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PREPARATION AND CHARACTERIZATION OF A SOLUBLE PRECIPITATING ANTIGEN FROM AFRICAN HORSESICKNESS VIRUS PROPAGATED IN CELL CULTURES (*)

A. HAZRATI AND H. MIRCHAMSY

Since the introduction of cell cultures for propagation of a variety of viral agents, these new host systems have extensively been used, as adequately potent sources, for the production of different viral antigens, including the precipitating antigens (1–3, 6, 7, 9, 11–13, 15).

The first attempt to produce African horsesickness (AHS) precipitating antigen in cell cultures was made by Hazrati, Mastan, and Bahrami (5). They demonstrated that ultrasonically disrupted cells of monkey kidney cell culture, as well as suckling mouse brain infected with AHS virus, when diffused towards homotypic and heterotypic specific antisera, reacted specifically and produced two distinct precipitin lines (5).

In the present communication the production of AHS precipitating antigen in monkey kidney cell cultures, and the results of studies undertaken to characterize the antigen are presented.

Materials and Methods

Horse sickness virus. The 7th passage of the neurotropic attenuated strain S2, type 9 of AHS virus, was used in the experiment. The virus was isolated during the 1959 epizootic of African horsesickness in Iran, and was attenuated through more than 100 intracerbral passages in adult mice (4).

Cell culture. Monkey kidney stable (MS) cells were used as the host system. The origin of the cells, their susceptibility to AHS virus and the technique of their cultivation have been described in a previous paper (10).

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Release and collection of precipitating antigen from infected cells. The virus-containing cells, harvested at desired interval periods postinfection, were suspended in a sufficient amount of the culture fluid as described in the text. The cell suspension, in a glass container held in an ice-bath, was then subjected to 4–min ultrasonication at 20 kC/sec in a Branson Sonifier Cell Disruptor tuned to 2.5 A. The preparation was partially clarified by centrifugation at 3,000 rpm for 30 min and was kept at –70°C until use.

Precipitation and concentration of the antigen in culture fluids. The content of precipitating antigen in culture medium of the infected cultures was concentrated by precipitation with saturated ammonium sulfate, pH 7.6, at 4°C.

Ammonium sulfate was added in equal volume to the infected tissue culture medium, drop by drop, while the fluid was being stirred by a magnetstirrer. The fluid pH was adjusted to 7.0– 7.2 and the mixture was kept at 4°C. overnight. Then it was centrifuged, and the precipitate which contained almost all the original infectivity and the precipitating activity was collected in an appropriate amount of Earle's balanced salt solution containing 0.005% yeast extract, 0.5% lactalbumin hydrolysate, 0.0015% phenol red, and antibiotics (YLE). The final product was freed from excess salts by dialysis against several changes of PBS at 4°C overnight. The techniquee provided the precipitation of the antigen and viral particles to the concentration of 100– to 300–fold.

Precipitating antibody. AHS hyperimmune rabbit serum was used as precipitating antibody. Rabbits were hyperimmunized using mouse-adapted AHS virus, strain S2, propagated in suckling mouse brain as antigen. The antiserum, however, was prepared according to the method previously described, and was kept without any preservative at –20°C until used (5).

Agar double-diffusion precipitation test. A medium consisting of 1.25–1.5% Difco purified agar, 1% sodium chloride in double-distilled water with a pH value of approximately 7.2, was found optimal in testing the AHS precipitating antigen and antibody in agar precipitation test. This medium with 0.5% phenol as preservative was used in these studies. The procedure of the test, examining, reading and recording of the precipitin reactions have been reported before (5).

Antigen titration. 0.2 log 10-fold dilutions of the antigen were prepared in veronal buffer solution. Then each antigen dilution was allowed to react against an optimal dilution of precipitating antiserum, in agar diffusion medium, using two separate antigen-antibody systems for each dilution. The highest dilution giving a visible precipitin line in at least one antigen antibody system was taken as the titer of the antigen. In titrating a treated antigen to evaluate the effect of the treatment more precisely, the location and density of the precipitin lines in each antigen dilution as compared to those in the corresponding dilution of nontreated antigen were also taken into consideration.

Virus infectivity titration. Serial 10-fold dilutions of the antigen to be titrated were prepared in YLE + 2% calf serum. The tubes containing virus dilutions were kept in an ice-water bath during the titration procedure. MS cell culture tubes, prepared as described previously (10), were used as host system for virus titration. Four culture tubes were employed per dilution. The growth medium of the culture tubes were discarded and then each was inoculated with 0.1 ml of the virus dilution. The virus was allowed to adsorb to the cells at 37°C for 45 min before 1.5 ml of YLE + 2% calf serum was added to each tube. The cultures were reincubated for 7 days and observed for presence or absence of cytopathic changes on the 4th and the last days. The infective virus titer was calculated by the Reed and Muench method (14).

High speed centrifugation. To study the effect of ultracentrifugation on the antigen, a standard AHS precipitating antigen was centrifuged for 60 min at 10,000 rpm in a Sorvall RC2-B automatic superspeed refrigerated centrifuge. The supernatant was carefully collected, and used as a semiclarified antigen in this experiment. A sample of the antigen was retained for testing and the remaining was subjected to 60-min centrifugation at 40,000 rpm in a Spinco model L50 ultracentrifuge.

The upper half of the supernatant fluid was carefully removed and the remaining supernatant was discarded. The sediment was reconstituted in YLE to one-fifth of the original volume and the product was homogenized in a sterile tissue grinder.

The semiclarified original standard antigen, the supernatant fluid and the reconstituted sediment obtained after ultracentrifugation were tested for virus infectivity and antigen activity almost immediately after preparation, while being kept at 4° C before and during titration.

Effect of alternate freezing and thawing. The standard antigen was distributed in 1.2-ml amounts in 12 × 75 mm tubes with rubber stoppers, and were alternatively frozen (in a deep freezer at – 70°C) and thawed in cold tap-water.

The samples subjected to 3, 5, 7, 9, 13, 15, 17 and 21 alternate freezings and thawings, as well as the original antigen, were tested for infective virus and antigen content.

Effect of temperature. A series of screw-capped bottles containing 1.2ml of standard AHS precipitating antigen was prepared and stored at 4, –20 and –70°C. At various intervals up to 1 year, a bottle stored at a different temperature was removed and tested.

The sensitivity of the antigen to the effect of 37, 45, 55, 60, 65 and 75°C temperatures was also determined by holding the tubes containing 1.2 ml of the standard antigen, at the selected temperature, in a water-bath for various periods of time. The tubes, after being removed from the water-bath, were placed in an ice-bath until titrated.

Production of the Precipitating Antigen in Tissue Culture

The development of AHS precipitating antigen in tissue culture was studied by virus inoculation of MS cell cultures and determining the infectivity and precipitating antigen content, of the infected cells and culture fluids, at various intervals. Roux bottles of MS cell cultures were inoculated each with 10 ml of the 8th MS passage of strain S2, type 9 AHS virus, containing $10^{7\cdot0}$ TCID 50/ml of the virus. Adsorption was allowed to proceed at 37°C for120 min. The inoculum was discarded and the infected cell sheets were rinsed with 70–80 ml of YLE before 65 ml of the same medium was added in each bottle. The bottles were reincubated at 37°C and at various time intervals, 30 bottles were randomly taken and tested for infectivity and precipitating antigen presented in cells and in tissue culture fluid.

The pooled infectious tissue culture fluid was freed from cells and cell debris by centrifugation, before its virus and antigen being precipitated and concentrated according to the procedure already described. Approximately 6–9 ml concentrated cell-released antigen was obtained from the total fluid of 30 culture bottles. A sample of the culture fluid before concentration was kept as cell-released nonconcentrated antigen.

The cells, at interval times before the appearance of complete virus specific cell destruction, were detached and harvested by means of sterile glass beads and, thereafter, by vigorous shaking of the bottles. Cells, however, were sedimented by centrifugation at 4°C and then subjected to ultrasonic disruption to obtain the intracellular (cell-associated) antigen, as described. Approximately 10–14 ml of the antigen were prepared from total cells of each series of 30 bottles.

Clarified infectious tissue culture fluids, concentrated cell-released antigens and cell-associated antigens were tested for viral infectivity and precipitating antigen content.

It was found that although the cell-released precipitating antigen was undetectable in tissue culture fluid even at the time of complete cell destruction, its presence could be demonstrated when it was concentrated 100–200 times.

The newly formed infective virus appeared in the medium approximately 8 h after infection, and the titer rose fairly rapidly. The precipitating antigen, on the other hand, was not detected before 20 h postinfection, and it took a much longer time to reach its high titer.

The cell-associated antigen could be detected 12 h after infection and reached a much higher titer compared with the cell-released antigen.

Properties of the Antigen

AHS precipitating antigen could be separated from infected virus particles by ultracentrifugation. Following centrifugation of a standard antigen

at 40,000 rpm for 60 min, only about 0.03% of the infective virus remained in the supernatant fluid, which was proved to retain almost all of its precipitating antigen. On the other hand, the sediment resuspended in one-fifth of the original volume, although containing almost 5 times as much virus as the original antigen, showed no precipitin activity.

The precipitating antigen and the infective virus were no longer detected after heating the preparation at 75 and 65°C for 5 min. At temperatures of 60 and 55°C, while the virus was completely destroyed after 30 and 60 min, the antigen was still demonstrable even after 45 and 120 min, respectively. The precipitating antigen was found to be relatively more stable at 45 and 37°C. Keeping the preparation at 45°C for 360 min and at 37°C for 7 days resulted only to a 2–to 3– fold decrease in its antigenic activity. The decrease of infectivity at these temperatures during the same periods, however, was 10,000–fold or more.

No difference was observed in the nature of the precipitin lines when samples of an antigen preparation which had undergone various cycles of alternate freezing and thawing, were tested against the standard precipitating antiserum and, in spite of some slight variation in the results of titration, it appeared that during 21 cycles of alternate freezing and thawing the infective virus and the antigen content of the antigen preparation remained practically constant.

It was found that various periods of sonication from 2 to 10 min effected neither the infectivity and antigen activity nor resulted in a better release of the antigen molecules and virus particles from infected cells.

Discussion

Of various established cell lines susceptible to AHS virus, MS was found to be the most suitable host system for various studies on the virus (10). The cell culture was also found suitable for production of AHS precipitating antigen (5).

Studies on replication of AHS virus in MS cell cultures, indicated that there was an 8–hour latent period before newly formed infective virus was demonstrable in the fluid of the infected cultures, and that the maximum titer achieved very rapidly (8, 10). This was confirmed in the present experiments and it was found that a longer period was needed to detect the first appearance of the precipitating antigen in the infected cells or its release into the culture fluid.

The intracellular precipitating antigen was first demonstrated in MS cells collected 12–16 h after infection, when approximately 5% of the cells showed the cytopathic changes. The antigen concentration increased progres-

sively and reached its highest titer before 50% of the cells showed CPE, and retained it up to the time of complete cell destruction.

The cell-released antigen, on the other hand, due to its low concentration was not detectable in culture fluid unless it was concentrated 100- to 300-fold. When the antigen was precipitated and then concentrated by using ammonium sulfate, its first appearance was found to be not sooner than 20–24 h postinfection.

No difference was noticed in the nature of the intracellular and cellreleased antigens. Both produced two distinct precipitin lines when they were allowed to react against AHS precipitating antiserum in a gel diffusion precipitation test.

The fact that infectious tissue culture fluids of high infectivity titers failed to produce a precipitin line in diffusing against AHS precipitating antibody, whereas preparations obtained from infected cells of corresponding infectivity had a good antigenic activity indicated that the precipitating antigen was not infective virus. This was well demonstrated by ultracentrifugation of an antigen preparation in which the soluble precipitating antigen was separated from infective virus particles and all remained in supernatant fluid. In this experiment the supernatant fluid with all its antigenic activity contained approximately 0.03% of infective virus, while the reconstituted sediment with an infectivity titer 5 times more than the original preparation produced no precipitin line with the precipitating antiserum.

Separation of precipitating soluble antigen from infective particles, in a similar fashion, has been reported for rinderpest and canine distemper viruses (15), hog cholera virus (12), bovine viral diarrhea viruses (3), paravaccinia viruses (11) and many other viruses.

The finding that the antigen could be precipitated by saturated ammonium sulfate suggested that the AHS precipitating antigen has a protein nature. Alternate freezing and thawing 21 times and various periods of sonication up to 10 min did not effect the precipitating antigen.

The precipitating antigen could no longer be detected in preparation after heating at 65 and 75°C for 5 min at 60°C for 60–90 min or at 55°C for 180 min. Heating the antigen at 45°C for 360 min and at 37°C for 7 days resulted in a 2- to 3-fold decrease of its antigenic activity. The virus was found, however, to be more labile to the effect of heat, so that at the above temperatures the decrease of virus titer was much more rapid.

The precipitating antigen, however, should be considered as a heat labile antigen and must be stored at a low temperature. It was found that AHS precipitating antigen could be kept at 4°C and at – 70°C without a considerable loss of its activity for at least a year.

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