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The study of African horsesickness virus by the agar double–diffusion precipitation test. II. Characterization of the precipitating antigen

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Introduction

In previous paper, the production of a precipitating viral antigen from ultrasonically disrupted cells of suckling mouse brain or monkey kidney stable (MS) cell cultures, infected with African horsesickness (AHS) virus, was reported. (2)

It was also demonstrated that the antigen, when diffused, through an appropriate diffusion medium, towards the homologous and heterologous AHS precipitating antibodies, existed in AHS convalescent serum from a recovered horse or sera of hyperimmunized rabbit and chicken, reacted specifically and produced two distinct precipitin lines. The factors affecting the sensitivity of the reaction were studied, and the technique of a double-diffusion test, which ensures the reliability and reproducibility of the reaction, was described. (2)

In the present communication the results of studies undertaken to characterize the AHS precipitating antigen are presented.

Materials and Methods

Precipitating antigen: — The standard precipitating antigen employed throughout the experiments, unless otherwise stated, was produced from ultrasonically disrupted cells of MS cultures infected by strain S2, type 9, of AHS virus. The method for preparation of the antigen was described in the previous paper. (2)

Precipitation and concentration of the antigen in 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 being stirred by a magnette-stirrer. The fluid pH was adjusted to 7.0-7.4 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 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 technique provided the precipitation of the antigen and viral particles to the concentration of 100-300 folds.

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 being used. [2]

Agar double-diffusion precipitation test: -- A medium consisting of 1.25 to 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 the precipitin reactions were reported before. [2]

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 titre 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, [4] were used as host system for virus titration. 4 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 adsorbe to the cells at 37°C. for 45 minutes 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 titre was calculated by Reed and Muench method, and expressed as $Log_{10}TCID_{50}$/ml; [7]

Experiments and Results

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 of AHS virus, containing $10^{7.0}$ $TCID_{50}$/ml. of the virus. Adsorption was allowed to proceed at 37°C. for 120 minutes. The inoculum was discarded and the infected cell sheets were rinsed with 70 to 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 to 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 non concentrated antigen.

The cells, at interval times before the appearance of complete virus specific cell destruction, were detached and harvested by means of sterile glass beeds, and thereafter, by vigorous shaking of the bottles. Cells, however, were sedimented by centrifugation at 4°C., and then subjected to ultrasonic distruption to obtain the intracellular (cell associated) antigen, as described before [2].

Table 1. Virus and precipitating antigen produced in MS cell cultures at various intervals after infection with strain S2 of African horsesickness virus.

Time in hours		CPE %	Culture fluids				Cells	
			Original.		Concentrated.			
			Virus	Antigen	Virus	Antigen	Virus	Antigen
Exp. 1	0	0	3.5^{++}	0	-	0	-	0
	16	5^{+}	5.5	0	8.0	0	4.5	0.8^{o}
	24	20	6.5	0	9.0	TR	6.5	1.0
	36	80	7.0	0	9.0	0.2	9.0	1.0
Exp. 2	0	0	3.75	0	-	0	4.3	0
	8	0	4.0	0	-	0	5.75	0
	20	10	6.0	0	7.75	TR	7.0	0.6
	24	20	6.0	0	7.75	TR	8.0	0.8
	42	50	6.25	0	9.0	0.6	9.5	1.0
	48	70	6.25	0	8.5	0.8	8.5	1.0
	72	100	6.5	0	9.0	1.0	8.5	1.0
Exp. 3	0	0	2.5	0	-	-	5.0	0
	8	0	3.0	0	-	-	6.5	0
	12	5	3.5	0	-	-	7.5	TR
	16	20	5.0	0	-	-	8.0	0.6
	24	50	5.5	0	-	-	9.0	1.0
	30	90	6.0	0	-	-	9.5	1.0

+ : % cells showing CPE.

++ : Infective virus titre ($Log_{10}TCID_{50}$/ml.)

0 : Neg. Log_{10} dilution giving visible precipitin line.

Approximately 10 to 14 ml. of the antigen was 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. The results obtained from 3 experiments were summarized in (Table 1).

It was found that although the cell released precipitating antigen was undetectable in tissue culture fluid even at the time of complete cells destructions, its presence could be demonstrated when it was concentrated to 100-300 times.

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

The cell associated antigen could be detected 12 hours after infection and reached to much higher titre as compared with the cell released antigen.

Effect of high-speed centrifugation on the antigen

A standard AHS precipitating antigen was centrifuged for 60 minutes at 10,000 r.p.m., in a Sorvall, RC2-B, automatic superspeed refrigerated centrifuge. The supernatant was carefully collected, and used as a semi-clarified antigen in this experiment.

A sample of the antigen was retained for testing, and the remaining was subjected to 60 minutes centrifugation at 40,000 r.p.m. in a Spinco, model L 50, ultracentrifuge.

The upper half of the supernatant fluid was carefully removed, and the remaining supernatant was discarded. The sediment was reconstituted in YLE to 1/5 of the original volume, and the product was homogenized in a sterile tissue grinder.

The semi claified 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.

It was found that following ultracentrifugation, only about 0.03% of the infective virus remained in the supernatant fluid, which was proved to retain almost all its precipitating antigen. On the other hand, the sediment being re-suspended in one fifth of the original volume, although contained almost 5 times as much virus as in the original antigen, showed no precipitin activity. (Table 2).

Effect of alternate freezing and thawing on the antigen

The standard antigen was distributed in 1.2 ml. amount in 12x75 mm. tubes with the rubber stoppers, and were alternatively frozen, in a deep freezer at -70°C., and thawed, in cool tap water.

The samples subjected to 3,5,7,9,13,15,17, and 21 alternate freezing and thawing, as well as, the original antigen were tested for infective virus and antigen content. (Table 3)

No difference was observed in the nature of the precipitin lines when the treated and untreated antigen samples were tested against the standard pre-

Table 2. Virus content and precipitating activity of the supernatant and deposit of an ultracentrifuged antigen.

	Virus	Antigen
Ultracentrifuged supernatant fluid.	5.75	1.0 (0.8)
Semi clarified original antigen	9.25+	1.0 (0.8)++
Virus deposit resuspended in 1/5 of the original volume	10.00	0 (0)

+ : Infective virus titre (Log_{10} $TCID_{50}$/ml.).

++ : Neg. Log_{10} dilution giving visible 1st, and (2nd.) precipitin line.

cipitating 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.

Table 3. Virus and antigen titration of precipitating antigen subjected to various cycles of freezing and thawing.

Alternate cycles of freezing and thawing	Virus	antigen
0	9.0+	0.8 (0.8)++
3	9.5	0.8 (1.0)
5	9.0	0.8 (0.8)
7	9.5	0.8 (0.8)
9	9.25	0.8 (0.8)
13	8.75	0.8 (0.8)
15	8.75	0.8 (0.8)
17	9.00	0.6 (0.6)
21	8.5	0.6 (0.6)

+ : Infective virus titre($Log_{10}TCID_{50}$/ml.)

++ : Neg.Log_{10}dilution giving visible ,1st,and(2nd)precipitin line.

Effect of sonication on the antigen

Infected cells, of MS cultures inoculated with strain S2 of AHS virus, while in a glass container, held in an ice bath, were subjected to five 2 minute periods of ultrasonication. At the end of each period a sample was removed and kept in ice bath, before being centrifuged at 2500 r.p.m for 15 minutes. The supernatant fluids were immediately tested for infectivity and antigenic activity.

All samples produced similar precipitin lines and various periods of sonication from 2 to 10 minutes neither effected the infectivity and antigen activity,

nor resulted to a better release of the antigen molecules and virus particles from infected cells (Table 4).

Table 4. Effect of sonication on AHS precipitating antigen.

Sonication period	Virus	Antigen
2 min.	8.75+	0.8 (0.6)
4 min.	9.0	0.8 (0.8)
6 min.	8.75	0.8 (0.8)
8 min.	9.0	0.8 (0.8)
10 min.	9.0	0.8 (0.8)

+ : Infective virus titre ($Log_{10}TCID_{50}$/ml.)

++ : Neg. Log_{10} dilution giving visible 1st, and (2nd) precipitin line.

Effect of temperature on the antigen

A series of screw capped bottles containing 1.2 ml. of standard AHS precipitating antigen was prepared and stored at 4°C., -20°C., and -70°C. At various intervals, up to one year, a bottle stored at the different temperatures, was removed and tested. (Table 5). The results indicated that both infectivity titre and the antigen content of the preparation stored at -20°C. decreased more rapidly than those of the antigen samples kept at 4°C. and -70°C. Precipitating antigen, however, could be stored at 4°C. and -70°C. for at least a year.

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 waterbath, for various periods of time. The tubes after being removed from the waterbath were placed in an ice bath until being titrated. (Tables 6,7,8)

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

No difference in heat stability or sensitivity was noticed between the two components of the precipitating antigen.

Table 5. Stability of AHS virus and precipitating antigen at 4°C., -20°C., and -70°C. temperature.

Time in weeks	+4°C.		-20°C.		-70°C.	
	Virus	Antigen	Virus	Antigen	Virus	Antigen
O	11.0[+]	0.6(0.6)[++]	11.0	0.6(0.6)	11.0	0.6(0.6)
1	11.0	0.6(0.6)	7.0	0.4(0.2)	11.0	0.6(0.6)
2	10.0	0.6(0.4)	7.0	0.2(0.2)	9.0	0.6(0.4)
4	8.5	0.6(0.4)	7.0	0.2(0.2)	8.0	0.6(0.4)
8	8.5	0.6(0.6)	5.0	TR.	8.0	0.6(0.6)
12	8.0	0.6(0.4)	4.0	0.2	7.0	0.6(0.4)
20	8.0	0.8(0.6)	4.0	0.2(0.2)	8.5	0.8(0.6)
40	6.5	0.6(0.4)	3.5	0.2	7.0	0.6(0.4)
54	6.0	0.6(0.4)	3.5	0.2(0.2)	6.5	0.6(0.4)

+ : Infective virus titre ($Log_{10}TCID_{50}$/ml.)

++ : Neg. Log_{10}dilution giving visible 1st, and (2nd) precipitin line.

Table 6. Sensitivity of AHS virus and precipitating antigen to the effect of 60°, 65°, and 75°C. temperature.

Time in minutes	60°C.		65°C.		75°C.	
	virus	Antigen	Virus	Antigen	Virus	Antigen
0	8.5[+]	0.8(0.8)[++]	8.25	1.0(0.8)	8.25	1.0(0.8)
5	2.5	0.8(0.8)	0	TR	0	0
10	2.25	0.8(0.8)	0	0	0	0
20	2.0	0.6(0.6)	0	0	0	0
30	0	0.4(0.4)	0	0	0	0
45	0	0.2(0.2)	0	0	0	0

+ : Infective virus titre ($Log_{10}TCID_{50}$/ml.)

++ : Neg. Log_{10}dilution giving visible 1st, and (2nd) precipitin line

Table 7. Sensitivity of AHS virus and precipitating antigen to the effect of 45°C., and 55°C. temperature.

Time in minutes	45°C.		55°C.	
	Virus	Antigen	Virus	Antigen
0	8.5+	1.0(0.8)o	8.5	0.8(0.6)
5	-	-	4.5	0.8(0.6)
10	8.25	1.0(0.8)	4.0	0.8(0.6)
20	-	-	3.25	0.6(0.6)
30	8.0	0.8(0.8)	2.0	0.6(0.6)
45	-	-	1.0	0.6(0.4)
60	8.0	0.8(0.8)	0	0.6(0.4)
90	7.5	0.8(0.8)	0	0.4(0.4)
120	7.0	0.8(0.8)	0	0.2(0.2)
180	6.5	0.8(0.8)	-	-
240	6.0	0.8(0.8)	-	-
300	4.5	0.8(0.6)	-	-
360	4.5	0.6(0.4)	-	-

+ ::: Infective virus titre ($Log_{10}TCID_{50}$/ml.)

o : Neg. Log_{10} dilution giving visible 1st, and ((2nd)) precipitin line.

-: Not tested.

Discussion

Of various established cell lines susceptible to AHS virus, monkey kidney stable (MS) cells, was found the most suitable host system for various studies on the virus. [4] The cell culture was also found suitable for production of AHS precipitating antigen. [2]

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 titre achieved very rapidly. [3,4]

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.

Table 8. Stability of AHS virus and precipitating antigen at 37°C.

Time in days	Virus	Antigen
0	7.5[+]	0.8(0.8)
1	5.75	0.8(0.8)[++]
2	4.75	0.8(0.6)
3	3.75	0.8(0.4)
4	2.75	0.8(0.4)
5	2.25	0.8(0.6)
6	-	0.8(0.6)
7	-	0.6(0.4)

+ : Infective virus titre($Log_{10}TCID_{50}$/ml.)

++ : Neg. Log_{10} dilution giving visible 1st, and (2nd)' precipitin line.

- : Not tested.

The intracellular precipitating antigen was first demonstrated in MS cells collected 16 hours after infection, when approximately 5% of the cells showed the cytopathic changes. The antigen concentration increased progressively and reached to its highest titre, 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 for 100 to 300 folds. When the antigen was precipitated and then concentrated by using ammonium sulfate, its first appearance was found to be not sooner than 20 - 24 hours post infection (Table 1).

No difference was noticed in the nature of the intracellular and cell released 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 titres 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 acitivty, (Table 1.), 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 titre 5 times more than the original preparation, produced no precipitin line with the precipitating antiserum (Table 2).

Separation of precipitating soluble antigen from infective particles, in a similar fashion, has been reported for rinderpest and canine distemper virus, [8] hog cholera virus, [6] bovine viral diarrhea viruses, [1] paravaccina viruses, [5] and many other viruses.

The finding that the antigen could be precipitated by saturated ammonium sulfate, suggested the AHS precipitating antigen to have a protein nature.

Alternate freezing and thawing for 21 times, and various periods of sonication up to 10 minutes did not effect the precipitating antigen.

The precipitating antigen could no longer be detected in preparation after heating at 65°C. and 75°C. for 5 minutes, at 60°C. for 60 to 90 minutes, or at 55°C. for 180 minutes. Heating the antigen at 45°C. for 360 minutes and at 37°C. for 7 days, resulted to a 2-3 folds 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 titre was much more rapid (Tables 6,7,8).

The precipitating antigen, however, should be considered as a heat labile antigen and must be stored at 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, (Table 5).

Summary

A precipitating antigen was produced when monkey kidney stable (MS) cells were infected with African horsesickness virus. The antigen could be demonstrated in the infectious culture fluid, when concentrated from 100 to 300 folds, by ammonium sulfate precipitation, approximately 20 hours after infection. The intracellular precipitating antigen was first detected in MS cells collected 16 hours post infection.

Both cell released and intracellular (cell associated) antigens were composed of at least 2 components, producing two distinct precipitin lines, when they were allowed to react against horsesickness precipitating antiserum. No difference was observed in the time of first appearance of the two antigen components in the infected cultures.

The antigen, separated from infective virus particles by ultracentrifugation, was precipitated by saturated ammonium sulfate, indicating that it was of a protein nature, entirely distinct from the virus particles.

Heating the antigen at 65-75°C. for 5 minutes, or at 60°C. for 60 - 90 minutes, or at 55°C. for 180 minutes resulted to the complete loss of its precipitating activity. A 2-3 fold decrease of its titre was also observed when the antigen preparation was heated at 45°C. for 360 minutes, or at 37°C. for 7 days. The

antigen was found, however, to be more stable to the effect of heat than the AHS infective virus.

The antigen resisted ultrasonication, for at least 10 minutes, and the alternate freezing and thawing, for 21 cycles. It was kept at 4°C. and -70°C., without a considerable loss of activity, for a period of one year.

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