Differences in Clinical and Convalescent-Phase Antibodies of Rhesus Monkeys Infected with Monkey Pox, Tanapox, and Yaba Poxviruses

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Complement-fixing and complement-fixing inhibiting (CFI) antibodies were demonstrated in the clinical and convalescent stages, respectively, of rhesus monkeys infected with either monkey poxvirus, Tanapoxvirus, or Yaba poxvirus. Specificity of the CFI antibody was confirmed by its failure to cross-react with heterologous poxvirus antigens and by experiments demonstrating the CFI test as being antigen dependent. Serum containing CFI antibody neutralized homologous poxvirus but failed to agglutinate antigen-coated, tanned red blood cells. The application of CFI test as a seroepidemiologic tool for studies of poxvirus infection of man and simian monkeys and the biologic role of CFI antibody in pathogenesis were discussed.

With the development of the immunoelectrophoresis technique by Grabar (6), subclasses of immunoglobulins (Ig) were identified in numerous animal species (17). The early work of Benacerraf, et al. (2) demonstrated distinguishing biological and serological properties between guinea pig IgG1 and IgG2 subclasses; the latter fixed complement, whereas the former mediated local anaphylaxis. The biological significance of immunoglobulin subclasses during the immune response to pathogenic microorganisms, however, remains unclarified.

We report here the finding of complement-fixing (CF) and complement-fixing inhibiting (CFI) virus-specific antibodies in rhesus monkeys experimentally infected with various simian poxviruses. The temporal appearances of these antibodies were studied in relation to clinical and convalescent phases of the diseases. Additional serological properties of CF and CFI antibodies were studied by serum neutralization and passive hemagglutination tests. Ig classes were characterized by two-step radi-immunoelectrophoresis. The biologic role of CF and CFI antibody during poxvirus infection and the applicability of the CFI test to seroepidemiologic studies of poxvirus diseases in primates are discussed. (R. D. H. used a portion of this work in partial fulfillment of the M.S. degree.)

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MATERIALS AND METHODS

Viruses. Monkey poxvirus (MPV) was isolated by Prior in 1959 (18) from a spontaneous outbreak of disease in a colony of monkeys in Pennsylvania. The virus was obtained in 1961 and has been propagated in rhesus monkey kidney cells. Virus was added to monolayers of LLC-MK2 cells grown in roller bottles and allowed to adsorb for 2 h at 37 C. Medium 199 in Earle balanced salt solution with 2% fetal calf serum, antibiotics (penicillin, streptomycin, and mycostatin), and sodium bicarbonate (medium adjusted to pH 7.2) was added, and cells were incubated at 37 C until maximum cytopathic effect (CPE) was observed. A pool of cells and medium from several bottles was frozen and thawed three times, sonically treated for three 1-min intervals, and centrifuged at 5,000 X g for 10 min.

The Tanapox virus (5) isolate used in this study was from an epizootic of naturally occurring skin tumors in rhesus monkeys housed at the Brooks Air Force Base, Texas. Tanapox virus was grown in LLC-MK2 cells as described above for monkey pox, except that virus adsorption and incubation of infected cells were performed at 33 C.

Yaba poxvirus, isolated from the only reported naturally occurring outbreak of disease (1), has been maintained by passage through rhesus monkeys (23). Yaba tumors stored at -70 C were thawed, minced, and blended in a Sorvall blender (Ivan Sorvall Inc., Newton, Conn.) with sufficient sodium citrate-buffered saline (pH 7.0) to make 10% tumor homogenate. The Yaba inoculum was sealed in 5-ml ampoules and stored at -70 C.

CF and CFI antigens. The preparation of virion and virus-associated antigens from Yaba
tumors has been described by Olsen and Yohn (15). Minced tumor was homogenized in a Sorvall blender with equal volumes of sodium citrate-buffered saline and fluorocarbon (Genetron 113, Razon Inc., Wichita, Kan.) to produce a 40% tumor homogenate. The fluorocarbon-saline mixture was centrifuged at 2,000 × g for 10 min, and the saline layer was decanted from the fluorocarbon. The saline extract was extracted twice more with fresh fluorocarbon and reduced in volume by dialysis against Aquacide III (Calbiochem, San Diego, Calif.). The dialyzed material was layered upon 36% sucrose and centrifuged in a Beckman L-2 Model 65B ultracentrifuge at 60,000 × g for 90 min to remove whole virus. After centrifugation, the saline layer above the sucrose was used as Yaba virus-associated, soluble (YS) antigens.

The virus pellet was resuspended in Veronal buffer (pH 7.4) and treated with an X-Press (Ab Biox-Nacka, Sweden) a total of 12 times at −70 C to disrupt the virions. Disrupted Yaba virus material was then thawed and centrifuged at 8,000 × g for 10 min. The supernatant fluid contained soluble virion (X) antigen previously referred to as YX antigen. The insoluble material also contained virion antigens but, because of the small quantity obtained, was not used in this study.

Two similar groups of antigens were prepared from monkey pox and Tanapox viruses. Virus-infected LLC-MK2 cells and tissue culture media were pooled when maximum viral CPE developed. The pooled material was frozen and thawed three times, sonically treated for three 1-min intervals, and reduced to 1/10 the original volume by dialysis against Aquacide III. The antigen preparation was sonically treated again for 1 min and centrifuged for 10 min at 5,000 × g before being layered onto 36% sucrose and centrifuged as described for Yaba poxvirus. The saline layer containing soluble- associated antigens was referred to as MS antigen and TS antigen for monkey pox and Tanapox antigens, respectively. The virus pellets were treated as described above for Yaba poxvirus, and the solubilized virion antigens were referred to as MX antigen and TX antigen, respectively.

Control antigens were prepared from uninfected LLC-MK2 cells by treating them in the same manner as monkey pox- or Tanapox-infected cells. Control S (CS) and control X (CX) antigens were prepared.

Animals. Young adult rhesus monkeys (Macaca mulatta) of both sexes, weighing 3 to 6 kg, were obtained from a colony of monkeys maintained by the Department of Veterinary Pathobiology, College of Veterinary Medicine, The Ohio State University, and from the Veterinary Animal Branch, Brooks Air Force Base, Texas.

Animal inoculation. Seven monkeys were injected subcutaneously with 1 ml of MPV containing 108 plaque-forming units (PFU) of virus. The dose of virus was divided into three injection sites on the upper abdomen. Serum samples were collected prior to virus injection, every third day during the clinical and early convalescent stages of disease, and every other week thereafter. All sera were inactivated at 56 C for 30 min and stored at −60 C until used for serologic testing.

Five monkeys were injected subcutaneously with 1 ml of 104 PFU of Tanapox virus as described above for monkey pox. Three monkeys were injected with Yaba virus by injecting 1 ml of serial 10-fold dilutions of 10% Yaba tumor homogenate subcutaneously into separate areas on the animals' abdomens. Serum samples were collected and handled as described above for monkey pox.

Serological tests. CF tests were performed by microtiter technique using microtiter needles, pipettes, and U-bottomed disposable plates (Cooke Engineering, Alexandria, Va.). Antigens were titrated against hyperimmune rabbit sera by "checkerboard" (block) titration. One complement-fixing unit (CFU) of antigen was the highest dilution that resulted in complete fixation (+4+) of 5 C/H40 units of guinea pig complement. Sera were tested using 10 CFU of associated (S) antigen and 4 CFU of virion (X) antigen. Test sera and antigen were incubated with 4 C/H40 units of guinea pig complement for 16 to 24 h at 4 C before the sensitized sheep erythrocytes were added. After an additional incubation for 30 min at 37 C, the plates were centrifuged at 800 × g for 1 min, and the test results were recorded. Fixation of complement was graded from 1+ to 4+. The CF antibody titer was expressed as the highest dilution of serum that gave a 3+ or greater complement-fixing reaction.

The CFI test, as originally described by Rice (20), was modified to the microtiter technique by Olsen and Yohn (16). Twofold serial dilutions of test sera were incubated with 2 CFU of antigen for 16 to 24 h at 4 C. Two units of reference serum and 5 C/H40 units of guinea pig complement were added to the test, and incubation at 4 C was continued for an additional 24 h. Antibody-sensitized sheep red blood cells were added, and the tests were incubated for 30 min at 37 C. The plates were centrifuged at 800 × g for 1 min, and the results were recorded using the same scoring as described above for the CF test. The CFI antibody titer was expressed as the highest dilution of test serum which blocked by greater than 50% the fixation of complement by reference antibody and antigen. In the CFI test, each serum was concomitantly tested alone and with the reference antibody for anticomplementary activity. A positive control was used to show that the reference antibody and the antigen both fixed complement.

CFI reference antigens were titrated against a homologous reference serum by the block titration method. The various reference sera were: (i) monkey pox antisera from rabbit no. 39 hyperimmunized with MS and MX antigens; (ii) Tanapox antisera prepared by pooling several monkey sera with high CF antibody titers; and (iii) Yaba antisera obtained from cynomolgous monkey no. 91 that had been hyperimmunized with YS
antigen. All reference antisera were tested for CF activity against fetal calf serum and control antigens. Those showing CF reactivity with control antigens or calf serum were adsorbed until free of such CF activity. All sera showed strong CF activity with homologous antigens.

Serum neutralization (SN) tests for monkey pox were performed by the plaque reduction test of Itoh and Melnick (9). Test sera were diluted in threefold steps from 1:10 through 1:270 with Hanks balanced salt solution (HBSS). One milliliter of virus, containing 100 to 300 PFU of virus per 0.2 ml, was incubated with 1 ml of diluted test serum for 16 to 24 h at 4 C. An amount (0.4 ml) of serum-virus mixture was added to LLC-MK2 cells grown in 60 by 15-mm wells of plastic Limbro plates (Limbro Chemical Co., Inc., New Haven, Conn.) and allowed to adsorb for 2 h. During the adsorption period the plates were shaken every 30 min to insure uniform infection. Excess virus-serum was removed by aspiration, and the monolayers were washed once with 4 ml of HBSS before addition of tissue culture maintenance medium. For optimal plaque counting, cells infected with MPV were incubated at 37 C for 3 days, and cells infected with Tanapox virus were incubated at 33 C for 18 days. At the end of the incubation period, the cells were washed with HBSS, fixed with methyl alcohol, and stained with May-Grünewald Giemsa stain. Plaques were counted with the aid of a dissecting microscope. The neutralization titer was expressed as the highest dilution of serum that caused a 50% reduction in the number ofplaques.

**Tanned cell passive hemagglutination.** The procedures used for the passive hemagglutination test were described by Kabet and Mayer (10). Tanned cells were coated with soluble poxvirus antigens (8 mg/ml, protein concentration) and subsequently stabilized with heat-inactivated rabbit serum diluted 1:100 in phosphate-buffered saline (PBS) (pH 7.2). All sera used in this test were heat inactivated at 56 C for 15 min and adsorbed with sheep red blood cells.

**Two-step radioimmunoelectrophoresis (RIE).** In principle the RIE technique (24) consists of diffusing soluble, radioiodine-labeled viral antigen against agar-gel immunoelectrophoresis-precipitated monkey serum globulins. The specific binding of radiolabeled virus antigen to monkey immunoglobulin precipitin arcs are viewed directly by subsequent autoradiography.

Immunoelectrophoresis was performed by the procedure of Grabar and Burtin (1). Veronal buffer (pH 8.2) with an ionic strength of 0.05 was used in this procedure. Electrophoresis of monkey serum proteins was for 1 h at 150 V (5 mA per slide).

Monkey gamma globulin precipitin arcs were developed by goat anti-monkey gamma globulin serum and rabbit anti-monkey gamma globulin serum. The preparation of the monkey gamma globulin-specific goat and rabbit sera was according to the procedure of Olsen et al. (14).

All unbound protein components were removed from the immunoelectrophorograms by washing in PBS (pH 7.2) for 24 h. After thorough washing, 125I-labeled antigen was placed in the immunoelectrophorogram troughs and allowed to diffuse against monkey-precipitated gamma globulins for 48 h. After the second immunodiffusion step, the immunoelectrophorograms were extensively washed for 72 h in PBS and for an additional 24 h in distilled water before air-drying and staining with Buffalo blue-black stain.

Autoradiograms were made by placing Kodak X-ray film (Electron Images Plates) against the dried agar for an appropriate period of time. The X-ray film was developed by Kodak Liquidol developer.

**RESULTS**

**Humoral antibody response in monkeys to MPV.** In monkeys infected with MPV (Fig. 1) CF antibodies were generally detectable by the first week postinfection (PI) and peak CF titers against both virus-associated MS and virion MX antigens were observed by day 9 PI. In nearly all six MPV-infected animals, peak CF titers corresponded to the period when the lesions were most prominent. As the lesions regressed, CF antibody titers decline, and in most monkeys such antibodies were not detectable after the 10th week PI. As illustrated in Fig. 1, the CF response to MS and MX antigens were quite similar.

Virus-neutralizing antibodies (Fig. 1), although they appeared in the serum of MPV-infected animals at the same time as did CF antibody, did not disappear throughout the observation period (36 weeks).

CFI antibodies were first detected in MPV-infected monkeys to MS antigen (Fig. 1) at approximately the 12th week PI. The CFI antibody titer reached peak titers nearly 24 weeks PI and remained relatively constant to the end of the observation period. CFI antibodies to MS antigen were demonstrated in convalescent sera of six of seven MPV-infected monkeys. The seventh monkey (no. 217) did not develop monkey pox lesions after MPV inoculation, but did develop CFI antibody titers of 1:16 against MS and MX antigens during the 3rd week PI. These titers persisted throughout the observation period (29 weeks PI).

CFI antibody to homologous virus antigen was demonstrated in the five Tanapox virus-infected monkeys and in the three Yaba tumor virus-infected monkeys. The time course of the CFI and CFI response to Tanapox virus infection was similar to the responses of MPV-infected monkeys. In the three Yaba poxvirus-infected animals, CFI antibody titers persisted until the tumors regressed or were surgically removed be-
INFECT. IMMUNITY

Fig. 1. Humoral antibody response of monkey no. 215, a monkey which developed primary and secondary monkey pox lesions after inoculation with MPV. CF antibodies against homologous MX and MS antigens reached peak titers (1:16) on day 9 PI and then declined to undetectable levels by the 5th and 12th weeks PI, respectively. CFI antibody titers against MS antigen were not detected before the 12th week PI but increased to 1:16 between the 12th and 24th weeks PI and remained at that level throughout the test period. Neutralizing antibody titers of 1:30 were detected within 2 weeks PI and persisted throughout the observation period.

Serologic specificity of the CFI test. Six convalescent monkey sera, two from each pox disease, were tested in the CFI test against varying units of homologous antigen (Table 1). A twofold increase in number of antigen units used in the CFI test resulted in a 2- to 16-fold decrease in the CFI antibody titer.

The detection of CFI antibodies was shown to be dependent upon the order in which reagents were added to the CFI test (Table 2). Two test sera with CFI antibody titers of 1:16 failed to inhibit the CF reaction when the reference antigen and reference antibody were preincubated under test conditions.

The specificity of the CFI test was confirmed by demonstrating the absence of reactions with heterologous poxvirus antigens (Table 3).
**DISCUSSION**

CF and CFI antibodies were demonstrated in the clinical and convalescent stages, respectively, of rhesus monkeys infected with either monkey poxvirus, Tanapoxvirus, and Yaba poxvirus. The early appearance of CF antibody in Yaba virus and MPV rhesus monkeys support the earlier findings of Metzgar et al. (12) and Wenner et al. (22), respectively. The presence of an antibody incapable of fixing complement, however, has not been heretofore demonstrated in rhesus monkeys.

Although specific CFI antibodies have been detected in the avian (19), equine (20), feline (16), and human (21) sera, we felt that it was first necessary to show serological specificity of the CFI test for monkey antibody. The possibilities that CFI-positive monkey sera nonspecifically blocked the reaction between antibody (CF anti-poxvirus serum) and poxvirus antigen and/or interfered with the fixing of complement were excluded when it was demonstrated that (i) CFI positive monkey serum had little or no effect on fixation of complement to reference poxvirus antigen-reference antibody complexes, and (ii) CFI-positive sera failed to block heterologous CF reactions. The demonstration that CFI titers were essentially inversely proportional to the units of viral antigen in the CFI test was further evidence that the activity in the monkey sera was directed toward the viral antigen.

The failure of the convalescent CFI positive sera to fix homologous (monkey) complement as

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**Table 1. CFI antibody titer of convalescent monkey sera determined with varying number of units of homologous reference antigen**

<table>
<thead>
<tr>
<th>Test sera</th>
<th>Homologous reference antigen</th>
<th>No. of reference antigen units</th>
<th>1</th>
<th>4</th>
<th>6</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>219-19</td>
<td>TS</td>
<td>128&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>279-16</td>
<td>TS</td>
<td>256</td>
<td>4</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>215-26</td>
<td>MS</td>
<td>&gt;256</td>
<td>&lt;2</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>215-31</td>
<td>YS</td>
<td>16</td>
<td>4</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3125-49</td>
<td>YS</td>
<td>32</td>
<td>4</td>
<td>2</td>
<td></td>
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</tbody>
</table>

<sup>a</sup> CFI antibody titers expressed as reciprocal of serum dilution.

**Table 2. CFI antibody titer of convalescent monkey pox sera determined by adding CFI test reagents in normal and reverse sequence**

<table>
<thead>
<tr>
<th>Test sera</th>
<th>Reagents added in normal sequence&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Reagents added in reverse sequence&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>215-28</td>
<td>1:16</td>
<td>1:2</td>
</tr>
<tr>
<td>215-30</td>
<td>1:16</td>
<td>1:2</td>
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</table>

<sup>b</sup> Reference MS antigen incubated with test serum 24 h before addition of reference antibody.

<sup>c</sup> Reference MS antigen incubated with reference antibody 24 h before addition of test sera.

**Characteristics of monkey CF and CFI antibody.** Globulin from serum samples of monkey no. 215 that demonstrated CF (4 weeks PI) and CFI activity (24 weeks PI) with MS antigen were analyzed for antigen-binding activity in the RIE test (Fig. 2). Both CF- and CFI-positive sera contained globulin with electrophoretic characteristics of IgG which bound 125I-labeled MS antigens.

A preimmune serum sample, an early clinical phase serum, and a late convalescent serum of monkey no. 215 were tested for direct CF reactivity with MS antigen using guinea pig, rabbit, and monkey complement (Table 4). Serum taken from monkey no. 215 during the acute phase of MPV infection (4 weeks PI) fixed all sources of complement. Convalescent serum from the same animal which had a CFI titer to MS antigen of 1:16 did not fix heterologous (guinea pig, rabbit) or homologous (monkey) complement. In addition, only clinical-phase serum agglutinated MS-coated, tanned cells in the passive hemagglutination test (PHA) (Table 4). As mentioned earlier, both CF and CFI sera neutralized MPV at a dilution of 1:30.
Fig. 2. Two-step RIE analysis (see text) of three sequential sera from monkey no. 215 infected with MPV. Sera tested included preimmune (top), serum 2 weeks PI (CF titer 1:8), and serum 24 weeks PI (CFI titer of 1:16). No binding of 125I-labeled MS was observed with preimmune globulin; however, both CF-positive (middle) and CFI-positive (bottom) globulin selectively bound labeled MS antigen. Antigen-binding globulin from both serums had electrophoretic characteristics of IgG.

Table 4. Comparison of acute phase serum and convalescent phase from a monkey infected with MPV

<table>
<thead>
<tr>
<th>Serum from three sequential bleeds</th>
<th>Antibody titers from various serologic testsa</th>
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<tbody>
<tr>
<td></td>
<td>CF test with complement from:</td>
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<tr>
<td></td>
<td>Monkey</td>
</tr>
<tr>
<td>Preimmune sera</td>
<td>&lt;1:2</td>
</tr>
<tr>
<td>Clinical phase (4 weeks PI)</td>
<td>1:4</td>
</tr>
<tr>
<td>Convalescent phase (25 weeks PI)</td>
<td>&lt;1:2</td>
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</table>

- Monkey no. 215 exhibited the classical disease response to MPV infection.
- CF tests used 3 C’Ha units of monkey, guinea pig, or rabbit complement.
- Passive hemagglutination tests used MPV-soluble (S) antigen.
- Serum neutralization test.

well as heterologous complement (guinea pig and rabbit) indicated that the CFI properties were not due to incompatibility between the IgG and complement used in the test. The inability to fix complement may be due to some physical attribute of the Fc portion of the CFI immunoglobulin. An additional point to consider, however, is that failure to demonstrate that CF activity in serum may be due to the heat lability of the immunoglobulin as suggested by Brumfield and Pomeroy (3). The lack of agglutinin activity in CFI antibody suggests that the monkey CFI Ig functions essentially as a univalent antibody. This may indicate that CFI antibody either remains rigid in the hinge area of the Ig or that the binding affinity of one or both antigen-binding sites are weak. In either case the efficiency of this type of antibody as a functional agglutinin would be lowered in the presence of the red blood cell zeta potential (repelling electrostatic charge). Lack of agglutinin activity in CFI sera diminishes the applicability of a PHA test in seroepidemiology studies.

The CF and CFI test as described in this paper
using a single poxvirus type-specific CF antigen and its corresponding (monospecific) reference antibody may be a useful tool in epidemiological studies of poxvirus infection of human and simians. In view of the reported SN cross-reaction between Yaba virus and Tanapox virus (5, 13), we feel that the CF and CFI test will more readily distinguish between these viruses.

Although the two-step RIE test demonstrated that both CF and CFI antibody have immunoelectrophoretic characteristics of IgG, additional studies are required to confirm whether two different subclasses of IgG are involved.

The persistence of CF antibody in infected monkeys varied between the three diseases. The time of appearance and disappearance of CF antibody in Tanapox- and MPV-infected animals was relatively short (approximately 10–12 weeks), whereas in Yaba-infected animals the CF antibody was detected up to 35 weeks PI. The presence of CF antibody in all three pox diseases appeared to depend on the presence of persistent viral antigen stimulation. The temporal relationship of CF and CFI antibody with respect to virus antigen stimulation has also been observed by McGuiire et al. (11) in horses infected with infectious equine anemia virus.

The biological role of CF antibody during the clinical phase of the disease is clearly defined in most viral diseases. It is well accepted that CF antibody plays a major role in virus neutralization as well as in promoting antigen clearance by augmenting a general inflammatory response. Although little is known about the CF subclasses of IgG in viral pathogenesis, our studies and reports by others (11) suggest that the host possesses a regulatory mechanism for shutting down CF antibody following immunogenic stimulation. From an evolutionary point of view, such a mechanism would have a positive selection value to the host in counteracting immunologic diseases associated with CF antibody–viral antigen complexes. Immune complex diseases are prevalent in chronic virus infections such as lymphocytic choriomenigitis virus infections in mice and the pathological affect of virus infection appears only to be moderated where the host is rendered tolerant (8), i.e., the host lacks virus-specific CF antibody but not non-CF fluorescent antibody.

It may be hypothesized, therefore, that the avoidance of immunologic disease due to autoantibodies or immune complexes is mediated by antigen-specific CFI antibody. The function of a CFI antibody may play a similar role as the noncytotoxic enhancing antibody described by Hellstrom et al. (7) in various tumor-bearing animals.

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