adsorption and virus multiplication in liquid medium, while chondroitin sulfate was completely ineffective as an inhibitor. No difference was noted in the behavior of the 2 variants with respect to their sensitivity toward the 4 sulfated polysaccharides.

1. Takemoto, K. K., Liebhaber, H., *Virology,* 1962, v17, 499.
2. Nahmias, A. J., Kibrick, S., *Bact. Proc.,* 1963, 145.
3. Takemoto, K. K., Liebhaber, H., *Virology,* 1961, v14, 456.
4. Kaplan, A. S., *ibid.,* 1957, v4, 435.
5. Roizman, B., *Cold Spring Harbor Symposia on Quantitative Biology,* 1962, 327.
6. Liebhaber, H., Takemoto, K. K., *Virology,* 1961, v14, 502.
7. Allison, A. C., Valentine, R. C., *Biochim. Biophys. Acta,* 1960, v40, 400.
8. Vaheri, A., Cantell, K., *Virology,* 1963, v21, 661.
9. Bengtsson, S., Dinter, Z., PROC. SOC. EXP. BIOL. AND MED., 1963, v113, 1019.

Received February 12, 1964. P.S.E.B.M., 1964, v116.

A Complement-Fixing Antigen for Varicella-Zoster Derived from Infected Cultures of Human Fetal Diploid Cells.* (29184)

NATHALIE J. SCHMIDT, EDWIN H. LENNETTE, CAROL W. SHON AND TAK T. SHINOMOTO

Viral and Rickettsial Disease Laboratory, California State Department of Public Health, Berkeley

General application of the complement fixation technic for serologic diagnosis of infections caused by the varicella-zoster (V-Z) virus has been limited by difficulties in obtaining sufficiently potent antigens free from anticomplementary activity.

While fairly potent antigens can be prepared from vesicular fluids(1), the source is too undependable to meet the requirements of the diagnostic laboratory. In early studies on the V-Z virus, Weller and Witton(2) showed that infected tissue cultures contained specific complement-fixing antigen, but concentration of the culture fluids by ultrafiltration was necessary to obtain antigens with sufficient reactivity. Concentrates prepared in this manner were anticomplementary, and while heating abolished this activity, it also reduced the antigenicity. Caunt *et al*(3) prepared complement-fixing antigens to the V-Z virus in cultures of human amnion cells; fluids from infected cultures were concentrated by drying from the frozen state, and the resulting antigens were free from anticomplementary activity.

The procedures described to date for preparation of V-Z complement-fixing antigens from infected cell cultures are cumbersome, involving replacement of the culture medium at relatively frequent intervals after infection as well as concentration of the antigenic material from large volumes of culture fluid. More importantly, despite such concentration the resulting antigens have been of low potency. This report describes a simple method for preparation of relatively high-titered antigens from the cellular phase of infected cultures of human fetal diploid skin and muscle cells.

Materials and methods. Human fetal diploid cell strains. Initiation of cell strains: Cultures of human fetal diploid skin and muscle (HFDSM) cells were initiated from fetuses 2 to 5 months of age. Tissue from the limbs and torso of the fetus was minced and washed in 3 changes of Hanks' balanced salt solution (BSS). Cells were dispersed by overnight trypsinization at 4°C in 200 ml of a 0.25% trypsin solution, pH 7.5. The dispersed cells were centrifuged at 600 rpm for 20 minutes and the trypsin solution was removed. The cells were then resuspended in 40 ml of Hanks' BBS, transferred to a 50 ml graduated, conical contrifuge tube and centrifuged at 600 rpm for 20 minutes. The volume of the cell pack was noted and, after

* Supported by grant from Nat. Inst. of Allergy and Infect. Dis., U.S.P.H.S.

removal of the wash fluid, the cells were resuspended in growth medium to give a 1:100 dilution of the cell pack. Growth medium consisted of 10% calf serum (inactivated at 56°C for 30 minutes) and 90 % Eagle's minimum essential medium prepared in Earle's BSS. The cell suspension was planted in 8 oz. prescription bottles in volumes of 10 ml and the cultures were incubated at 36°C for 2 days, at which time an additional 5 ml of growth medium was added to each bottle. When the cultures contained confluent monolayers of cells (usually 4-5 days), the cells were subpassaged.

Continuous cultivation of HFDSM cell strains: The growth medium was removed from cultures containing confluent monolayers of cellular growth, and 10 ml of a 0.25% trypsin solution were added to each bottle culture. The trypsin solution was spread over the monolayer and the culture was held at room temperature for 30 seconds. The trypsin was then removed from the culture, taking care not to disrupt the cell sheet, and the culture was held at room temperature for 2-3 minutes, until microscopic observation revealed rounding and separation of the cells. The cells from each bottle were dispersed in 10 ml of growth medium, and were finally diluted in 4 times the original volume of growth medium (a 4-1 split). Ten ml volumes of the suspension were planted in 8 oz. prescription bottles, and after 3 days' incubation at 36°C, the cultures were fed an additional 5 ml of growth medium. The cultures were employed for viral propagation or subpassage 4-5 days after initiation. The cell strains employed in these studies were at the 2nd to the 12th passage level.

Virus strain. The Batson strain of the V-Z virus, isolated in this laboratory, was employed for these investigations. This strain was isolated in human fetal kidney cell cultures from vesicular fluid from a case of zoster, and was utilized at the 3rd to 21st passage level in HFDSM cells (Table I).

Complement fixation (CF) technic. All CF tests were conducted by the microtiter procedure(4), employing our standard complement fixation technic(5,6). The potency of the antigens was assayed in block titrations against a pool of convalescent-phase human sera from known cases of varicella or zoster.

Results. Demonstration of specific complement-fixing antigen in HFDSM cell cultures infected with the Batson strain of V-Z virus. In selecting a cell culture system for preparation of CF antigen, consideration was given to both the availability and susceptibility of the cells. Human diploid cell strains are more readily available than primary cell cultures, as they can be carried in serial passage and stored in the frozen state. Diploid cell strains of human fetal skin and muscle tissue were chosen for these studies as they were found to be highly susceptible to the cytopathic effects of the V-Z virus. Also, it was considered desirable, from the standpoint of eliminating time-consuming manipulations and reducing risk of contamination, to avoid frequent medium changes after infection of the cultures. In preliminary investigations it was found that HFDSM cell cultures could be maintained without a fluid change for at least 2 weeks on a medium consisting of 2% fetal bovine serum (inactivated at 56°C for 30 minutes) and 98% Leibovitz Medium No. 15 (L-15)(7). This maintenance medium was employed for all of the studies described herein.

The following procedure was employed throughout these investigations for cultivation of V-Z virus and production of CF antigens. Cultures of HFDSM cells in 8 oz. prescription bottles were infected with virus when they contained confluent monolayers of cells (usually 4-5 days after initiation). The growth medium was removed, the cell sheets were washed 3 times with 10 ml of Hanks' BSS, and 10 ml of maintenance medium was added to each culture. The cultures were then inoculated with 1 ml of cells and fluids freshly harvested from V-Z virus-infected cell cultures showing 3-plus or 4-plus degeneration, and incubated at 36°C. Scattered foci of specific degeneration were seen on the 3rd day, and complete cellular degeneration of the culture was present by the 10th to the 12th day after infection, at which time the infected cells and fluids were harvested. The maintenance medium was not changed during the entire period of incubation, thus the har-

vest contained all of the antigen produced in the cultures.

The pooled cells and fluids from infected cultures were sonicated for 3-6 minutes at 10 kc/second in a Raytheon oscillator, and the materials were examined for specific complement-fixing activity. Control antigens were prepared in the same manner from uninoculated cultures incubated for 10 to 12 days.

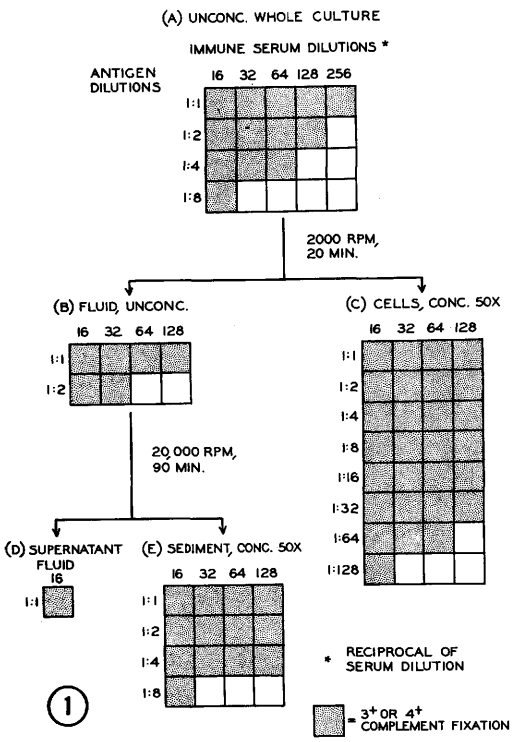
Unconcentrated V-Z antigens prepared as described above had specific titers of 1:2 or 1:4, and occasionally 1:8, on the basis of block titrations with human convalescent-phase serum. While the antigens were not strongly anticomplementary, some of the lots were slightly so at dilutions containing 2 units of antigen, and thus were unsuitable for diagnostic purposes. Consequently, ensuing studies were aimed at concentrating the antigens and freeing them from anticomplementary activity.

Distribution of CF antigen in infected cell cultures. Weller and Witton(2) prepared CF antigens principally by ultrafiltration of the fluid component of primary cultures of human embryonic skin and muscle cells infected with V-Z virus, but reported that on one occasion a satisfactory antigen was obtained from the cellular component. Caunt *et al*(3) prepared CF antigens exclusively from the fluid phase of infected human amnion cell cultures; they were unable to obtain satisfactory antigens from infected cells. The experiments described below, however, show that most of the V-Z complement-fixing antigen produced in HFDSM cultures is cell-associated at the time of harvest of the cultures.

Infected cultures were harvested when the cells showed 4-plus degeneration. A portion of the whole culture was withdrawn and sonicated; this represented the unconcentrated, whole antigen (A). The remainder of the culture was centrifuged at 2000 rpm for 20 minutes, after which the fluid phase (B) was removed, and the cells were resuspended in 1/50th the original volume of Hanks' BSS and sonicated; this constituted the cellular antigen, concentrated 50-fold (C). A portion of the unconcentrated supernatant fluid (B) was set aside and the remainder was centrifuged at 20,000 rpm for 90 minutes in a Spinco machine. A portion of the supernatant fluid from this centrifugation (D) was saved for examination, and the pellet was resuspended in 1/50th the original volume of Hanks' BSS; this represented the fluid phase, concentrated 50-fold (E). All of these fractions were assayed in block titrations for specific CF activity. Results of a representative experiment are shown in Fig. 1. Most of the antigen present in the whole culture was recovered in the cellular component; little was present in the fluid phase, and virtually all of the antigen in the fluid phase was sedimented by centrifugation at 20,000 rpm for 90 minutes. Thus, there was no good evidence of the presence of a "soluble" antigen, separable from the virus, in the fluid component of the cultures.

Preparation of CF antigen from the cellular phase of infected HFDSM cultures. The experiments described above demonstrated the importance of utilizing infected cells from HFDSM cultures to obtain V-Z complement-fixing antigens of maximum potency. Initially, antigens were prepared from whole cultures which were sonicated and then centrifuged at 20,000 rpm for 90 minutes. The pellets resuspended in 1/50th the original volume of Hanks' BSS were used as antigens. Antigens prepared in this manner had fairly high titers (1:32 or 1:64) and usually were not anticomplementary, but their preparation involved handling large volumes of material for sonic oscillation and also for high-speed centrifugation. As comparatively little antigen was found in the fluid phase of infected cultures, it was considered that a simpler method might be employed for preparation of antigens, using only the cellular phase of infected cultures. The following method was finally devised for the routine preparation of V-Z complement-fixing antigens.

Infected HFDSM cell cultures are harvested when the cells show 4-plus degeneration, and the whole culture is centrifuged at 2000 rpm for 20 minutes. After removal of the supernatant fluid, the cells are resuspended in 1/50th the original volume of Hanks' BSS and placed in a lusteroid tube. The tube, sealed with adhesive tape, is placed in an oscillator cup, water is added to fill the



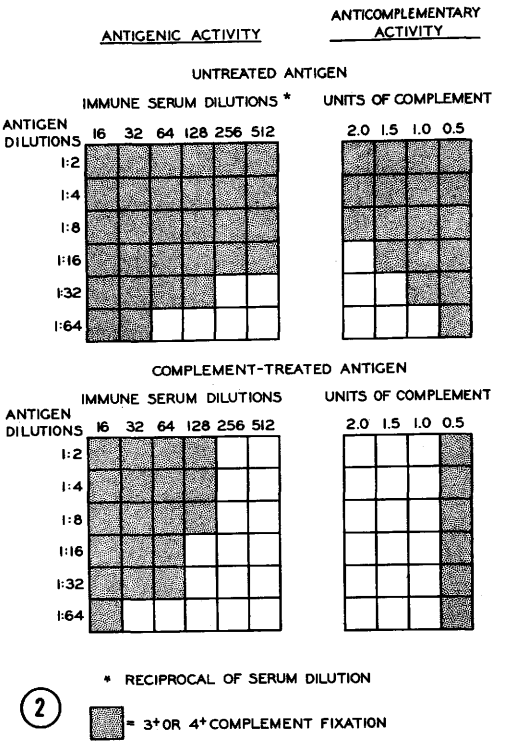


FIG. 1. Distribution of complement-fixing antigen in human fetal diploid skin and muscle cell culture infected with varicella-zoster virus.

FIG. 2. Elimination of anticomplementary activity of varicella-zoster antigen by treatment with inactivated guinea pig complement.

cup almost to the top of the tube, and the suspension is sonicated for 6 minutes at 10 kc/second. The disrupted cell suspension constitutes the antigen. Uninfected control antigens are prepared in the same manner from uninoculated cultures. Antigens are stored at —20°C prior to use, and if particulate material appears in the antigen upon thawing, it is resuspended rather than sedimented and discarded.

Table I gives the titers of several lots of V-Z antigen prepared in the above manner from the Batson strain of virus at several different passage levels. The titers of the unconcentrated, whole infected cultures (sonically disintegrated) ranged from 1:2 to 1:8, and titers of the cellular concentrates from these cultures were 1:32 and 1:64. The passage level of the virus had no apparent effect on the potency of the complement-fixing antigens.

Elimination of anticomplementary activity from antigens. A single lot of antigen produced from the cellular phase of infected HFDSM cultures was anticomplementary; however this activity was abolished by treatment of the antigen with inactivated guinea pig complement as suggested by von Ziepel

TABLE I. Titers of Some V-Z Complement-Fixing Antigens Derived from the Cellular Phase of Infected HFDSM Cultures.

Antigen lot No.	Passage level of virus*	CF titer of antigen†	
		Uncone whole culture‡	Cellular phase (50×)‡
9562	4	1:2	1:32
9589	5	1:8	1:64
9670	14	1:4	1:64
9687	15	1:4	1:64
9702	15	1:4	1:64
9770	20	1:4	1:32
9774	22	1:2	1:32

* First passage of virus in primary human fetal kidney cells, subsequent passages in HFDSM cells.

† Titer of 1 unit of antigen, based on block titration. Unit = highest dilution of antigen giving 3-plus or 4-plus fixation with highest dilution of immune serum.

‡ Sonically disintegrated.

(8). Commercial guinea pig complement was reconstituted and heated at 56°C for 30 minutes. The heated complement was added to the antigen to give a final concentration of 5%, the treated antigen was held at room temperature for 30 minutes, then stored at —20°C. Fig. 2 shows the antigenic and anticomplementary activity of this antigen before and after treatment with complement. For use in serologic diagnosis, a dilution of antigen containing 2 antigenic units should have no anticomplementary activity with 2.0, 1.5 or 1.0 units of complement. The untreated antigen is seen to be anticomplementary at dilutions up to and including 1:32. However, treatment of the antigen with complement abolished its anticomplementary activity without reducing its specific antigenic activity. (The higher serum titers obtained with the untreated antigen are seen to be attributable to the anticomplementary activity of the antigen.)

Discussion. There are several advantages in preparing V-Z complement-fixing antigen by the procedure outlined. The use of diploid cell strains provides a more readily available source of host tissue than does the use of primary or secondary cultures of human cells. The HFDSM cell strains employed in these studies require a relatively short period of incubation (4-5 days) before they produce well-developed monolayers suitable for viral cultivation. In contrast, cultures of primary human fetal skin and muscle tissue or human amnion cells require 7 to 20 days' incubation before they can be utilized for antigen production(2,3). HFDSM cell strains would appear to be as susceptible as primary fetal skin and muscle cultures to the cytopathic effect of the V-Z virus, and they have been found to be considerably more susceptible than primary or secondary human amnion cell cultures. Another advantage to the use of this cell system lies in the fact that the cultures can be maintained on 2% fetal bovine serum and 98% L-15 medium for the entire incubation period after infection without a fluid change, and the resulting antigens are not markedly anticomplementary.

Since most of the V-Z antigen remains cell-associated in HFDSM cultures, it can be easily concentrated by low-speed centrifugation, eliminating the cumbersomeness of processing large volumes of material by high-speed centrifugation or other concentration procedures. Antigens prepared by 50-fold concentration of the cellular phase of infected HFDSM cultures showed higher titers than those reported by other investigators(2,3) for antigens prepared by a similar degree of concentration of pooled fluids harvested at intervals from infected cultures. There would thus appear to be no advantage in preparation of antigens from multiple harvests of fluids collected from infected cell cultures over a long period of incubation.

The complement-fixing antigen of the V-Z virus appears to resemble that of herpes simplex virus(9) in that a large amount of the antigen remains associated with the host cells. These 2 viruses have been found to be alike morphologically(10), and ostensibly may be assembled and released from host cells in similar fashion.

Summary. A procedure is described for preparation of complement-fixing antigens for the varicella-zoster (V-Z) virus in human fetal diploid skin and muscle cell strains. These cells are highly susceptible to the cytopathic effect of the V-Z virus, and can be maintained without a fluid change for the entire period required by the virus to produce complete cellular degeneration. Most of the specific antigen elaborated in the cultures was found to be cell-associated, and relatively high-titered antigens (1:32 and 1:64) were prepared by 50-fold concentration of the cellular phase of infected cultures. Antigens were usually free from anticomplementary activity, but when they were not, the activity could be abolished by treatment with inactivated guinea pig complement.

The authors are indebted to Miss Ann T. Bradley for conducting some of the serologic tests.

1. Taylor-Robinson, D., Downie, A. W., *Brit. J. Exp. Pathol.*, 1959, v40, 398.

2. Weller, T. H., Witton, H. M., *J. Exp. Med.*, 1958, v108, 869.

3. Caunt, A. E., Rondle, C. J. M., Downie, A. W., *J. Hyg. Camb.*, 1961, v59, 249.

4. Sever, J. L., *J. Immunol.*, 1962, v88, 320.

5. Schmidt, N. J., Lennette, E. H., *J. Exp . Med.*,

1955, v102, 133.
6. Schmidt, N. J., Lennette, E. H., Doleman, J. H., Hagens, S. J., *Am. J. Hyg.*, 1957, v66, 1.
7. Leibovitz, A., *ibid.*, 1963, v78, 173.
8. von Zeipel, G., *Arch. ges. Virusforsch.*, 1958, v8, 246.
9. Schmidt, N. J., Lennette, E. H., Shon, C. W., *Am. J. Hyg.*, 1960, v72, 59.
10. Almeida, J. D., Howatson, A. F., Williams, M. G., *Virology*, 1962, v16, 353.

Received February 13, 1964. P.S.E.B.M., 1964, v116.

Induction of Neoplasms in Salivary Gland Rudiments Recovered from Frozen Storage. (29185)

C. J. DAWE, M. S. SLATICK, W. D. MORGAN AND E. HENSLEY
Laboratory of Pathology, National Cancer Institute, Bethesda, Md.

Preservation of semen, cell cultures, and neoplasms in the frozen state is now widely practiced by application of well standardized procedures(1,2,3,4). The frozen preservation and storage of more highly organized biologic materials such as tissues, organs, or whole organisms has had more limited application, partly because of technical difficulties(1).

We recently found it possible to freeze and store the embryonic rudiments of mouse submandibular salivary glands by the same technique that is used to freeze and store cell cultures. It has, furthermore, been shown that such frozen rudiments are capable of responding qualitatively to infection with polyoma virus (PV) in the same way that fresh rudiments do; *i.e.*, neoplasms of charactertistic morphology and biologic behavior develop in the rudiments when they are thawed, infected with PV, and grafted to isologous newborn mice.

Procedures. Submandibular salivary gland rudiments were dissected from 13- and 14-day embryo mice of strain C3H/Bi. They were kept for 2 hours at room temperature in a medium of adult human serum (30%), Morgan, Morton, and Parker's mixture 199 (65%), glycerol (5%), penicillin in concentration of 100 U/ml, and phenol red in final concentration of 0.001%.

Dishes containing rudiments in the medium were kept in an atmosphere of 95% air and 5% CO_2 to maintain a pH 7.0 to 7.4. After 2 hours, 6 to 12 rudiments in 1.0 ml of medium were transferred by capillary pipette into individual ampoules. The ampoules were sealed and placed in a refrigerator and cooled to 3 to 5°C. They were then slowly frozen under alcohol at a temperature decline rate of one degree per minute or less by placing in a vermiculite-insulated double-walled container immersed in 95% ethyl alcohol and CO_2 ice, pre-cooled to the starting temperature of 4°C. When a temperature below —20°C was achieved, the frozen ampoules were rapidly transferred to canes and placed immediately in a liquid nitrogen refrigerator, above the liquid level, at a temperature of approximately —150°C.

At intervals varying upwards from one hour after placing in the N_2 refrigerator, ampoules were withdrawn and rapidly thawed by agitating in a running water bath held at 38°C.

Rudiments were removed from the glycerol medium, washed once in 1.0 ml of similar medium lacking glycerol, and then transferred to a dish of virus suspension or to an equal volume of virus-free control medium. They were held then at room temperature in a 95% air-5% CO_2 atmosphere for 2 hours, and their condition was evaluated by: (1) histologic examination of serial sections; (2) observation of development in culture; (3) transfer (with and without PV) to subcutaneous tissue of new-born C3H/Bi mice.

Results. Histologically the rudiments showed little alteration as a result of the freezing and thawing procedure. The basic form and the relationships between epithelium and the capsular mesenchyme were retained, but in some preparations it was noted