72 hr. The initial hypersensitivity tests were conducted by instillation of PPD in the right eye and histoplasmin in the left. Blastomycin and coccidioidin were applied to the left and right eye, respectively, 10 days later.

The animals were housed in individual cages in a modern constant-temperature animal room containing no other animals.

Preparation of antisera. The monkeys were immobilized, and an area between the scapulae was shaved and cleansed with an aqueous iodine solution. Disposable syringes (2-ml) fitted with 20-gauge 1.5-inch (3.81-cm) needles were used to introduce the appropriate antigen emulsion under the right scapula of each animal. The antigen was deposited in two pockets, approximately 1 inch (2.54 cm) from the point of insertion, by delivering 0.5 ml before repositioning the needle and discharging the remaining 0.5 ml.

Serum specimens. Serum was obtained from each monkey prior to inoculation and periodically thereafter by immobilizing the animal and withdrawing approximately 10 ml of blood from the femoral vein. Serum was harvested from the clot and stored at -70°C until at least six bleedings were available for testing at one time. Serial bleedings from uninoculated monkeys were not obtained.

Serological tests. All sera were assayed for antibodies by use of a CF test and an LA test. In addition, a coccidioidin-precipitin test and histoplasmin immunodiffusion tests were performed on selected sera.

The CF test employed in this laboratory was devised by Kent and Rein and previously reported in detail (1, 3, 4). The test consists of three 50% units of complement incubated for 18 hr at 4°C for fixation, and for an additional 30 min at 37°C for the hemolytic system. Serum dilutions having less than 50% hemolysis are considered positive. Twofold serum dilutions were prepared from the initial 1:8 dilution. Four antigens were used in the test: histoplasmin, whole yeast-form (WYF) *Histoplasma*, coccidioidin, and ground yeast-form *B. dermatitidis*.

The LA test, employing a histoplasmin antigen, was performed according to the instructions accompany-

ing the antigen (Colab Laboratories, Chicago Heights, Ill.).

Immunodiffusion tests were conducted by Paul Klite, National Institutes of Health, using a matrix of 1% Difco Special Agar (Noble) in 0.22% NaCl. The histoplasmin antigens were prepared by flash evaporation to fivefold concentration, precipitation with acetone, and resuspending the precipitate in distilled water to one-half the original volume (5). Plates were incubated for 72 hr at 37°C .

The coccidioidin-precipitin test described by Smith et al. (6) was employed. The test consists of layering 0.2-ml quantities of undiluted 1:10 and 1:40 dilutions of antigen over 0.2 ml of undiluted serum. The 6 by 50 mm tubes were incubated for 5 days at 37°C and were observed for precipitate at the antigen-serum interface or at the bottom of the tube as a "button". Merthiolate (1:10,000) was added to both serum and antigen to diminish bacterial growth during incubation.

RESULTS

None of the 15 monkeys were hypersensitive to the intraocular instillation of PPD, histoplasmin, blastomycin, or coccidioidin. Slight erythema noted in one monkey, 24 hr after the instillation of PPD, was subsequently considered to be non-specific when a repeat test failed to elicit a similar reaction.

Serum from each of the monkeys, prior to inoculation with one of the fungal antigens, was nonreactive ($< 1:8$) in the CF, LA, coccidioidin-precipitin, and immunodiffusion tests.

The results obtained in the CF and LA tests with serum from the five monkeys inoculated with *H. capsulatum* (1.20 mg of protein N per ml) are presented in Fig. 1A. The response of each of the monkeys to their respective antigen was quantitative rather than qualitative; therefore, the titers are presented as the geometric mean of the five animals.

Results obtained with the *Coccidioides* antigen in the CF test are omitted from the figures since they were negative in 14 of the 15 monkeys. The one exception, a monkey inoculated with *H. capsulatum*, demonstrated a 1:8 titer with coccidioides antigen at both the 6- and 8-week bleedings.

Titers obtained with the sera of monkeys inoculated with *B. dermatitidis* (1.06 mg of protein N per ml) are shown in Fig. 1B, and those inoculated with *C. immitis* (1.40 mg of protein N per ml), in Fig. 1C.

The first 12 bleedings from all 15 monkeys, representing preinoculation to 36 weeks post-inoculation, were screened in immunodiffusion tests with a 10-fold concentration of histoplasmin antigen. Sera from three of the five monkeys inoculated with *H. capsulatum* produced precipitin lines from the 2nd through the 36th week.

FIG. 1. Geometric mean titers of sera from five rhesus monkeys inoculated with nonviable *Histoplasma capsulatum* (A), *Blastomyces dermatitidis* (B), and *Coccidioides immitis* (C).

Antibodies were demonstrable in the remaining two monkeys, from the 2nd until the 12th week in one and until the 20th week in the other. Two of five monkeys inoculated with *B. dermatitidis* produced precipitin lines, but not until the 16th week postinoculation. Sera from monkeys inoculated with *C. immitis* failed to produce precipitin lines with the histoplasmin antigen.

Additional immunodiffusion tests performed to show the relationship of antibodies produced in human histoplasmosis to antibodies produced in monkeys inoculated with *H. capsulatum* or *B. dermatitidis* are illustrated in Fig. 2 and 3. The immunodiffusion test illustrated in Fig. 2 effectively demonstrated the similarity of precipitins in human histoplasmosis, monkey antisera, and anti-*Histoplasma* mouse ascitic fluid. There also appears to be an identical precipitin line shared by the two patients with histoplasmosis which was not present in the monkey serum and may or may not have been present in the mouse ascitic fluid. A third precipitin line observed with mouse ascitic fluid was not found in the human or the monkey sera. Figure 3 shows the dissimilarity of anti-*Blastomyces* monkey sera with both anti-

Histoplasma monkey serum and serum from human histoplasmosis.

Precipitin test. Anti-*Coccidioides* monkey sera were assayed in the standard tube precipitin test with dilutions of coccidioidin as antigen (Table 1). Precipitins were demonstrable in all five monkeys shortly after inoculation, and, like precipitins observed in human *Coccidioides* infection, were transitory in nature.

DISCUSSION

The serological patterns obtained with monkey sera, after inoculation with nonviable *H. capsulatum*, *B. dermatitidis*, or *C. immitis*, closely parallel those found with human infections. The cross-reactions and patterns of detectable antibody response seen in human histoplasmosis, blastomycosis, and coccidioidomycosis were reviewed by Campbell in 1960 (1) and will not be discussed in detail.

CF reactions with *Histoplasma* antisera. The monkeys inoculated with *H. capsulatum* responded quickly to the antigen. CF antibodies were detectable with both WYF *Histoplasma* and *Blastomyces* antigen in two of five monkeys

at 2 weeks, and in all five by the 4th week. The cross-reactions with *Blastomyces* antigen are a common occurrence in pulmonary cases of both histoplasmosis and coccidioidomycosis, and frequently CF titers with blastomyces antigen will exceed those obtained with the homologous

TABLE 1. *Coccidioidin-precipitin titers among monkeys inoculated with Coccidioides immitis antigen*

Monkey	Titers at week ^a								
	2	4	6	8	12	16	20	24	28
B-145	U	10	40	40	10	U	U	—	—
B-152	U	10	U	—	—	—	—	—	—
N-604	U	10	40	10	—	—	—	—	—
N-618	U	10	40	10	—	—	—	—	—
N-619	—	10	40	40	40	10	10	U	—

^a Minus sign represents no reaction; U represents undiluted antigen.

FIG. 2. Diagrammatic representation of immunodiffusion plate with histoplasmin antigen (10-fold concentration) in the center well; identical anti-Histoplasma monkey serum in wells A and D; human sera from patients with histoplasmosis in wells B and C (different patients); and anti-Histoplasma mouse ascitic fluid in well E.

FIG. 3. Diagrammatic representation of immunodiffusion plate with histoplasmin antigen (10-fold concentration) in the center well; anti-Blastomyces monkey sera in wells A, B, and C (serial collections at 1-month intervals); anti-Histoplasma monkey serum in well D; and human serum from patient with histoplasmosis in well E.

antigens. In the monkey anti-*Histoplasma* sera, the titers with heterologous *Blastomyces* antigen exceeded the WYF *Histoplasma* titers only at the 2nd week. Thereafter, they were equalled or more frequently were exceeded by titers obtained with the homologous WYF *Histoplasma* antigen. This tendency for titers with homologous antigens to exceed those with heterologous antigens is used in the interpretation of CF results in mycotic infections.

The antibodies detected by the histoplasmin (mycelial filtrate) CF antigen were delayed by at least 2 weeks beyond those detected with the WYF histoplasma antigen. They reached a peak at 6 weeks and quickly receded until four of five animals were nonreactive at the 24th week. The remaining monkey maintained a low titer throughout the 104 weeks of observation. In human infections, this antigen has proved most valuable in the diagnosis of long-standing chronic infections; however, reactivity is not restricted to these cases.

Another similarity with human infection was the low titer cross-reaction obtained with *Coccidioides* antigen. This reaction is usually observed in patients with histoplasmosis, but only when the homologous CF titers are very high. CF titers of 1:8 were obtained with *Coccidioides* antigen at the 6th and 8th week when WYF *Histoplasma* titers were 1:512 and 1:2,048, respectively.

CF reactions with Blastomyces antisera. The complement-fixing antibody response to *B. dermatitidis* was similar to the response with *H. capsulatum* in that antibodies were demonstrable in two monkeys at the 2nd week and in all five by the 4th week. In each reactive serum, the CF titers with homologous antigen (*Blastomyces*) exceeded those with heterologous antigen (*Histoplasma*). However, the serological response to the inoculation of *Blastomyces* was not as uniform as that obtained in the monkeys inoculated with *Histoplasma*. All five monkeys inoculated with *Histoplasma* had CF titers with both WYF *Histo*-

plasma and *Blastomyces* antigens at the end of the 2-year observation period. Only three of the monkeys receiving *Blastomyces* were reactive at the end of 2 years, whereas one became non-reactive at the 20th and the other at the 40th week. There is no reason to believe there is a correlation, but it is interesting to note the transitory response in two of the monkeys and the observation that sera from approximately 60% of proven cases of blastomycosis are serologically nonreactive (1).

The value of the CF test in the diagnosis of blastomycosis is limited; however, in those verified cases producing complement-fixing antibodies, the titers are frequently of prognostic value. The *Blastomyces* antigen is retained in the battery of CF antigens, because titers are of value in the serological detection of histoplasmosis and coccidioidomycosis as well as blastomycosis.

CF reactions with Coccidioides antisera. The serological response of monkeys inoculated with *C. immitis* could not be differentiated from the patterns observed with monkeys inoculated with *B. dermatitidis*. This similarity is also seen with human serum when the differential diagnosis between blastomycosis and coccidioidomycosis must be made on the basis of clinical symptoms.

The failure to obtain titers with *Coccidioides* antigen is frequently observed in human cases of acute pulmonary coccidioidomycosis, even though CF titers are obtained with *Histoplasma* and *Blastomyces* antigens.

LA and coccidioidin-precipitin tests. The LA test is used as an adjunct to the CF test for the detection of agglutinins which are normally demonstrable in serum before the appearance of complement-fixing antibodies. The agglutinins are usually transitory in acute cases of histoplasmosis, blastomycosis, and coccidioidomycosis, and they serve only to signal the necessity for exhaustive diagnostic cultures. One surprising finding was the persistence of LA titers in one monkey in each of the groups inoculated with *H. capsulatum* and *B. dermatitidis*. In the remaining four monkeys in each group and in the five inoculated with *C. immitis*, the LA titers were demonstrable by the 2nd week, but quickly receded and were less than 1:8 by the 28th week. The coccidioidin-precipitin test serves the same purpose as the LA test in that precipitins are usually demonstrable early in the course of coccidioidomycosis but quickly diminish to a nondetectable level.

Serological patterns in histoplasmosis, blasto-

mycosis, and coccidioidomycosis have been established by studying serial sera from thousands of verified cases. There is, however, little information on the influence of subsequent mycotic infections on the serological response in persons who are hypersensitive to *H. capsulatum*, *B. dermatitidis*, or *C. immitis*. Initial plans were to hold all animals until they were nonreactive in the serological tests and then to determine the anamnestic antibody production stimulated by the introduction of a heterologous antigen (e.g., monkeys previously receiving *H. capsulatum* would be inoculated with nonviable *Cryptococcus neoformans*, *Aspergillus fumigatus*, or *Mycobacterium tuberculosis*). The persistence of CF titers, in some animals for as long as 2 years, precluded accomplishment of this aspect of the protocol.

A natural extension of this work would be a comparative serological study on monkeys infected with *H. capsulatum*, *B. dermatitidis*, or *C. immitis*. Pulmonary infections could be controlled with antibiotics to prevent the development of fatal infections and to allow time for the appearance of complement-fixing antibodies. It might also be possible to arrest the disease, permitting later infection with another mycotic agent to determine the influence of multiple mycotic antigens on the serological patterns.

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