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Studies of Marek's Disease Herpesvirus and Turkey Herpesvirus Specific Common Antigen which Stimulates the Production of Neutralizing Antibodies

By

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With 6 Figures

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Summary

An agar-gel precipitation antigen prepared from the skin (feather-Ag) of chicken infected with JM strain of Marek's disease herpesvirus (MDHV) and cell extracts of cultures infected with either Type 2 plaque producing agent (PPA) of MDHV or turkey herpesvirus (HVT) (Type 2-Ag, HVT-Ag) had 1 precipitation line in common. Hyperimmune sera to the common antigen (common-Ag) neutralized both cell-free virus of Type 2 PPA and HVT. However, these viruses were neutralized to a greater extent by homologous antiserum. Absorption of HVT antiserum or hyperimmune serum to Type 2 PPA with common-Ag reduced their neutralizing activity. This reduction was almost complete with homologous antigen but less complete with heterologous antigen. The location of antigen in Type 2 PPA and HVT infected cells was determined using hyperimmune serum to the common-Ag in fluorescent antibody tests. Antigen mainly occurred in the cytoplasm of cells corresponding to the rounded refractile cells in the plaques, whereas unfixed cells showed antigen on the cytoplasmic membrane. The common-Ag associated with MDHV or HVT infections might be a virus structural component which is associated with the envelope.

Introduction

In chicken kidney or Japanese quail embryo fibroblast (QEF) cultures, virus-induced soluble antigen is detectable by the agar gel precipitin (AGP) test. The existence of a common precipitation antigen (common-Ag) has been reported

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among antigens obtained from feather tips of Marek's disease (MD) infected chickens, cultures infected with Marek's disease herpesvirus (MDHV) and those infected with turkey herpesvirus (HVT) (1, 16, 18, 22). Recently, we reported the chemical and physical properties of this common-Ag which possesses characteristics of glycoprotein (16). Other viruses including herpes simplex virus (HSV) and vesicular stomatitis virus produced a number of virus-induced soluble antigens. These antigens are, in part, glycoprotein and induced virus neutralizing antibody (5, 6). COHEN *et al.* (5) suggested that this glycoprotein is associated with the envelope. The common-Ag is MDHV- or HVT-induced antigen but it is not clear whether this antigen is associated with the structural component(s) of the virion.

The purpose of the present study is to investigate the neutralizing capacity of hyperimmune serum against the common-Ag using cell-free viruses of HVT or Type 2 plaque producing agent (PPA) derived from Cal-1 strain of HDHV (8). Further, this hyperimmune serum was used for detection of virus-specific antigen in cultures infected with MDHV and HVT by using fluorescent antibody (FA) tests.

Materials and Methods

Viruses and Cell Cultures

Sources of the JM strain of MDHV (passage 10—17) and HVT (passage 18—23) have been described previously (12). The Type 2 PPA (passage 40—45) derived from Cal-1 strain of MDHV (8) was used. Preparation of the QEF cultures and culture media (MEM) used for growth and maintenance of the cultures was described previously (16). QEF cells were propagated in Roux bottles and cultures in 35 mm plastic plates for virus assay.

Agar Gel Precipitation Test

The AGP test was performed as previously described (9). For detection of the AGP antigen in fractions obtained after gel filtration or sucrose gradient described in the following section, each fraction was tested against a constant amount of anti-MDHV chicken serum. This serum is a pooled serum from 5, 60 to 116-day-old chickens infected with the JM strain of MDHV (4).

Antigen Preparations for Immunization

Common-Ag was prepared from skin of MD-infected chickens from which the feathers had been removed by a previously described technique (13) with a slight modification. The supernatant obtained after centrifugation at $100{,}000 \times g$ for 90 minutes was further purified by gel filtration on Sephadex G-150 as previously described (16). Fractions having antigenic activity were pooled and designated as feather-Ag. Antigen was also prepared from normal chickens by the same procedure. Fractions in gel filtration corresponding to those of skin extracts of infected chickens were pooled and designated as feather (—).

Common-Ag from disrupted culture cells infected with Type 2 PPA or HVT was prepared by sucrose gradient (12—52 per cent) centrifugation, by which the common-Ag was separated from both the naked virus particles and cell debris (16). Fractions having antigenic activity were pooled and dialysed against phosphate buffered saline (PBS pH 7.2). These antigens were designated as Type 2-Ag and HVT-Ag.

Antisera

The hyperimmune serum against the common-Ag (feather-Ag, Type 2-Ag and HVT-Ag) was prepared in 7-week-old chickens (resistance inducing factor free and specific-pathogen free). Each of the 3 common-Ags was mixed with an equal volume of complete Freund's adjuvant and injected intramuscularly. The same volume of

these antigens without adjuvant were inoculated intramuscularly on the 21st, 25th, and 29th days. The chickens were bled 7 or 10 days after the last inoculation. None of the chickens were infected with MDHV; attempted virus isolation by direct chicken kidney-cell culture and search for MD antigens from feather tips by AGP test yielded negative results.

The hyperimmune serum against feather (—) was prepared in rabbits. The feather (—) antigen was mixed with an equal volume of adjuvant and injected intramuscularly. Then, 3 weeks later, the same volume of antigen without adjuvant was inoculated intramuscularly. The rabbits were bled 7 or 10 days after the last inoculation.

Hyperimmune serum to Type 2 PPA was obtained from a 7-week-old chicken which was immunized without adjuvant with the supernatant fluid of disrupted chicken kidney cultures infected with Type 2 PPA (9). The antiserum to HVT was obtained from infected chickens. The hyperimmune sera against 3 common-Ags were absorbed with normal QEF cells before using in AGP test and virus neutralization (VN) test. After absorption of these sera with normal QEF cells, a single precipitation band was obtained against common-Ag but none was obtained against normal QEF cells. Reactions of identity were obtained between those sera and with HVT antiserum or hyperimmune serum to Type 2 PPA.

All sera were inactivated at 56° C for 30 minutes before use.

Virus Neutralization Test

Because preliminary experiments indicated a linear relationship between the amount of cell-free virus of Type 2 PPA or HVT inoculated in QEF cultures and the number of plaques counted, the plaque assay was performed without an agar overlay. Similar dose-response relationships were reported for cell-associated MDHV and HVT (2, 7, 22).

The preparation of cell-free viruses for VN test was performed by the method described previously (9, 10). Cultures infected with Type 2 PPA and HVT were subjected to 3 cycles of freezing and thawing in MEM containing 10 per cent dimethylsulfoxide (DMSO) and 10 per cent calf serum [significantly, this menstruum was found to protect cell-free MDHV (10)] and then the mixture was centrifuged at $1000 \times g$ for 10 minutes. The supernatant was filtered through 0.45 μ filter (Millipore Filter Co., Bedford, Mass.) to obtain cell-free virus.

Samples of cell-free viruses containing about 6×10^2—1.5×10^3 PFU/ml were mixed with an equal volume of antisera (usually 1/10 dilution). After incubation for 60 minutes at room temperature, the serum-virus mixture was assayed for residual infectivity in replicate QEF cultures. The reduction of PFU of each serum-virus mixture was given as percent of the control MEM-virus mixture. The neutralization contant, k, of an antiserum was determined by the formula described by Watson and Wildy (21) with minor modification: $k = (2.303/60\,c) \times (\log V_0/V)$, where c = (volume antiserum in the test mixture)/(total volume test mixture), V, V_0 = residual infectivities of mixture with antiserum and MEM after 60 minutes.

Absorption of Antisera

HVT antiserum and hyperimmune serum to Type 2 PPA were absorbed with both HVT-Ag and Type 2-Ag having AGP titer of 1:8 and 1:6, respectively. These sera were absorbed by adding 9 ml of antigen to 1 ml serum and incubating overnight at 4° C. The absorbed sera were centrifuged at $15{,}000 \times g$ for 20 minutes, the supernatant fluid concentrated to the original volume of the sera by forced dialysis against polyethylenglycol No. 6000, and tested for residual neutralization activity.

Fluorescent Antibody Test

An indirect FA test was performed according to the procedure described by Naito *et al.* (14). All test sera were diluted 1:10 or more with PBS. Acetone fixed coverslip cultures infected with Type 2 PPA, JM strain and HVT were treated with these test sera and then stained with fluorescein isothiocyanate (FITC)-conjugated anti-chicken gamma globulin rabbit serum. Tests for cell membrane antigen in cultures infected with

Type 2 PPA and HVT at a multiplicity of about 8×10^4 PFU/ml and 5×10^5 PFU/ml, respectively, was conducted as follows. At 48 hours postinoculation, the cells were washed twice with PBS and dispersed with trypsin-versene solution, rewashed three times with PBS then treated with hyperimmune serum to common-Ag at 37° C for 1 hour. These mixtures were washed three times with PBS, stained vitally with FITC-conjugated anti-chicken gamma globulin rabbit serum at 37° C for 1 hour, washed three times with PBS and then resuspended in one drop of 90 per cent (v/v) glycerol. A drop of the suspended cells were placed on a slide under a cover slip for microscopic examination (11).

Results

VN Tests

Using the anti-common-Ag sera, VN tests with cell-free viruses of Type 2 PPA and HVT were performed to determine whether hyperimmune serum to common-Ag neutralized these viruses. Table 1 shows the inhibition of plaque formation after incubation of HVT or Type 2 PPA with various antisera. The hyperimmune sera to common-Ags (feather-Ag, HVT-Ag and Type 2-Ag) neutralized both HVT and Type 2 PPA, although the homologous strain was neutralized to a greater extent. Antiserum to HVT neutralized homologous HVT (over 90 per cent) and heterologous Type 2 PPA (84—87 per cent). The hyperimmune serum to Type 2 PPA also neutralized Type 2 PPA and HVT (over 90 and 83—85 per cent, respectively). Furthermore, hyperimmune serum to feather (—) also neutralized both viruses and the neutralization activity was higher than that of pre-immune sera (39—55 vs. 6—21 per cent) (Table 1). It should be noted that this serum had not been absorbed by normal QEF cells.

Table 1. *Neutralization Activity of Different Antisera in Tests against Cell-Free Viruses of Type 2 PPA and HVT*

	Reduction of PFU (%)			
	Type 2 PPA		HVT	
Antisera[a] against	Trial 1	Trial 2	Trial 1	Trial 2
Feather-Ag	79	82	73	85.5
Type 2-Ag	99	97	84.6	88.4
HVT-Ag	87	87.5	100	99
Type 2 PPA	93	98	85	82.5
HVT	87	83.5	98	100
Feather (—)	39.2	48	54.8	45
Pre-immune sera[b] (control)	21	15.2	13.5	5.8
MEM (control)	0	0	0	0

[a] All test sera were diluted 1:10 with PBS.
[b] Two different sera were used and the results were expressed by average.

In order to examine the neutralizing activity of hyperimmune sera to common-Ags in more detail, VN tests on HVT and Type 2 PPA were performed with serial 2-fold dilutions of hyperimmune serum to HVT-Ag and type 2-Ag and mean values of neutralization constants were calculated. Anti-HVT-Ag gave the values 2.1 and 0.73 for HVT and Type 2 PPA, respectively. Corresponding values for anti-Type 2-Ag were 0.59 and 0.95 (Fig. 1A and B). In identical tests with HVT and

Type 2 PPA antisera, the following values were recorded (the same order as above): 1.47 and 0.72 : 0.56 and 1.0 (Fig. 2A and B).

These results indicate that the common-Ag is able to stimulate the production of virus neutralizing antibodies.

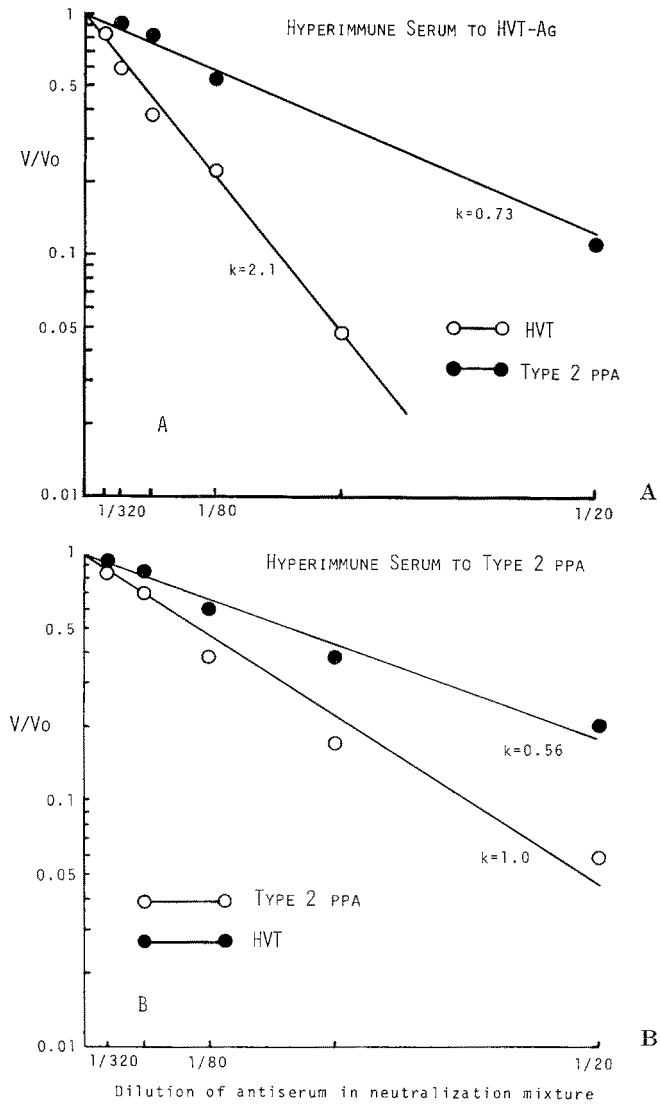

Fig. 1. Neutralizing activity of hyperimmune serum to common-Ag
Cell-free viruses of HVT and Type 2 PPA were treated with serial 2-fold dilution of hyperimmune serum to common-Ag. V and V_0 were residual infectivity of mixtures with antiserum and MEM after 60 minutes, respectively. The neutralization constant, k, of hyperimmune serum was defined as $k = \frac{2.303}{60c} \log \frac{V_0}{V}$, where c = (volume antiserum in the test mixture) / (total volume test mixture)
A. Hyperimmune serum to HVT-Ag. B. Hyperimmune serum to Type 2-Ag

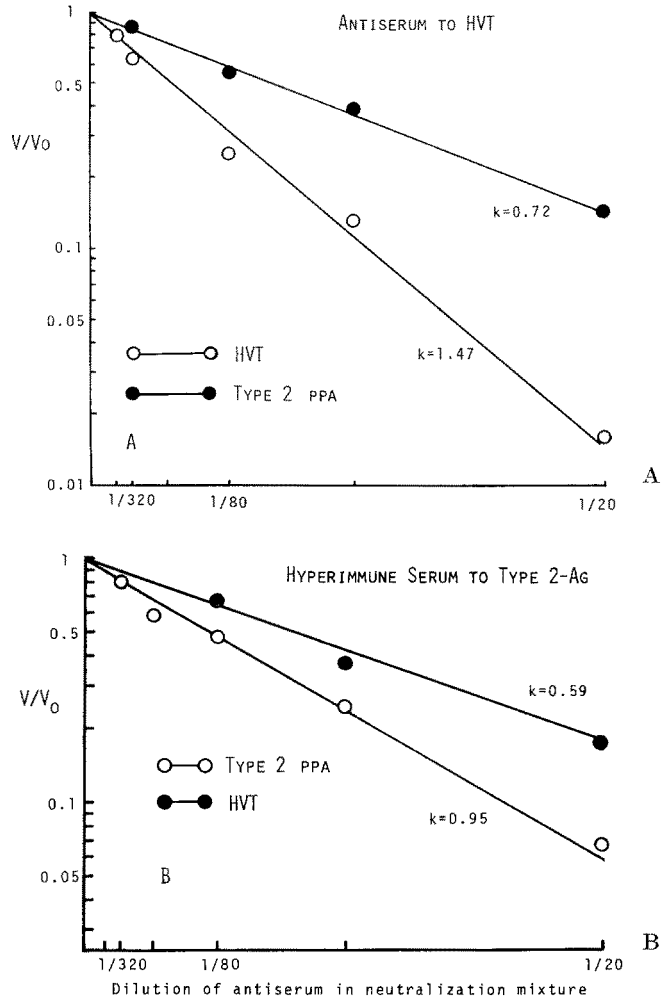

Fig. 2. Neutralizing activity of HVT antiserum and hyperimmune serum to Type 2 PPA

Procedure and calculation of neutralization constant, k, of antiserum were described in Figure 1

A. Antiserum to HVT

B. Hyperimmune serum to Type 2 PPA

Effect of Absorption of Sera on VN Test Activities

HVT antiserum and hyperimmune serum to Type 2 PPA were found to neutralize homologous virus to more than 90 per cent. Experiments were designed to show whether common-Ags have a capacity to block the neutralizing activity in these sera. Antisera to HVT or hyperimmune serum to Type 2 PPA were absorbed with HVT-Ag and Type 2-Ag, separately, and the absorbed sera were examined for residual neutralizing activity to HVT or Type 2 PPA. By estimating

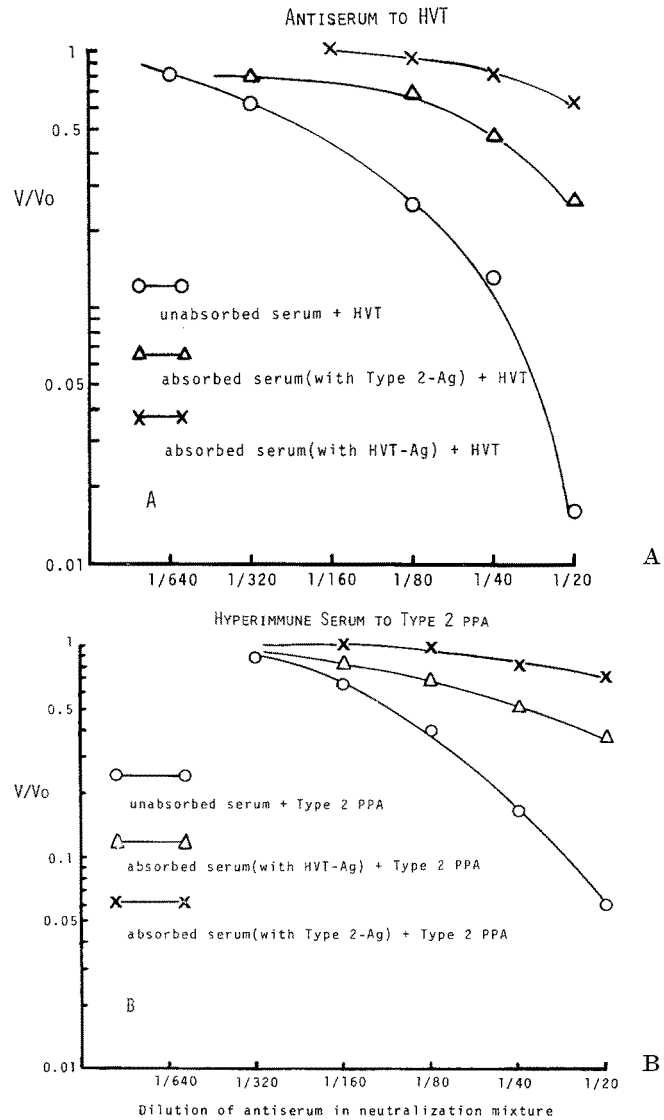

Fig. 3. Inhibition of neutralizing activity of HVT antiserum and hyperimmune serum to Type 2 PPA

Antiserum to HVT or hyperimmune serum to Type 2 PPA was absorbed with HVT-Ag and Type 2-Ag, separately, and the absorbed sera were examined for the residual neutralizing activity to HVT or Type 2 PPA. By estimating the value of V/V_0 for the virus treated with absorbed and unabsorbed sera, neutralization capacity of the former was compared with that of the latter

A. Antiserum to HVT. B. Hyperimmune serum to Type 2 PPA

the value of V/V_0 for the viruses treated with absorbed and unabsorbed sera, neutralization capacity of the former was compared with that of the latter (Fig. 3A, B). The absorption of HVT antiserum with HVT-Ag almost completely

removed its neutralizing activity against HVT. Absorption of the HVT antiserum with Type 2-Ag also considerably reduced the activity against HVT (Fig. 3A). At the lowest dilution tested, the serum absorbed with HVT-Ag had a 40 times reduction in neutralizing activity compared to that of the unabsorbed serum, whereas the reduction in activity after absorption with Type 2-Ag was about 16 times.

Reciprocal experiments using Type 2 PPA were performed with hyperimmune serum to Type 2 PPA before and after absorption with HVT-Ag and Type 2-Ag. The results were shown in Fig. 3B. Absorption of serum with HVT-Ag had plainly reduced its neutralizing activity but absorption with Type 2-Ag completely removed activity against Type 2 PPA. These results similar to those of HVT were obtained in Type 2 PPA with the lowest dilution of the absorbed sera.

These experiments indicate that HVT antiserum and hyperimmune serum to Type 2 PPA contained specific antibodies against common-Ag and the neutralizing activity of these antibodies was stronger to homologous virus than to heterologous virus.

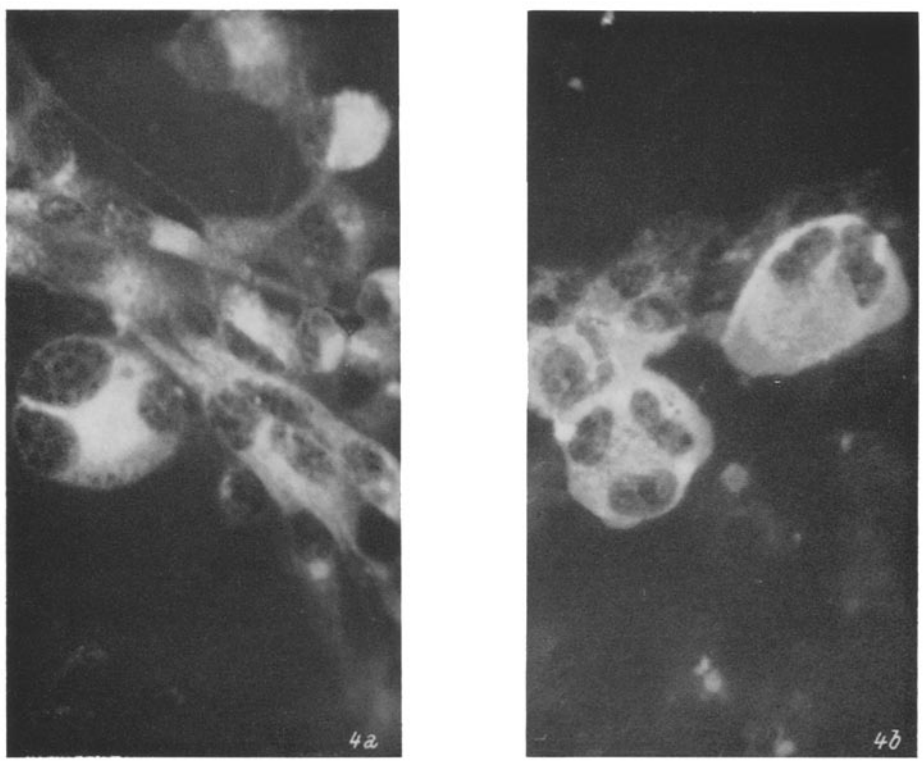

Fig. 4. Location of immunofluorescent antigen by using hyperimmune serum to common-Ag
The HVT and Type 2 PPA infected cells were fixed in acetone and stained with homologous hyperimmune serum to common-Ag. An immunofluorescent antigen showed mainly in the cytoplasm of cells ($\times$ 1200)
A. HVT infected cells
B. Type 2 PPA infected cells

FA Tests

When the location of immunofluorescent antigen in MDHV and HVT infected cells was examined using hyperimmune serum to common-Ag by FA tests, the cells fixed in acetone showed immunofluorescent antigen mainly in the cytoplasm of cells corresponding to the rounded refractile cells in the plaques (Fig. 4A, B), whereas those using HVT antiserum and hyperimmune serum to Type 2 PPA showed the antigen in both nucleus and cytoplasm (Fig. 5). Uninfected control cells did not become stained with these sera and control sera did not stain any of the cell preparations.

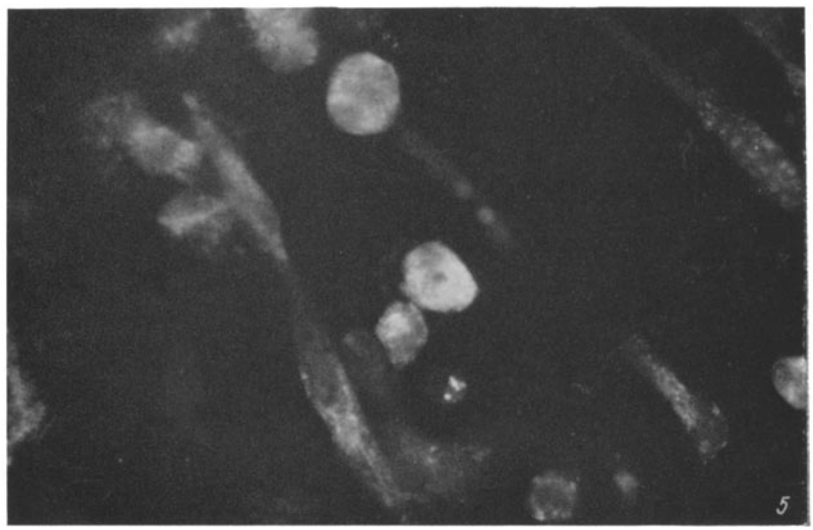

Fig. 5. Location of immunofluorescent antigen by using hyperimmune serum to Type 2 PPA. The Type 2 PPA infected cells were fixed in acetone and stained with hyperimmune serum to Type 2 PPA. Both nucleus and cytoplasm fluorescence could be seen (×1200)

To examine whether membrane antigen is detectable by hyperimmune serum to common-Ag, culture cells infected with Type 2 PPA or HVT were directly treated with hyperimmune serum to common-Ag without fixation. When unfixed cells were treated with homologous hyperimmune serum to common-Ag, about 6 to 8 per cent of the cells were positive for membrane antigen (Fig. 6A, B). Control serum and infected cells on the one hand and hyperimmune serum to common-Ag and uninfected cells on the other, gave negative results. Similarly, when the culture cells infected with HVT or Type 2 PPA were treated with HVT antiserum or hyperimmune serum to Type 2 PPA, respectively, similar membrane antigen staining was observed. However, absorption of HVT antiserum or hyperimmune serum to Type 2 PPA with homologus common-Ag had almost completely removed antibody reacting with membrane antigen.

These results indicate that the common-Ag may be located in the cytoplasm as well as in the cell membrane of infected cells.

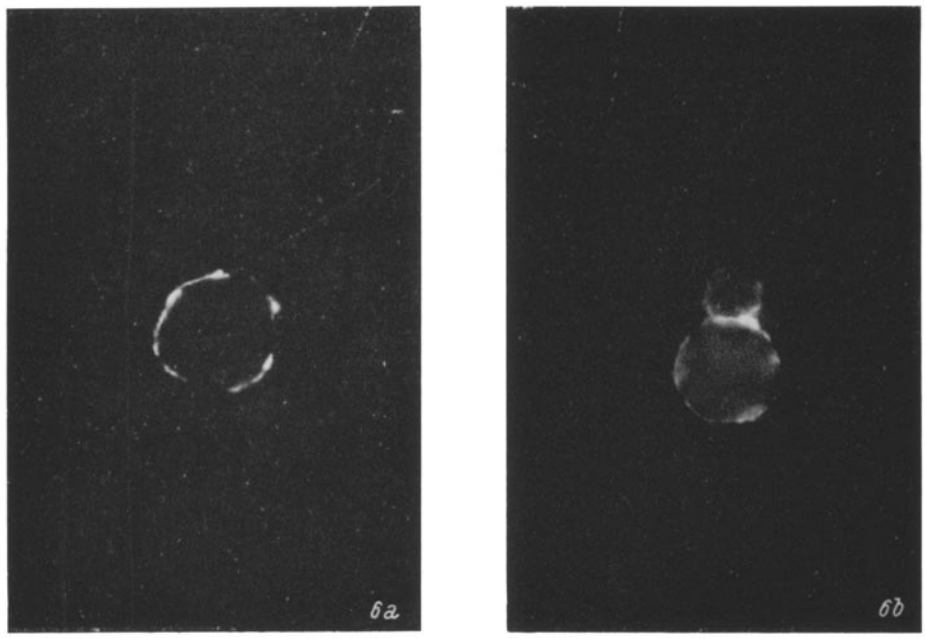

Fig. 6. Detection of membrane antigen by using hyperimmune serum to common-Ag The HVT and Type 2 PPA infected cells were directly treated with homologous hyperimmune serum to common-Ag without any fixation
A. HVT infected cells
B. Type 2 PPA infected cells (× 1200)

Discussion

Recently, we reported the properties of the common-Ag, indicating that this antigen is, in part, a glycoprotein and the molecular weight ranges from 33,000 to 46,000 (16). In the present studies, we examined the relationship between hyperimmune serum to common-Ag and virion by using VN tests.

Because of the highly cell-associated nature of MDHV in cell culture (2), VN tests have been difficult to perform. Nevertheless, MIKAMI and BANKOWSKI (9) have performed neutralization of Type 2 PPA. The plaque forming capacity of cell-free Type 2 PPA was reduced by 90 per cent or more by homologous antiserum but not by heterologous antiserum to Type 1 PPA of Cal-1 strain of MDHV. WITTER *et al.* (22) reported that infectivity of cell-free HVT was reduced by 50 per cent or more by both HVT and MDHV antiserum but to a greater extent by homologous antiserum. Similar results were obtained in the present experiments. Plaque formation by the cell-free viruses of Type 2 PPA and HVT was reduced by 90 per cent or more by homologous antisera and by from 73 to 88 per cent by heterologous antisera. Furthermore, it is interesting to note that HVT and Type 2 PPA were neutralized by anti-feather (—) rabbit hyperimmune serum by 39 to 55 per cent. This considerable virus neutralizing effect of the serum believed to be due to antibodies against host cell-derived components. Since enveloped viruses budding from the cell surface or the endoplasmic reticulum carry host material

which is incorporated in the virion, it cannot be excluded that antibodies against such host components may have a virus neutralizing activity. However, further work will be required to determine the role of such antibodies in virus neutralizing effect. These results indicate that JM, HVT and Type 2 PPA are antigenically related but not identical, and suggest that the neutralizing activity of antiserum to MDHV or HVT should be determined by at least 90 per cent plaque reduction (9, 10).

The hyperimmune serum to common-Ag neutralized both cell-free Type 2 PPA and HVT but the neutralization occurred to a greater extent by homologous antiserum. Furthermore, the common-Ag had plainly removed neutralizing activity of HVT antiserum and hyperimmune serum to Type 2 PPA by absorption of these sera. These results suggest that the common-Ag is a structural component associated with the virus envelope. However, the possibility that the common-Ag originates from altered cellular membranes of infected cells can not be ruled out (19). Since MDHV is one of the cell-associated group B herpesviruses, it is very difficult to separate the virus envelope from altered cellular membranes. Therefore, whether the antigen detectable by AGP-test originated from the virus alone or together with altered cellular membrane is not clear.

The ratio of k value against HVT and Type 2 PPA respectively, with hyperimmune serum to HVT-Ag and the ratio of k value against Type 2 PPA and HVT respectively with hyperimmune serum to Type 2-Ag were 2.88 and 1.16 respectively. Although the neutralizing activity of the HVT antiserum or hyperimmune serum to Type 2 PPA was greatly reduced by absorption with heterologous common-Ag, the residual activity still remained significant. These experiments suggested that the residual neutralizing activity after absorption might be type specific. However, this assumption is still open question, because we could not detect a reaction of partial identity or spur formation in AGP test when HVT-Ag and Type 2-Ag were tested with hyperimmune serum to Type 2-Ag. Therefore, we should not rule out the possibility that common-Ags may contain several distinct determinants, the same species in all antigens, but in different proportions. Further work will be required to find the nature of type differences. Nevertheless, MIKAMI and BANKOWSKI (9) reported that using hyperimmune serum to Type 1 PPA or Type 2 PPA, at least 2 antigenic components were found by the AGP test. One of the components was common to both viruses whereas another appeared to be specific for each virus. SIM and WATSON (20) clearly showed that the hyperimmune sera to type 1 and 2 HSV contain type specific and cross-reacting or type common neutralizing antibodies.

Although the results of FA tests suggested that the common-Ag is located in the cell membrane as well as in the cytoplasm of infected cells, the relation between common-Ag and membrane antigen is still obscure. In the present experiment, hyperimmune serum to common-Ag reacted with membrane antigen. This result suggests that the common-Ag might be related to membrane antigen. NAZERIAN (15) reported that membrane antigen may be related to the 'A' antigen (3). Recently, existence of membrane antigen in arginine deprived cultures infected with MDHV was presented by MIKAMI *et al.* (11) who showed that two peaks were obtained in the appearance of membrane antigen, early appearing membrane antigen reached a peak at 8 hours postinoculation, and late appearing membrane

antigen reached a plateau at 20 hours, but it dropped to a low level at 48 hours. Preliminary experiments have shown that the common-Ag was related to the late appearing membrane antigen but not to early appearing membrane antigen by AGP test and FA test. Antibodies against EB virus-induced cell membrane antigen demonstrated by FA technique were apparently responsible for neutralization of viral infectivity (17). Investigation concerning the relation of membrane antigen to the common-Ag is now in progress.

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