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# Complement-Fixing Antigen from BHK-21 Cell Cultures Infected with Lymphocytic Choriomeningitis Virus

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Infection of BHK-21 cells with lymphocytic choriomeningitis (LCM) virus resulted in the production of significant titers of complement-fixing (CF) antigen. The antigen was spontaneously released from the cells, but the highest titer of 1:16 was recovered by disruption of the infected cells by freeze-thawing in tryptose phosphate broth. The antigen could be partially separated from infectious virus by centrifugation. Furthermore, it was possible to detect LCM virus infection of cell cultures by the production of the CF antigen, but this method proved less sensitive than titration by intracerebral inoculation of mice. The CF antigen from cell cultures was at least as sensitive and specific as the reference antigen prepared from infected guinea pig spleen.

The production of lymphocytic choriomeningitis (LCM) virus complement-fixing (CF) antigen was first reported in infected animals by Smadel et al. (12). Subsequently, spleen tissue from infected guinea pigs has been the source of this antigen. Although the occurrence of LCM virus CF antigen in cell cultures has been reported, recovery of the antigen in quantity has been difficult. Pederson and Volkert (7) were able to recover a maximum 1:2 titer of antigen from LCM virus-infected Earle's L cells. Lehmann-Grube and Hesse (5) reported significant titers of CF antigen from cultures of infected L cells but only examined the medium for antigen. Furthermore, their system required special procedures to maintain the cells for sufficient lengths of time for antigen production.

In this paper, we describe (i) the rate and sensitivity of LCM virus CF antigen production, (ii) the relationship of antigen to infectious virus, and (iii) the comparison of the cell culture antigen with antigen prepared from the spleens of infected guinea pigs.

## MATERIALS AND METHODS

**Viruses.** The Armstrong strain of LCM virus (VR134) was obtained from the American Type Culture Collection. The WE strain was received from J. F. Winn of the National Communicable Disease Center (NCDC), Atlanta, Ga. Stock virus of both strains was prepared by pooling infected mouse brains 6 days after intracerebral inoculation of LCM virus. A 20% suspension of brain tissue in a mixture containing equal volumes of skim milk (Difco) and tryptose phosphate broth (TPB, Difco) was stored at −70 C. When preparing a stock suspension for use, the 20% suspension was diluted to twice the volume with TPB.

**Mycoplasma.** Mycoplasma could not be cultivated by a standard method (2) from stocks of either strain of virus.

**Virus titration.** Randomly bred, white, Swiss mice (4- to 6-weeks-old) were purchased from Hilltop Lab Animals, Inc., Scottdale, Pa. Latent LCM virus infection was not detected in these mice.

Serial 10-fold dilutions of virus suspensions were made in TPB. Mice were inoculated intracerebrally with 0.03 ml of the virus dilution using five mice per dilution. Mice were observed for 13 days after injection. Titers based on death were calculated by the method of Reed and Muench and are expressed as the number of $LD_{50}$ per 0.03 ml of the 10% suspension of brain tissue.

**Cell culture.** Baby hamster kidney cells (BHK-21, clone 13; 16) were obtained from Flow Laboratories, Rockville, Md. Monolayer cultures of cells were grown in Eagle basal medium (BME; Grand Island Biological Company, Grand Island, N.Y.) containing Hanks' salts, 0.028% sodium bicarbonate, 10% fetal calf serum, 10% TPB, 0.29 μg of glutamine per ml, 100 units of penicillin per ml, and 200 μg of streptomycin per ml. Maintenance medium consisted of BME plus Earle's salts, 0.22% sodium bicarbonate, and 2% fetal calf serum, with glutamine and antibiotics as above. Only cultures which were free of

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*Mycoplasma* were used. After infection, cultures were incubated at 35 C.

**Freeze-thaw technique.** Cell-associated virus was extracted from cells by a double freeze-thaw procedure. At appropriate intervals after infection of the cells, the medium was decanted and the cells were washed three times with TPB (2 ml/tube for each washing). Unless otherwise indicated, 1.5 ml of TPB was added to each tube. Tubes were placed in a −20 C freezer so that the cells were covered with the fluid. After 20 min, at which time the fluid was frozen, the tubes were thawed in a 37 C water bath. This procedure was repeated so that the cells underwent two freeze-thaw cycles.

**Alkaline extraction technique.** Tubes of cells were washed three times with TPB (2 ml/tube for each washing). After 1.5 ml of *p*H 9.0 borate buffer (3) was added per tube, the cells were incubated on roller drums at 35 C for 30 min. The fluids representing pooled contents of five tubes are referred to as the alkaline extraction samples.

**Complement fixation test.** The small volume complement-fixation technique recommended by the Laboratory Branch of NCDC was used (1). For this method, five 50% hemolytic units of complement were added per tube. The end point of titrations was the highest dilution showing 30% or less hemolysis. The LCM immune serum, LCM CF reference antigen, and negative tissue control antigen were obtained from NCDC.

TABLE 1. *Comparison of methods for extracting the WE strain of lymphocytic choriomeningitis virus and complement-fixing antigen from BHK-21 cells*

| Days after infection | Culture fluid | | Alkaline extraction of cells[c] | | Freeze-thaw extraction of cells | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | TPB[d] | | Borate[e] | |
| | CF Ag[a] | Virus[b] | CF Ag | Virus | CF Ag | Virus | CF Ag | Virus |
| 1 | 0 | 2.33 | 0 | 1.50 | 0 | 2.67 | 0 | 2.50 |
| 2 | 0 | 4.77 | 0 | 3.90 | 1:4 | 5.00 | 1:2 | 3.50 |
| 3 | 1:2 | 6.50 | 1:2 | 4.25 | 1:16 | 6.75 | 1:8 | 5.50 |
| 4 | 1:4 | 6.23 | 1:4 | 5.50 | 1:16 | 6.50 | 1:8 | 5.00 |
| 5 | 1:8 | 5.33 | 1:8 | 5.00 | 1:16 | 5.50 | 1:8 | 4.50 |
| 6 | 1:4 | 4.83 | 1:4 | 3.83 | 1:8 | 4.50 | 1:4 | 4.68 |
| Control, day 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Control, day 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

[a] Complement-fixing antigen titer determined by the LBCF test.

[b] Log 10 mouse $LD_{50}$ per 0.03 ml.

[c] Intact cells extracted with *p*H 9.0 borate buffer.

[d] Cells extracted by freezing in tryptose phosphate broth.

[e] Cells extracted by freezing in *p*H 9.0 borate buffer.

TABLE 2. *Effect of inoculum dose on production of lymphocytic choriomeningitis virus complement-fixing antigen in BHK-21 cells infected for 5 days*

| Virus strain | Inoculum dose[a] | Titer of CF antigen produced[b] |
|---|---|---|
| WE | 4.50 | 1:16 |
| | 3.50 | 1:16 |
| | 2.50 | 1:16 |
| | 1.50 | 1:16 |
| | 0.50 | 1:16 |
| | 0.05 | 1:4 |
| | 0.005 | <1:2 |
| Armstrong | 3.68 | 1:16 |
| | 2.68 | 1:16 |
| | 1.68 | 1:4 |
| | 0.68 | <1:2 |

[a] $Log_{10}$ mouse $LD_{50}$ added per tube.

[b] Complement-fixing antigen extracted from cells with TPB by the freeze-thaw method and titered by the LBCF test.

## RESULTS

**Virus and CF antigen production.** BHK-21 cells grown in roller tubes were infected with the WE strain of virus at a multiplicity of 0.1 $LD_{50}$ per cell. Infected cultures were incubated at 35 C and the medium was replaced daily. On the 6 succeeding days the culture fluid, alkaline extraction fluid, and freeze-thaw samples from infected cells were tested for infectious virus and CF antigen (Table 1). Virus was demonstrable both intra- and extracellularly 1 day after infection. However, CF antigen appeared intracellularly on the 2nd day and extracellularly on the 3rd day. The highest titer of antigen was 1:16 which was recovered by extracting cells with TPB by the freeze-thaw technique. Virus titers and CF antigen titers started decreasing in all samples on the 6th day.

**Effect of inoculum dose on antigen production.** Stock virus which had been titered in mice was diluted (Table 2) and used to infect BHK-21 cells for the production of CF antigen. Five roller tubes of cells were inoculated with 0.03 ml of each dilution of virus. Medium changes were made after 2 and 4 days. On the 5th day after infection, the medium was replaced with 1.5 ml of TPB and the cells were frozen and thawed twice as described above. The contents of the five tubes were pooled and assayed for CF antigen. The maximal CF antigen titer was 1:16 even with the most concentrated virus inoculum. The minimal inoculum of the WE strain which resulted in a 1:16 titer of CF antigen was $10^{0.50}$ $LD_{50}$, whereas the minimal inoculum of the Armstrong strain producing a comparable titer was $10^{2.68}$

$LD_{50}$. This difference in ability of the two strains to induce synthesis of CF antigen is reflected in the data below.

**Virus assay by CF antigen production.** A test method for quantitating LCM virus based on the production of CF antigen as an index of infection was evaluated in BHK-21 cell cultures. Five tubes of cells and five mice were inoculated with each dilution of virus. After incubating the culture for 5 days, a complement fixation test was run on the freeze-thaw extract of each tube to determine if antigen was present. The cell culture titer of the WE strain was 79% lower than the titer determined in mice, whereas the cell culture titer of the Armstrong strain was 97% lower than its mouse titer (Table 3).

**Attempts to separate CF antigen from virus.** Table 4 contains the results of centrifugation of a sample containing both virus and CF antigen. The sample obtained by freeze-thawing BHK-21 cells infected with the WE strain of virus was centrifuged at 110,000 × *g* for 3 hr, after which the supernatant fluid was decanted and recentrifuged. The results show that almost all the virus could be sedimented, whereas a large part of the CF antigen remained in the supernatant fluid. However, it was not possible to separate all the CF antigen from the infectious virus by this method.

TABLE 3. *Comparative infectivity titration of lymphocytic choriomeningitis virus by intracerebral inoculation of mice and by infection of BHK-21 cells as determined by appearance of complement-fixing antigen*

| Virus strain | Mouse titer | Cell culture titer |
|---|---|---|
| WE | 5.50[a] | 4.83[b] |
| Armstrong | 4.68 | 3.16 |

[a] $Log_{10}$ mouse $LD_{50}$ per 0.03 ml.
[b] $Log_{10}$ cell culture $ID_{50}$ per 0.03 ml. Five tubes per dilution were tested separately for antigen by the LBCF test.

TABLE 4. *Differential centrifugation of lymphocytic choriomeningitis virus and complement fixing-antigen from BHK-21 cells*

| Sample | Virus titer | Complement-fixing antigen titer |
|---|---|---|
| Original | 6.33[a] | 1:16 |
| Sediment # 1[b] | 6.67 | 1:8 |
| Supernate # 1 | 1.20 | 1:16 |
| Sediment # 2[c] | 1.62 | 1:4 |
| Supernate # 2 | <0.16[d] | 1:8 |

[a] $Log_{10}$ mouse $LD_{50}$ per 0.03 ml.
[b] Centrifugation at 110,000 × *g* for 3 hr.
[c] Supernatant fluid # 1 recentrifuged at 110,000 × *g* for 3 hr.
[d] One of five mice inoculated with undiluted fluid died.

TABLE 5. *Comparison of antibody titers to lymphocytic choriomeningitis virus using the reference guinea pig spleen complement-fixing antigen and the BHK-21 cell complement-fixing antigen*

| Serum sample | Complement fixing antigen | |
|---|---|---|
| | Guinea pig spleen | BHK-21 cells |
| NCDC guinea pig antiserum | 1:32[a] | 1:64[a] |
| Guinea pig, pre-immunization | <1:2 | <1:2 |
| Guinea pig, first bleeding[b] | 1:16 | 1:32 |
| Guinea pig, second bleeding[c] | 1:8 | 1:32 |
| Guinea pig, third bleeding[d] | 1:2 | 1:4 |
| Pooled mouse serum | 1:2 | 1:2 |
| Immune mouse serum | 1:32 | 1:32 |
| Human pre-exposure | <1:2 | <1:2 |
| Human immune serum | 1:4 | 1:8 |
| Seven additional human sera | <1:2 | <1:2 |

[a] Antibody titer determined by the LBCF test.
[b] Two months after immunization was begun.
[c] Eight months after immunization was begun.
[d] Eleven months after immunization was begun.

**Heat lability of CF antigen.** A reduction in the cell culture CF antigen titer from 1:16 to 1:2 was observed after incubation at 56 C for 1 hr. No further decrease in titer was noted after an additional hour of heating. However, autoclaving the antigen at 121 C for 15 min destroyed all detectable CF antigen.

**Comparison of cell-culture antigen with guinea pig spleen reference antigen.** The reactivity of the NCDC reference CF antigen and the BHK-21 cell CF antigen were compared using the reference antiserum to LCM virus as well as several sera collected in this laboratory. Doubling dilutions of sera were mixed with 1:8 dilutions of both the reference CF antigen prepared from spleens of infected guinea pigs and the antigen extracted from infected BHK-21 cells (Table 5). This dilution of both antigens was found to be optimal by previous titration. In several instances, titers determined for reactive sera using the cell-culture antigen were higher than comparable titers using the reference antigen. Also, sera which were nonreactive with one antigen were nonreactive with both antigens. In addition, the cell-culture antigen was shown to be capable of fixing complement with immune guinea pig, mouse, and human sera.

## DISCUSSION

These studies have shown that LCM CF antigen can be obtained from both the fluid and cellular phases of infected BHK-21 cells. Although the fluid-phase antigen is easier to obtain, the cellular antigen titers are sufficiently higher to justify their use.

In contrast to reports with rubella virus in BHK-21 cells (4, 10), the complement-fixing antigen of LCM virus was not recovered optimally by alkaline extraction. The alkaline extraction procedure was also unsatisfactory for recovery of CF antigen from cell monolayers infected with cytomegalovirus (9). With the system employed in our study, the highest titers of CF antigen were found by disruption of the cells by freezing in TPB.

CF antigen production was not found to parallel virus production during the early part of the infection. Although relatively high titers of virus were produced during the first 2 days, little CF antigen appeared during this period. This may be explained by a low sensitivity of the complement fixation test for detection of antigen. However, it seems more likely that a large part of the CF antigen is separate from the virus. When titers of extracellular and intracellular virus were comparable, the titer of CF antigen was higher in the cellular phase than in the culture fluids. The separateness of the CF antigen is further indicated since the antigen preparation could be nearly freed of infectious virus by centrifugation. These results are similar to those obtained with the LCM virus CF antigen from infected guinea pig spleen (12).

Work with rubella virus has revealed two CF antigens which were antigenically identical but were separable by centrifugation (11). The larger antigen was associated with the virion, whereas the smaller antigen was not. Similarly, on high-speed centrifugation of LCM virus and CF antigen preparations in this study, part of the CF antigen activity was sedimented with the virus. Thus, it seems that the LCM virus CF antigen system may be composed of a large and small particle component similar to the rubella system.

The CF antigen prepared from BHK-21 cell cultures seems to be fully as satisfactory as the reference antigen prepared from infected guinea pig spleens. Of the lots of antigen prepared to date, few have had any detectable anticomplementary activity. No attempt was made to concentrate antigen, and the maximum titer of 1:16 may reflect this fact. However, the ease and relative safety of antigen preparation by the cell-culture method makes this system the more desirable. Furthermore, the cell-culture CF antigen was shown to be at least as sensitive and specific as the reference antigen in the fixation of complement with immune sera. Thus, the cell-culture antigen was shown to be applicable to diagnostic work since it reacted with CF antibody from several animal species.

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