Relation between the Neutralization of Herpesvirus of Turkeys and the Antibody to Late-appearing Membrane Antigen Induced by the Virus

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SUMMARY

The relation between the neutralization of herpesvirus of turkeys (HVT) and the antibodies to early appearing membrane antigen (EMA), late appearing membrane antigen (LMA) and intracellular antigen (IA) induced by the virus was examined by blocking of direct immunofluorescence using 38 sera from chickens infected with HVT. Our results provide evidence that anti-LMA is significant in the neutralization of the infectivity ($P < 0.001$) since the neutralizing capacity of the serum was related to its blocking index (BI) and not to its anti-EMA titre (BI). Furthermore, the anti-IA titres (BI) of the sera were also related to their neutralizing activity to a lesser extent ($P < 0.025$). However, no relationships among any two of the titres of EMA, LMA and IA were observed. The implication of these results on the relationship of LMA to infectious virus particles is discussed.

Herpesvirus of turkeys (HVT) is antigenically related to Marek’s disease herpesvirus (MDV; Witter et al. 1970). Several antigens have been detected in cells infected with HVT or MDV by immunofluorescence (IF; Purchase, 1969; Naito et al. 1970; Witter et al. 1970), agar gel precipitation (AGP; Chubb & Churchill, 1968; Witter et al. 1970) and immunoferritin tests (Nazerian & Chert, 1973). These antigens were shown to be specifically related to the viruses and were partially characterized. Two types of antigen have been demonstrated by IF techniques in cell cultures infected with MDV or HVT. Intracellular antigen (IA) has been detected in both the nucleus and the cytoplasm of acetone-fixed cells (Purchase, 1969; Purchase et al. 1971). The antigen was found only in cells that produced MDV particles (Nazerian & Purchase, 1970). Membrane antigen (MA) was detected on the surface of live cells (Chen & Purchase, 1970; Ishikawa et al. 1972). Most cells positive for the MA of MDV were shown by immunoferritin studies to contain virus particles, but a few cells were positive for MA but did not contain any virus particles or show any other signs of virus replication (Nazerian & Chen, 1973). Recently, MA was subdivided into two subclasses, designated early MA (EMA) and late MA (LMA) which differ with respect to their sensitivity to inhibitors of DNA synthesis, their appearance in arginine-deficient Japanese quail embryo fibroblast (QEF) cultures and their antigenic specificity (Ishikawa et al. 1972; Mikami et al. 1973; Onuma et al. 1976; Inage et al. 1979). Two peaks in the percentage of cells producing MA were observed at 8 and 24 h after virus inoculation and the antigens appear to be coded either by an early or a late function of the virus genome (Ishikawa et al. 1972; Mikami et al. 1973; Inage et al. 1979). These results suggested that MA-producing cells are composed of a mixture of cells at different stages of infection expressing either EMA or LMA. Antibody against LMA of HVT is apparently responsible for neutralization of virus infectivity (Inage et al. 1979). The purpose of the present study was to investigate further the relationship between the neutralization of HVT and the antibodies to antigens induced by the virus using IF.

The QEF cell cultures were prepared from 8- to 9-day-old Japanese quail embryos (Onoda et al. 1970). Eagle’s minimal essential medium (MEM) was used in the present study.
The routine Eagle's MEM will be referred to as Arg⁺MEM, and medium lacking arginine will be referred to as Arg⁻MEM. Two kinds of cultures were prepared as previously described (Mikami et al. 1973, 1974). First, cells \((1 \times 10^6/ml)\) were propagated in 35 mm plastic plates using Arg⁺MEM supplemented with 10% tryptose phosphate broth (TPB) and 10% calf serum (CS). The monolayers were washed three times before virus inoculation. These monolayers will be referred to as Arg⁺ monolayers. Secondly, cells \((2 \times 10^6/ml)\) were propagated in plates using Arg⁻MEM supplemented with 10% CS for the first 2 days. These monolayers were then maintained for an additional day in Arg⁻MEM containing 10% dialysed CS before virus inoculation. These monolayers will be referred to as Arg⁻ monolayers. After virus inoculation, Arg⁺ monolayers were fed with Arg⁺MEM supplemented with both 5% CS and 10% TPB and Arg⁻ monolayers were fed with Arg⁻MEM supplemented with 5% dialysed CS.

The source of the FC-126 strain of HVT and the method of propagation of the virus in QEF cultures have been described (Okada et al. 1972; Onuma et al. 1974). Both cell-associated and cell-free viruses were used as inocula. Trypsinized cells of infected QEF cultures (2nd to 11th passage) having \(1.0 \times 10^6\) p.f.u./ml were used as cell-associated virus. These cells were suspended into Arg⁺MEM. Cell-free viruses were prepared according to the method described by Mikami & Bankowski (1970). HVT-infected QEF cells were scraped off culture plates or bottles and subjected to three cycles of freezing and thawing in different solutions for different experiments. These mixtures were then centrifuged at 1000 g for 10 min and the supernates were used as the cell-free virus preparation. For the experiment preparing the source of LMA-positive cells for determination of anti-LMA titre, the solution was Arg⁻MEM containing 10% dimethyl sulphoxide (DMSO) and 10% dialysed CS. For the serum neutralization (SN) test and the experiment preparing the source of EMA-positive cells for determination of anti-EMA titre, the solution was Arg⁺MEM containing 10% DMSO and 10% CS. The SN test was performed according to procedure described by Onuma et al. (1975).

Thirty-eight sera were tested. Nineteen specific pathogen-free chickens were inoculated with 0.2 ml of commercial HVT vaccine (Merck Co. Ltd) at one day of age, and sera from these chickens were obtained at 8 or 12 weeks after inoculation. The titres of anti-EMA, anti-LMA and anti-IA antibodies were measured by the blocking test against direct IF (Ishikawa et al. 1972; Inoue et al., unpublished data). For the titration of anti-EMA and anti-LMA antibodies, test sera at 1:5 dilution were mixed with \(1 \times 10^6\) target cells and incubated for 30 min at 37 °C. After incubation, the cells were washed three times with phosphate-buffered saline (PBS, pH 7.2) and stained with fluorescein isothiocyanate (FITC)-conjugated anti-HVT chicken serum for 30 min at 37 °C. The cells were then washed three times with PBS, resuspended in 1 drop of 90% (v/v) glycerol and placed on a slide with a coverslip for microscopic examination. For the titration of anti-IA antibody, smears of target cells were fixed in cold acetone for 30 min and then treated with test sera of 1:5 dilution for 30 min at 37 °C in a humidified chamber. After incubation, the smears were washed three times with PBS and stained with FITC conjugated anti-HVT chicken serum for 30 min at 37 °C. The smears were then washed three times with PBS and covered with a coverslip. In microscopic examination, 500 cells were counted and the percentage of positive cells was calculated. The blocking index (BI) was calculated as follows:

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BI = \frac{\% \text{ positive cells with conjugate alone} - \% \text{ positive cells with test serum and conjugate}}{\% \text{ positive cells with conjugate alone}}
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Target cells used for the detection of antibodies to EMA, LMA and IA by the IF test were prepared separately from two kinds of QEF monolayer cultures infected with either cell-free or cell-associated HVT at various titres. These monolayers were washed twice with PBS and trypsinized with trypsin versene solution. The dispersed cells were re-washed
Fig. 1. The relationship of the serum titres (BI) to each antigen determined by the blocking tests and SN titres. Regression lines (r) were calculated by the method of least squares. For the calculation, the SN titres were converted to logarithmic values (e.g. 1:5 as 1; 1:10 as 2...1:640 as 8). (a) Lack of correlation between levels of anti-EMA and anti-IA antibodies; r = 0.60 + 0.19 (0.54 - x), correlation coefficient = -0.15; P > 0.25. (b) Lack of correlation between levels of anti-IA and anti-LMA antibodies; r = 0.50 + 0.08(0.88 - x), correlation coefficient = 0.15; P > 0.25. (c) Lack of correlation between levels of anti-EMA and anti-LMA antibodies; r = 0.60 + 0.29(0.62 - x), correlation coefficient = -0.29; P > 0.05. (d) Lack of correlation between levels of neutralization titres and anti-EMA antibodies; r = 6.00 + 0.90(0.04 - x), correlation coefficient = -0.11; P > 0.25. (e) Presence of correlation between levels of neutralization titres and anti-LMA antibodies; r = 6.00 + 4.59(x - 2), correlation coefficient = 0.54; P < 0.001. (f) Presence of partial correlation between levels of anti-IA antibodies and neutralization titres; r = 0.50 + 0.04(x - 6), correlation coefficient = 0.42; P < 0.025.
twice with PBS and then used as target cells. For detection of antibodies to IA, the dispersed cells of Arg+ monolayers from 2 to 3 days after inoculation of cell-associated virus were used. For the detection of anti-EMA antibodies, the dispersed cells of Arg+ monolayers from 8 h after inoculation of cell-free virus (7 to 19×10³ p.f.u./ml) were used. For the detection of anti-LMA antibodies, the dispersed cells of Arg- monolayers from 24 h after inoculation of cell-free virus (1 to 3×10⁴ p.f.u./ml) were used. Correlation coefficients among pairs of the titres of EMA, LMA, IA and SN were calculated by Pearson's formula.

The relationships of anti-IA titres to anti-EMA titres (Fig. 1a) and anti-LMA titres (Fig. 1b) and of anti-EMA titres to anti-LMA titres (Fig. 1c) were examined. No apparent relationship was seen between any level of anti-IA antibody and that of the other antibodies; however, a slight negative correlation was observed between the anti-EMA and anti-LMA titres: sera with high BI for EMA had low BI for LMA in some sera and vice versa. Several lines of evidence indicate the distinction between IA and EMA or LMA: (1) the synthesis of IA in cultures infected with MDV or HVT is apparently affected by a DNA inhibitor but that of EMA is not (Ishikawa et al. 1972; Mikami et al. 1973, 1974); (2) the synthesis of IA is restricted in arginine-deficient cultures infected with the viruses but that of EMA or LMA is not (Mikami et al. 1973, 1974; Inage et al. 1979); (3) the absorption of anti-HVT serum by MA (EMA and LMA) positive cells results in a marked reduction of its homologous MA reactivity but not IA reactivity (Inage et al. 1979); (4) HVT and the low-passaged MDV induce both IA and MA in respectively infected cells, whereas the high-passaged MDV induces the production of IA but not MA (Nazerian, 1973). The present experiments provide further evidence of the antigenic difference between IA and any of EMA and LMA. Furthermore, the difference in antigenic specificity between EMA and LMA was previously demonstrated by differential absorption of an MA reactive antiserum (Inage et al. 1979). The negative correlation tendency between anti-EMA and anti-LMA titres observed in the present experiment further supports the antigenic difference between EMA and LMA.

When the titres of the neutralization of HVT infectivity were compared with different levels of anti-EMA (Fig. 1d), anti-LMA (Fig. 1e) and anti-IA (Fig. 1f) antibodies, the titres of SN antibodies were not correlated with those of anti-EMA antibody (P > 0·25) but were correlated with those of anti-LMA antibody (P < 0·001) and anti-IA antibody (P < 0·025). It is known that the envelope of most herpes viruses is derived from a part of the membrane of the infected cells (Darlington & Moss, 1969; Roizman et al. 1969) and circumstantial evidence suggests that virus-induced MA becomes part of the mature virus particle (Pearson et al. 1970). An antigenic relationship between their envelopes and the MAs of herpes simplex virus and Epstein–Barr virus (Roizman & Spear, 1971; Silvestre et al. 1971) has been reported. It has also been shown that hyperimmune sera prepared in rabbits with plasma membranes from MDV- or HVT-infected cells neutralize HVT (Kaaden & Dietzschold, 1974). By using serum hyperimmune to major HVT precipitin antigen, only LMA was detected in QEF cultures infected with HVT (Onuma et al. 1976) and the serum showed a high neutralizing activity to HVT (Onuma et al. 1975). These results provide indirect evidence of a relationship between LMA and the envelope of HVT. Recently, LMA, but not EMA, of HVT was shown to have a relationship with the envelope of the virus from the results of absorption experiments (Inage et al. 1979). The demonstration that antibody against HVT-induced LMA but not EMA can neutralize HVT infectivity in the present experiment further strengthens the idea that the antigen is closely related to the virus envelope antigen.

Furthermore, some relationship between the anti-IA titre and the SN titre was observed. The results suggest that some envelope components present in the cytoplasm are antigenically related to the envelope of HVT. In the immunoferritin test, not only the entire membrane system but also virus particles at various stages, including envelope virions in the
cytoplasm of cells infected with MDV, were tagged specifically with ferritin labelled antibody (Nazerian & Chen, 1973). If our interpretation is correct, then the question arises of how to explain the lack of relationship between IA and LMA. The exact explanation for this phenomenon is not known. However, the LMA and the envelope components in the cytoplasm may be antigenically different, but they may both still participate in constituting at least part of the envelope of HVT. Nevertheless, the previous results that LMA- but not IA-producing cells were observed in arginine-deficient cultures infected with MDV or HVT (Mikami et al. 1973, 1974; Inage et al. 1979) and that anti-HVT serum absorbed with LMA positive cells reduced its LMA reactivity but not its IA reactivity (Inage et al. 1979) support the difference between IA and LMA.

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Short communications


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