Effect of the Purification of Virus Antigens on the Production of Specific Complement-Fixing Antibodies

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The effect of viral purification procedures on the antibody response of guinea pigs to immunization with reovirus type 2 and echovirus type 19 was investigated. Three grades of antigens were employed: (i) infectious monkey kidney tissue culture fluid (TCF), (ii) virus sedimented in the ultracentrifuge and suspended in phosphate-buffered saline, and (iii) virus purified by centrifugation in CsCl density gradients. The antibody response of the guinea pigs was studied by the hemagglutination inhibition, complement fixation, and serum neutralization tests. Only sera produced from virus purified by CsCl density gradients reacted specifically with homologous antigen in the complement fixation test. Sera from animals receiving tissue culture fluid virus or sedimented virus cross-reacted with heterologous antigens such as tissue culture fluid from uninfected monkey kidney cells. All sera, however, reacted specifically in hemagglutination inhibition and serum neutralization tests. Sera from intranasally infected animals (reovirus type 2), even though reacting specifically in the complement fixation test, had much lower titers than sera from animals inoculated intramuscularly.

To produce virus antisera useful in the complement-fixing (CF) reaction, special precaution has to be taken to avoid emergence of antibodies against host tissue antigens. This usually is accomplished by using the same animal species for serum production as for the propagation of antigen [e.g., monkeys for virus antigens grown in homologous kidney tissue cultures or mice for viruses grown in suckling mice (8)]. Antigens were also purified by treatment with fluorocarbon solvents to remove much of the host protein (6). Such procedures, however, are not entirely satisfactory since the elimination of host antigens may not be complete, or antibodies against tissue debris of the homologous animal may be formed.

The separation of extraneous host cell material and virus antigens, however, should also be possible by physical means. We therefore studied the effect of purification by differential centrifugation and density gradient banding of virus antigens to arrive at an antiserum which contains only antibodies against the virions. Echovirus type 19 and reovirus type 2 were chosen as antigens because they can withstand purification in the centrifuge. The method, however, may not be applicable to viruses which cannot be as easily purified in a CsCl gradient. The virus antigens were propagated in monkey kidney cells; for the antiserum production, guinea pigs were used because such a combination of unrelated animal species should be more sensitive in showing the presence or absence of extraneous host cell material in the antigens.

MATERIALS AND METHODS

Viruses. Reoviruses type 1 (Lang), type 2 (D-5, Jones), and type 3 (Abney) and echovirus type 19 (Burke) were propagated in rhesus monkey kidney tissue cultures (MKTC). (Reovirus types 1 and 3 were used without purification as antigens in serological tests.) During virus multiplication, the cells were maintained in high cysteine-altered Eagle medium without serum (1). After the cytopathic effect was complete, the cells were frozen and thawed three times to release the virus. Suspensions of echovirus 19 were stored at −20 C; reovirus suspensions were kept at 4 C.

Antigens and their purification. Crude virus antigen (reovirus type 2 and echovirus type 19) was prepared by removing cell debris at 1,600 × g for 25 min. The supernatant fluid was designated as infectious tissue culture fluid (TCF).

One batch of antigen was further purified by sedimenting the virus in a Spinco model L centrifuge at a performance index (pi) (5) of 13.0 for 120 min or at pi 35 for 45 min. The sediment was suspended in phosphate-buffered saline (PBS), one fourth of the original volume of TCF centrifuged being used. This virus suspension was designated as sedimented antigen.

A portion of the sedimented antigen was further

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purified in a CsCl gradient. For the reovirus, 0.48 g of CsCl was added per ml of virus suspension; for echovirus 19, the amount was reduced to 0.4 g/ml. The CsCl-virus mixtures were centrifuged in a Spinco model L centrifuge at a pi of 7.0 for 18 hr (SW 25 head). The gradients were fractionated into 10 to 12 fractions. To locate the virus in the gradient, the average density of each fraction was determined by measuring its refractive index (7), and a small portion of each was dialyzed against PBS and titered for hemagglutinin (HA) activity. The fractions containing the bulk of HA and having the appropriate density of 1.34 g/cm³ for echovirus 19 and 1.38 g/cm³ for reovirus (3, 4) were dialyzed overnight against PBS.

TCF antigen from uninfected MKTC in high cysteine-altered Eagle medium was prepared by freezing and thawing the cultures three times, followed by removal of large cell debris as described for infectious TCF.

Antisera and immunization of guinea pigs. The preinoculation sera of all animals were tested by hemagglutination inhibition (HI) for antibodies against the three reovirus types; half of the presera were also checked for neutralizing antibodies, and all sera were found to be free of reovirus antibodies. The animals were then inoculated intramuscularly (im) with antigen mixed 1:1 with Freund's incomplete adjuvant (1 ml in each thigh muscle). Two to three animals were used for each antigen; they received a total of three injections at intervals of 2 weeks. One week after the last inoculation, they were exsanguinated by heart puncture and the sera were stored at −20°C.

Two animals were inoculated at the same time intervals by dropping 2 or 3 drops of TCF of reovirus type 2 in each nostril.

HI, CF, and SN. For both HI and CF tests, the microtiter technique was employed (12). The HI test was performed in PBS (0.01 M phosphate buffer), pH 7.4, with human type O red blood cells and 4 HA units. For the HI test, the antisera was treated with kaolin and human type O red blood cells (10); for CF tests, the antisera was inactivated at 60°C for 1 hr. The CF tests were performed in block titrations, by using 5 units of complement and allowing the fixation of complement to occur overnight at 4°C (12). Serum titers were expressed as reciprocal values of the highest serum dilution showing fixation of complement or HI, respectively. Serum neutralization (SN) was carried out by incubating 100 TCID₅₀/0.1 ml of virus with serial twofold dilutions of serum at 37°C for 1 hr and inoculating 3 or 4 MKTC tubes with each virus-serum combination. The serum dilution end point was calculated by the method of Reed and Muench (9); the neutralization titers are expressed as the reciprocal value of the serum dilution end point.

Virus titration. Reovirus type 2 and echovirus type 19 were titrated by the plaque assay method as previously described (2, 3).

**RESULTS**

**Effect of antigen purity on antisera specificity.**

Guinea pigs were inoculated (im) with TCF grade, sedimented, and density gradient-purified reovirus type 2 and echovirus type 19 or intranasally with TCF reovirus type 2. The antibody titers were determined by CF and HI reactions by using TCF antigen of both viruses and MKTC (Table 1). The assay values represent averages of four independent titrations, which never showed more than a twofold difference in titer. Serum titers of duplicate animals receiving the same antigen also were very consistent and did not vary more than by a factor of two. The HI tests, serving as a control, showed that all sera reacted only with the homologous antigen. In the CF tests, however, antisera from animals immunized

| Animal no. | Antigen for immunization | Route of inoculation | HI titer* against | CF titer* against |
|-------------|--------------------------|----------------------|------------------|------------------|
|             |                          |                      | Echo-virus 1₀     | Reo-virus 2₀     |
|             |                          |                      | Monkey kidney ₀   | Echo-virus 1₀    | Reo-virus 2₀ |
| 76, 78, 83  | Reovirus 2 TCF           | Intramuscular        | <2               | 640             | 1,024        | 1,024        | 2,048        |
| 160, 161    | Reovirus 2, sedimented   | Intramuscular        | <2               | 640             | 256          | 256          | 1,024        |
| 156, 157    | Reovirus 2, density      | Intramuscular        | <2               | 640             | <16          | <16          | 512          |
|             | gradient purified        |                      |                  |                 |              |              |
| 164, 165    | Reovirus 2, TCF          | Intranasal           | <2               | 80              | <16          | <16          | 16           |
| 166, 167    | Echovirus 19, TCF        | Intramuscular        | 1,280            | <2              | 1,024        | 1,024        | 1,024        |
| 158, 159    | Echovirus 19, sedimented | Intramuscular        | 640              | <2              | 1,024        | 2,048        | 1,024        |
| 162, 163    | Echovirus 19, density    | Intramuscular        | 1,280            | <2              | <8           | 1,024        | <8           |
|             | gradient purified        |                      |                  |                 |              |              |

*Titers are expressed as reciprocal value of highest serum dilution showing CF or HI, respectively.

The titers represent averages of at least four replicate tests.

b Tissue culture fluid (TCF) of these antigens were used in both HI and CF tests.
It has been shown that all three reovirus types cross-react in the CF reaction and that reovirus types 1 and 2 also may cross-react to a small degree in the HI test (10). We therefore investigated whether the serological cross-reactions may have been eliminated by the progressive purifica-
tion of the antigens. CF tests were performed only with sera from animals receiving density gradient-
infected virus im or TCF intranasally; HI and SN tests, however, were performed on all groups of
immune sera. The results are summarized in Table 3. The range of serum titers for all animals in each
group is listed; where only one figure appears, no
variation among the serum titers of that group
was detected. The sera showed a type-specific
response in both HI and SN tests; the CF titer,
however, was exactly the same against any of the
three reovirus antigens. Low heterotypic titers
against reovirus type 1 were observed in 4 out of
a total of 11 animals concurrently by the HI and

**TABLE 2. Effect of antigen purity on the success
of the complement fixation (CF) reaction**

| Antigen                                      | CF serum titer* of guinea pig immunized with reovirus 2 tissue culture fluid (83) |
|----------------------------------------------|----------------------------------------------------------------------------------|
| Reovirus 2, density gradient purified        | 512                                                                              |
| Echovirus 19, density gradient purified       | <16                                                                              |
| Reovirus 2, tissue culture fluid             | 2,048                                                                            |
| Echovirus 19, tissue culture fluid           | 1,024                                                                            |

*Titers are expressed as reciprocal value of highest serum dilution showing CF; values are averages from four tests.

**TABLE 3. Comparison of complement fixation (CF), hemagglutination inhibition (HI), and serum neutral-
tion (SN) titers of reovirus 2 immune sera against reovirus 1, 2, and 3**

| Antigen used for immunization | Route of inoculation | CF* against | HI* against | SN* against |
|-------------------------------|----------------------|-------------|-------------|-------------|
|                               |                      | R1 | R2 | R3 | R1 | R2 | R3 | R1 | R2 | R3 |
| Tissue culture fluid          | Intramuscular        | ND | ND | ND | <10 | 1,200 | <10 | <20 | 800 | <20 |
| Sedimented                    | Intramuscular        | ND | ND | ND | <10 | 1,200 | <10 | <20 | 1,000 | <20 |
| Density gradient purified     | Intramuscular        | 256 | 256 | 256 | <10 | 1,200 | <10 | <20 | 500 | <20 |
| Tissue culture fluid          | Intranasal           | 512 | 512 | 512 | <10 | 2,000 | <10 | <20 | 1,000 | <20 |

*Titers are expressed as reciprocal values of highest serum dilution showing CF, HI, or neutraliza-
tion of 100 TCD50. Two figures listed indicate the range of serum titers of a particular group of animals;
one figure indicates that all sera had the same titer.

* TCF antigen of all three reoviruses were used in the serological tests.

* ND = not done. As shown above, these sera fixed complement with antigens other than reovirus.
SN tests. It was also apparent that progressive purification of the antigen could not prevent the emergence of these low-level heterotypic responses. Table 3 as well as Table 1 further shows that CF, HI, and SN titers of the specific sera were in the same order of magnitude; however, the CF titers were generally lower than the SN titers, which in turn were somewhat lower than the HI titers.

CF and HA antigen in TCF. The CF reaction may be useful to demonstrate viral antigens, especially those which may not be associated with the virion or empty capsids and therefore cannot be detected by the HA reaction or infectivity titration for that matter. We titered and compared the infectivity and the HA and CF antigens of reovirus type 2 and echovirus type 19 TCF. The results are shown in Table 4. By means of CF titration, the virus antigen was detectable only at a dilution up to 1:16 and occasionally 1:32, whereas HA activity could be demonstrated at antigen dilution of 1:256 or 1:512. The efficiency of detection of virus antigen was much inferior by the CF reaction as compared with the HA reaction.

DISCUSSION

If antiserum against a virus is produced in an animal not highly susceptible to that virus, a massive inoculation is necessary. Inoculating crude virus suspensions (TCF) will result in production of antibodies against the whole spectrum of antigens present. Such sera, even though specific in reactions directed against the virus surface (HI and SN), will react with antigens other than the virus proteins in the CF reaction. It seems preferable to purify the virus antigen prior to its use for immunization.

The first purification step (pelleting) employed in this investigation was designated to precipitate most of the virus (echovirus type 19) based on a sedimentation constant of 160S (3); no excess centrifugation time was allowed. The CF tests revealed that there were tissue proteins present in TCF with similar sedimentation properties as the virus, which gave rise to antibodies. Density gradient purification of the virions was necessary to obtain an antigen which was essentially pure virus and yielded antiserum which did not cross-react with MK-TCF. Purification of the antigen used for inoculation may appear to be cumbersome but in certain procedures could be well worth the effort. No additional treatment of the assay antigen used later in CF reactions is necessary with antisera from such highly purified antigens, and uncertainties of nonspecific cross-reactions due to extraneous tissue antigens will be alleviated. The described procedure may also be useful in preparing vaccines, especially multivalent ones, or in situations of repetitive vaccinations in which omission of contaminating tissue culture antigens is highly desirable to avoid immunological side effects. The data also confirmed the existence of a common CF antigen for the three types of reovirus (11) and the occasional low-titer heterotypic HI response against reovirus type 1. There seemed to be a trend in which only sera with homologous HI titers of \( \geq 2,500 \) showed a heterotypic titer. Since the extensive purification of some reovirus type 2 preparations used as antigens did not eliminate the CF cross-reaction among the three types nor the partial cross-reaction of types 1 and 2 in the HI test, it seems more probable that the antigens responsible for these crossovers are part of the virion and not soluble proteins. The possibility that soluble antigens may have been synthesized by the inoculated animal, however, cannot be entirely discarded.

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