Archives of Virology 67, 31—43 (1981)

Archives of Virology

The Detection of Influenza A Virus Antigens in Cultured Cells by Enzyme-Linked Immunosorbent Assay

By

H. Watanabe and J. S. Mackenzie

University Department of Microbiology, Queen Elizabeth II Medical Centre, Nedlands, Western Australia

With 4 Figures

Accepted August 20, 1980

Summary

An enzyme-linked immunosorbent assay (ELISA) was employed to investigate the expression of influenza A/Hong Kong/68 (H3N2) virus structural proteins on the surface of infected MDCK cells, and to detect viral antigens in culture media and cell extracts. Infected cells were fixed with 0.1 per cent glutaraldehyde before being examined for the presence of cell-surface antigens. Viral antigens were first observed on the surface of cells 4 hours after infection and reached a maximum 10—12 hours after infection, when measured by haemadsorption with chicken erythrocytes and by ELISA and immunofluorescence with hyperimmune antiserum to Hong Kong virus. A good correlation was found between the three assay systems. The presence of individual virion structural proteins on the cell surface was determined by ELISA using specific antibodies purified by differential affinity chromatography. Either or both of the internal matrix and nucleoprotein antigens were expressed from 2 to 6 hours after infection, with maximum expression after 2 hours, and the strain-specific and common antigenic determinants of haemagglutinin were observed on the cell surface from 4 hours after infection, and reached a maximum 8 to 10 hours after infection. Low levels of neuraminidase were detected between 4 and 8 hours after infection. Culture media and cell extracts were titrated by infectivity and haemagglutination assays, and by ELISA. Titres obtained from the culture media showed a close correlation between the three assay methods, with peak titres being attained 24 hours after infection. Viral antigens were first observed in cell extracts by ELISA 4 hours after infection, and infectious virions and haemagglutinin 2 hours later, but whereas maximum titres of infectious virus and haemagglutinin were found 10 hours after infection, the ELISA titre continued to rise until 24 hours after infection, which suggested that virus structural proteins were being accumulated in the cells after most of the progeny virions had been released. The results are discussed in terms of the potential use of ELISA in rapid virus diagnosis.

0304-8608/81/0067/0031/$ 02.60

Introduction

Influenza A virus particles contain seven structural proteins; two external glycoproteins, haemagglutinin (HA) and neuraminidase (NA), and five internal proteins (reviewed in 33). The HA, which is responsible for virus attachment to susceptible cells and for the ability of the virus to agglutinate erythrocytes, displays several antigenic determinants (15), some of which are common to all viruses of the same antigenic drift series (HA-CM), whereas others are specific to each individual strain (HA-ST) (21). The function of NA is unknown, but it is able to hydrolyse sialic acid from the HA receptors on the surface of cells. Matrix and nucleoprotein are the most abundant internal proteins and both confer type specificity, whereas the three other internal proteins, P1, P2 and P3 are present in very small amounts and are believed to be involved in RNA transcriptase activity and possibly in virus maturation. During the replicative cycle of influenza, HA and NA are inserted into the cytoplasmic membrane of the infected cell where they can be detected by a variety of techniques including haemadsorption, immunofluorescence, immunoferritin and erythrocyte elution (23, 24, 29). Recent evidence has suggested that the internal matrix and nucleoprotein antigens may also be expressed on the surface of infected cells (1, 5, 6, 16, 28).

In recent studies, it has been shown that an enzyme-linked immunosorbent assay (ELISA) provided a sensitive method for titrating antibodies to influenza A virus structural proteins (20, 30) and for measuring the height and specificity of the immune response to influenza infection in mice (31). The purpose of the present study was to investigate the use of ELISA for detecting influenza viral antigens expressed on the surface of infected tissue culture cells, in cell extracts, and released into the culture medium during the viral replication cycle.

Materials and Methods

Virus and Cell Culture

MDCK cells were grown in 75 cm^2 plastic tissue culture flasks (Falcon, Maryland) with Dulbecco's modified Eagle's medium, as described previously (14). At confluency, each flask contained approximately 8×10^6 cells. Influenza A/Hong Kong/1/68 (H3N2) virus was grown in allantoic cavities of 10-day embryonated eggs. After 40 hours incubation at 37° C, the allantoic fluids were harvested, clarified by centrifugation at $1000 \times g$, and stored in aliquots at −70° C as stock preparations. The MDCK cells were infected with 240 haemagglutinating (HA) units of Hong Kong virus (HK) per flask, the virus allowed to adsorb for 1 hour at 37° C, and the monolayers overlaid with 20 ml Eagle's medium supplemented with 1 per cent foetal calf serum. At intervals between 2 and 48 hours after infection, one flask was harvested, the medium removed, and the monolayer rinsed three times with phosphate-buffered saline (PBS). The cells were detached by treatment with 0.02 per cent ethylenediamine tetraacetic acid containing 0.25 per cent trypsin. Dead, infected cells in the removed medium were sedimented at $450 \times g$, and pooled with the dispersed cells from the monolayer. A 10 per cent aliquot of the cell suspension (about 8×10^5 cells) was frozen and thawed twice, centrifuged at $1000 \times g$, and the supernatant collected as a cell extract. The rest of the cell suspension was fixed with 0.1 per cent glutaraldehyde in PBS at room temperature for 1 hour, washed in PBS by centrifugation at $450 \times g$, and resuspended to 2×10^6 cells/ml in PBS containing 0.5 per cent bovine serum albumin fraction V (BSA).

Virus Titrations

Monolayer cultures of MDCK cells were employed to titrate infectious virions in culture media and cell extracts. The titres were calculated as the 50 per cent tissue culture infectious dose ($TCID_{50}$).

Haemagglutination Assay

HA assays of culture media and cell extracts were performed as described previously (12).

Haemadsorption Assay

A 100 µl aliquot of the fixed cell suspension containing 2×10^5 cells was mixed with an equal volume of 0.05 per cent chicken erythrocytes in PBS, and incubated at room temperature for 1 hour with occasional shaking. Haemadsorption was observed under a low power objective and was considered positive if 3 or more erythrocytes were bound to each MDCK cell.

Antisera

Hyperimmune antisera to purified HK, A/Port Chalmers/73 (PC) and A/Victoria/75 (VIC) were raised in rabbits. The rabbits received three multi-site subcutaneous injections of 50—200 µg of virus suspended in Freund's complete adjuvant 5 days apart, followed by a fourth injection of the same dose one month later. They were bled 7 days after receiving the last injection. Goat anti-rabbit and goat anti-mouse γ-globulins were purchased from Grand Island Biological Co., New York. The globulin fractions were precipitated from the antisera with 35 per cent saturated ammonium sulphate and purified by ion exchange chromatography on DEAE-Sephadex A-50 (Pharmacia, Sweden) equilibrated with 0.01 M Tris-HCl buffer containing 0.1 M sodium chloride pH 8.0. Mouse convalescent antisera were obtained by intranasal inoculation of C3H/HeJ mice with a sublethal dose (0.26 HA units) of HK virus, and collecting the sera 30 days later. Sheep anti-rabbit immunoglobulin labelled with fluorescein isothiocyanate was purchased from Wellcome Reagents Ltd., England.

Affinity Chromatographic Separation of Rabbit Hyperimmune Antibodies to HK Virus Structural Proteins

Antibodies to the strain-specific determinants of HK virus haemagglutinin (anti-HA-ST), to the common determinants of haemagglutinin (anti-HA-CM), to the neuraminidase (anti-NA), and to the internal matrix and nucleoprotein antigens (anti-INT) were purified from rabbit hyperimmune anti-HK γ-globulin by differential affinity chromatography on cyanogen bromide-activated Sepharose 4B (Pharmacia, Sweden). The technique and the purity of the separated antibody preparations have been described previously (30). In brief, purified HK, PC and VIC viruses, together with influenza strains A/England/42/72, A/South Africa/64 and recombinant A/England/72—A/Bellamy/42 (H3N1) abbreviated as ENG, SA and ENG-BEL respectively, were disrupted with 0.5 per cent sodium deoxycholate in 0.02 M Tris-HCl buffer, pH 8.0. After overnight dialysis at 4° C against 0.1 M bicarbonate buffer, pH 9.0, containing 0.5 M sodium chloride, the disrupted viruses were coupled to cyanogen bromide-activated Sepharose 4B.

Initially, antibodies to HK virus were separated from the immunoglobulin fraction of the homologous rabbit antiserum by adsorption to the coupled HK virus, the gel washed with PBS, and the virus-specific antibodies eluted in 0.17 M glycine-HCl buffer, pH 2.3, containing 0.5 M sodium chloride. The eluate was dialysed overnight against PBS at pH 7.2. Antibodies to each of the structural proteins of HK virus were then individually separated by cycles of affinity chromatography using heterologous disrupted viruses. Thus anti-HA-ST antibodies were removed by passing the first eluate through gels coupled to ENG, PC and VIC viruses which retained anti-HA-CM, anti-NA and anti-INT antibodies but not anti-HA-ST antibodies. The anti-HA-CM, anti-NA and anti-INT antibodies were eluted with glycine-HCl buffer at pH 2.3, and after overnight dialysis against PBS, were loaded onto a gel coupled to disrupted SA virus, which allowed anti-HA-CM antibodies to pass, but which retained anti-NA and anti-INT antibodies. The anti-NA and anti-INT antibodies were again eluted with

glycine-HCl buffer, dialysed as before, and loaded onto a final gel coupled to disrupted ENG-BEL virus which retained anti-INT antibodies, but allowed anti-NA antibodies to pass through. The anti-INT antibodies were then eluted with glycine-HCl buffer.

Antibodies to the strain-specific haemagglutinin determinants of influenza PC and VIC strains were also separated from their respective rabbit hyperimmune antisera, and employed as controls.

Fluorescent Antibody Assay

A 100 μl aliquot of the fixed cell suspension containing 2×10^5 cells was mixed with an equal volume of rabbit anti-HK antiserum which had been diluted to 1:20 with PBS containing 0.5 per cent BSA. After incubation at room temperature for 1 hour with occasional shaking, the cells were washed three times with PBS containing 0.5 per cent BSA and mixed with an equal volume of sheep anti-rabbit fluorescein conjugate diluted to 1:30 with PBS. The cells were incubated and washed as before, mounted in neutral glycerol, and observed under a fluorescent microscope.

Enzyme-Linked Immunosorbent Assay (ELISA)

The immunoglobulin fractions of goat anti-rabbit and goat anti-mouse γ-globulins were coupled to alkaline phosphatase Type VII (Sigma Chemicals, U.S.A.) by the method of ENGVALL and PERLMANN (10).

Cell extracts and tissue culture media were titrated by ELISA as described previously (30). In brief, the wells of a polyvinyl microtitre plate (Cooke Engineering, U.S.A.) were coated with rabbit anti-HK immunoglobulins diluted to 10 μg/ml in 0.05 M carbonate buffer, pH 9.6. Fourfold dilutions of the test specimens diluted in PBS containing 0.05 per cent Tween 20 were added to the wells and the plate incubated for 1 hour at 37° C. After three washes with 0.05 per cent Tween 20 in 0.85 per cent saline, convalescent mouse antibodies to HK virus were adsorbed to the immobilized viral antigens, and the plates incubated for 1 hour at 37° C. After a further wash, 50 μl of enzyme-linked goat anti-mouse γ-globulin was added to the wells, and the plate again incubated for 1 hour at 37° C. The plates were then washed with 0.05 per cent Tween 20 in 0.85 per cent saline and 50 μl of p-nitrophenyl phosphate (Sigma Chemicals, U.S.A.) diluted to 3 mg/ml in 0.05 M bicarbonate buffer, pH 9.8, containing 0.001 M magnesium chloride, was added to the wells. The enzyme reaction was allowed to proceed for 100 minutes at room temperature, and then stopped by the addition of 25 μl 0.4 M sodium hydroxide. The yellow colour change in the substrate was measured by absorbance at OD 400 nm.

To titrate the cell surface antigens, 100 μl of the fixed cell suspension containing 2×10^5 MDCK cells were mixed with an equal volume of either rabbit anti-HK immunoglobulins diluted to 8 μg/ml in PBS containing 0.5 per cent BSA, or affinity chromatographically purified rabbit antibodies to each of the virion structural proteins diluted to 2 μg/ml in PBS. The mixtures were incubated for 1 hour at room temperature with occasional shaking, and washed three times with PBS containing 0.5 per cent BSA. The cells were resuspended to 100 μl, mixed with an equal volume of enzyme-linked goat anti-rabbit γ-globulin, and incubated and washed as above. The cells were resuspended with 50 μl of p-nitrpohenyl phosphate diluted to 3 mg/ml in 0.05 M bicarbonate buffer, pH 9.8, containing 0.001 M magnesium chloride, and the enzyme reaction allowed to proceed for 100 minutes. The reaction was stopped by the addition of 25 μl 0.4 M sodium hydroxide, and the yellow colour change of the substrate measured by absorbance at 400 nm. The ELISA titres were calculated as the absorbance at OD 400 nm of the test specimen over the absorbance of uninfected negative control specimen (P/N ratio).

Results

Comparative Assays of Viral Antigens and Whole Virions Released from HK-Infected MDCK Cells

Flasks of MDCK cells infected with HK virus were harvested at various intervals from 2 to 48 hours after infection, and the culture media assayed for released

virus particles and antigens by ELISA, HA and infectivity. The results are shown in Fig. 1. Viral antigens were detected in the medium 8 hours after infection by ELISA, the absorbance at OD 400 nm of the test specimen over the absorbence of an uninfected negative control specimen (P/N ratio) being 1.3. The P/N ratio then rose gradually to 2.20 14 hours after infection, and reached a peak of 5.00 after 24 hours. Virus particles were first identified by HA and infectivity assays 10 hours after infection, with peak titres of 3.8×10^4 HA units/flask and 2.88×10^4 $TCID_{50}$/flask after 24 hours. Infectivity titres exhibited a three-fold decrease between 24 and 48 hours after infection, but HA and ELISA titres did not vary greatly over this period.

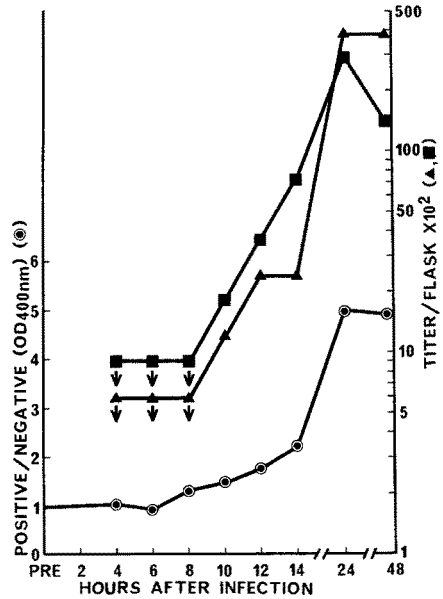

Fig. 1. Virus particles and viral antigens released into the culture media during infection of MDCK cells, titrated by ELISA (●), haemagglutination (▲) and infectivity (■)

The degree of similarity between the titres obtained by the three assay methods for samples harvested at different times from 10 to 24 hours after infection was assessed by their correlation coefficients (r). The correlation coefficients were found to be 0.986 for ELISA to HA, 1.000 for ELISA to infectivity, and 0.987 for HA to infectivity.

Comparative Assays of Intracellular Virus and Viral Antigens in Infected Cell Extracts

Newly synthesized viral antigens were first detected in cell extracts of infected MDCK cells 4 hours after infection by solid-phase ELISA using convalescent mouse anti-HK antiserum, the P/N ratio being 2.20 (Fig. 2). The P/N ratio then continued to increase to 5.80 24 hours after infection, at which time extensive cytopathic effects were observed in the infected monolayers. HA and infectious

virions were first identified 6 hours after infection, and maximum titres of 2.57×10^3 HA units/flask and $7{,}68 \times 10^3$ $TCID_{50}$/flask respectively were attained between 10 and 12 hours after infection. Whereas HA and infectivity titres fell significantly between 12 and 24 hours after infection, the ELISA titres continued to rise as a measure of the accumulation of the total structural and non-structural viral proteins in the cells. This continued accumulation was further emphasized by the low correlation coefficients with ELISA, which were, for all samples titrated from 6 to 48 hours after infection, 0.040 for ELISA: HA, 0.241 for ELISA : infectivity, and 0.855 for HA : infectivity.

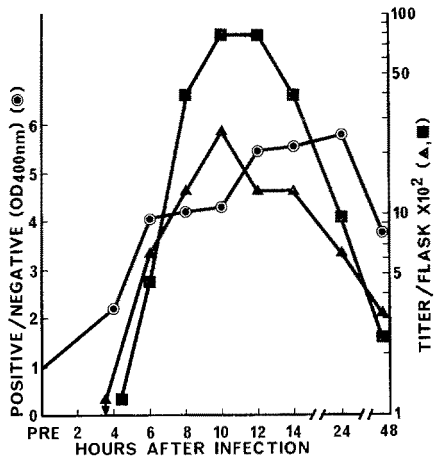

Fig. 2. Virus particles and viral antigens in extracts of infected MDCK cells, titrated by ELISA (⊙), haemagglutination (▲) and infectivity (■)

Assays of Cell Surface Antigens

Uninfected glutaraldehyde-fixed MDCK cells were examined initially by ELISA using affinity chromatographically-purified hyperimmune rabbit antibodies to HK virus and to the HA-ST, HA-CM, NA and INT antigens of HK virus, and to the HA-ST antigenic determinants of PC and VIC viruses. A weak non-specific reaction was observed between the uninfected cells and each of the antibody preparations, the average reading for all the antisera at OD400 nm being 0.083 with a standard deviation of 0.024. Uninfected cells were therefore considered suitable as controls in ELISA titrations of influenza antigens on the surface of infected MDCK cells.

MDCK cells infected with HK virus were harvested at various intervals from 2 to 48 hours after infection and fixed with 0.1 per cent glutaraldehyde as described in materials and methods. The viral antigens exposed on the surface of the infected cells were then titrated by ELISA using affinity chromatographically-purified hyperimmune rabbit anti-HK antibodies. The results are shown in Fig. 3. When antiserum containing antibodies to all the virion structural proteins was employed in ELISA, viral antigens were first observed on the surface of cells 4 hours after infection. The ELISA P/N ratio at this time was 3.71, and it con-

tinued to rise thereafter to a peak of 13.86 at 10 hours after infection, and then dropped slowly to a ratio of 10.00 at 48 hours. However, if purified anti-INT antibodies were used in ELISA, matrix and/or nucleoprotein antigens were detected on the cell surface even earlier, at 2 hours after infection, with an ELISA P/N ratio of 3.17. The P/N ratio using anti-INT antibodies then decreased gradually to be undetectable 8 hours after infection. No other viral antigens were observed at 2 hours. Haemagglutinin HA-ST and HA-CM determinants appeared on the cell surface 4 hours after infection, and reached a plateau 8—10 hours after infection, the P/N ratios for both determinants being essentially similar over the 48 hour period. HA-ST antibodies against heterologous virus strains of the same subtype, anti-PC HA-ST and anti-VIC HA-ST, only exhibited a weak reaction in ELISA against HK antigens with an average P/N ratio over 48 hours of 1.24 ± 0.21. A slight reaction was observed with anti-NA antibodies between 4 and 8 hours after infection, with P/N ratios rising from 1.12 to 1.63 but at later times in the growth cycle the P/N ratios were similar to the heterologous anti-PC HA-ST and anti-VC HA-ST control ratios. The average P/N ratios over 48 hours were 1.54 ± 0.21. However, although low, NA was consistently observed in all experiments.

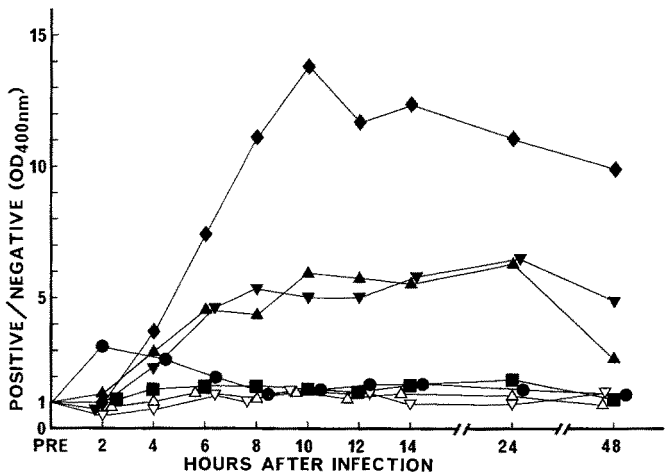

Fig. 3. Virus structural proteins on the surface of HK virus-infected MDCK cells measured by ELISA using anti-HK antiserum (◆), anti-HK HA-ST antibodies (▲), anti-HA-CM antibodies (▼), anti-NA antibodies (■), anti-INT antibodies (●), anti-PC HA-ST antibodies (△) and anti-VIC HA-ST antibodies (▽)

If the glutaraldehyde-fixed cells were stored at 4° C, identical ELISA P/N ratios could be obtained up to 1 week later, providing considerable flexibility to the assay system.

Immunofluorescence and haemadsorption were employed as comparative assays for the detection of viral antigens on the surface of infected, fixed MDCK cells. The results, depicted as the percentage of cells fluorescing with hyperimmune rabbit anti-HK antibodies, or haemadsorbing with chicken erythrocytes, are shown in Fig. 4. Viral antigens were first observed on the surface of a few cells 4 hours

after infection by both techniques. The maximum number of cells exhibiting viral antigens on their surface was attained 10 hours after infection by immunofluorescence (85 per cent) and 12 hours after infection by haemadsorption (84 per cent). These results were therefore consistent with those obtained by ELISA, although the maximum number of cells with viral antigens of their surface occurred 2 hours later in the less sensitive haemadsorption assay. The correlation coefficients for cells harvested from 4 to 48 hours after infection were 0.885 for ELISA : immunofluorescence, 0.712 for ELISA : haemadsorption, and 0.847 for immunofluorescence : haemadsorption.

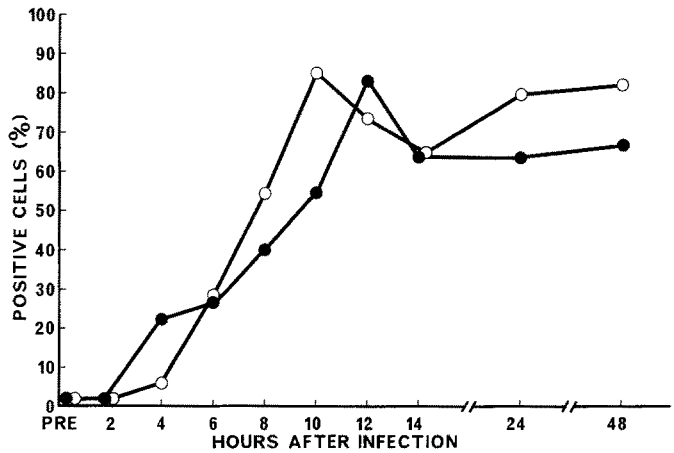

Fig. 4. Viral antigens on the surface of HK virus-infected MDCK cells detected by immunofluorescence (○) and haemadsorption (●)

Discussion

Previous studies have shown that ELISA provides an accurate and sensitive method for titrating influenza viral antigens (30) and for measuring the immune response to influenza infection in mice (31). This report extends the earlier findings by describing the use of ELISA in detecting influenza viral antigens on the surface of infected cells, in cell extracts, and released into the culture media during infection.

Viral antigens were first detected on the surface of infected cells 4 hours after infection by haemadsorption with chicken erythrocytes and by immunofluorescence and ELISA with a hyperimmune rabbit antiserum containing antibodies to all of the HK virion structural proteins. The maximum expression of surface antigens was attained 10—12 hours after infection as defined by the ELISA P/N ratio or by the percentage of cells exhibiting fluorescence or haemadsorption. However, despite high pairwise correlation coefficients, haemadsorption was the least sensitive of the three assay systems employed and only provided evidence of the presence of HA on the cell surface, whereas ELISA and immunofluorescence were also able to measure NA and internal antigens on the cell surface.

It is well established that the two external virion glycoproteins, HA and NA, are present on the cell surface as part of the virus maturation process, and can be

detected by a variety of techniques (23, 24, 29). In addition, recent evidence has indicated that the internal matrix and nucleoprotein antigens may also be expressed on the surface of infected cells. Thus matrix antigen has been demonstrated on the surface of infected P815 cells by cell lysis with anti-matrix antibody and complement (5, 6) and by radio-labelled monoclonal anti-matrix antibody binding to infected P815 cells (16), and matrix protein has been precipitated from the outer surface of influenza-infected L929 cells with purified anti-matrix antibody (1). Nucleoprotein antigen has been detected on the surface of infected cells 2 hours after infection by immunofluorescence (28), but it could not be demonstrated by cell lysis with anti-nucleoprotein antibody and complement (6). In the present study, the expression of individual antigenic determinants was investigated by ELISA using specific antibody preparations purified by differential affinity chromatography (30). Although no distinction could be made between matrix and nucleoprotein antigens, either or both of the two internal proteins were shown to be expressed on the cell surface from 2 to 6 hours after infection, but not thereafter. From experiments of cell lysis in the presence of specific antibody and complement (6), and from the results obtained by REGINSTER *et al.* (27) in which matrix protein rather than nucleoprotein was exposed on the surface of intact and proteolytically-cleaved spikeless particles, it would appear likely that matrix protein rather than nucleoprotein was being expressed. However, the highest ELISA P/N ratio was found 2 hours after infection and it then decreased, whereas in an earlier study the peak expression of matrix antigen measured by antibody and complement-mediated cell lysis occurred 6—7 hours after infection (5). The reason for this discrepancy in the time course of expression is unknown, but it should be noted that the type of infection was different in each study; in one case it was a productive infection in MDCK cells, and in the other, a non-productive infection in P815 mastocytoma cells.

The relevance of either matrix or nucleoprotein antigens on the surface of infected cells early in the replication cycle to protection is uncertain: both are type-specific and immunogenic. Antibodies to nucleoprotein have frequently been observed after virus infection and after immunization, but they did not confer protection on subsequent virus challenge (26). Antibodies to matrix protein, however, have rarely been detected after either infection or immunization (26, 32), and the protective capacity of anti-matrix antibodies raised by immunization with purified matrix protein have given conflicting results. Immunization with purified matrix protein did not confer protection against clinical disease in ferrets (25) or against a lethal infection in mice (4) on subsequent virus challenge, nor did it reduce the severity of lung lesions in mice (32). In contrast, anti-matrix antibodies have been shown to neutralize infectivity and to inhibit haemagglutination *in vitro* (27), and mice immunized with purified matrix protein were able to clear challenge virus more rapidly from their lungs than unimmunized control animals (32). In the latter study, no reduction in the severity of pneumonia was observed in the immunized mice despite the more rapid clearance of virus, and furthermore, an increased delayed-type hypersensitivity reaction was demonstrated after foot-pad incoulation with purified matrix protein. The authors suggested, therefore, that these results were indicative of a cell-mediated response rather than a humoral immune response. Of cell-mediated immune responses to influenza

infection, cytotoxic T-lymphocytes have engendered the most interest. Two populations of cytotoxic T-lymphocytes are generated by immunization with type A influenza viruses; one population is highly specific for the HA of the virus used to immunize, and the other is highly cross-reactive for all type A viruses (8, 9). In some studies, only the former have been recognised (7, 11), whereas in other studies both populations have been described (8, 9). It was generally believed that the cross-reactive T-lymphocytes recognised a type-specific antigen on the surface of infected target cells, the antigen probably being matrix protein. More recent evidence, however, has suggested that cross-reactive T-lymphocytes may recognize a common sequence on HA molecules. Thus cross-reactive T-lymphocytes have been shown to lyse L929 target cells abortively infected with fowl plague virus in which matrix protein synthesis is specifically inhibited, and to lyse artificial target cells composed of lipid vesicles reconstituted with HA and NA but which lacked matrix protein (18). Furthermore, monoclonal antibodies to matrix protein, or anti-matrix protein antibody from hyperimmunized rabbits, did not block lysis of target cells by cross-reactive T-lymphocytes either alone or in combination with anti-H-2 monoclonal antibodies (17), and some anti-HA monoclonal antibodies (but not all) were able to act synergistically with anti-H-2 monoclonal antibodies to inhibit lysis by cross-reactive T-lymphocytes (2). However, although these results suggest that cross-reactive T-lymphocytes recognise a common determinant on HA rather than matrix protein, they do not preclude the presence of a separate subpopulation of cells specific for matrix protein. Indeed the results of KOSZINOWSKI *et al.* (18) with reconstituted lipid vesicles and with the abortive fowl plague infection reflected a negative specificity for matrix protein because of its exclusion. The experiments with anti-INT antibodies in the present communication, together with the sensitivity of ELISA (20, 30), suggest that the expression of matrix protein on the surface of productively infected cells, if it is the antigen detected by anti-INT antibodies, may be inversely related to the expression of HA. Thus maximum expression occurred 2 hours after infection and then decreased, whereas HA was first observed 4 hours after infection and it increased thereafter. In an abortive infection in P815 cells, the ratio of HA to matrix was 100:1 by 16 hours after infection (17). It would be of interest, therefore, to determine whether cross-reactive T-lymphocytes exhibit any cytolytic activity to target cells during the first 3 hours after infection, or to glutaraldehyde-fixed target cells after proteolytic removal of the glycoprotein peplomers.

HK virus HA-ST and HA-CM determinants were found to be expressed on the cell-surface from 4 hours after infection, and the ELISA P/N ratios reached a peak 8—10 hours after infection. Similar ELISA P/N ratios were observed for both determinants. Anti-VIC HA-ST and anti-PC HA-ST antibodies were included as a second control, but ELISA titres were not significantly different to the uninfected cell control. The second external virion glycoprotein, NA, was detected at low levels between 4 and 8 hours after infection in all experiments. It is uncertain whether the low level of detection was due to steric hindrance imposed by the HA peplomers on the anti-NA antibodies, to low avidity of the anti-NA antibodies, or to a significant degree of antigenic drift between HK virus and the A/South Africa/64 (H2N2) strain employed in the differential affinity chromatographic separation (30).

The results of comparative assays on cell extracts harvested at various times after infection indicated that virion structural proteins continue to be accumulated in the cell even after most progeny particles have matured. Moreover, the high correlation coefficients between infectivity, haemagglutination and ELISA of virus particles and antigens released into the culture media strongly suggest that only complete virus particles are released, and very little leakage of individual antigens occurred, even from dead cells.

ELISA has also been successfully employed to detect other cell-associated viral antigens, including rabies virus in brain tissue (3) and feline oncornavirus-associated cell membrane antigen in FL74 cells (22). In the latter study, the ELISA titres showed a close correlation in sensitivity with indirect fluorescence tests, but had the added advantage of being quantitative rather than qualitative. The results presented in this report, together with those using other viruses (3, 19, 22) suggest that ELISA is a potentially useful technique for the detection of specific virus infections in tissue culture, and as a tool for rapid virus diagnosis. Problems have been encountered using peroxidase rather than alkaline phosphatase as the enzyme in ELISA due to endogenous peroxidase in clinical material (13), but no evidence of non-specific staining was observed in this study, probably because the cells had been fixed initially with glutaraldehyde. We believe, therefore, that this study has demonstrated that ELISA can be employed as a rapid technique to detect and to distinguish subtype specificity of influenza A virus in tissue culture, and can potentially be applied to other viruses with cell surface-associated antigens. Moreover, the use of glutaraldehyde-fixation allows considerable flexibility in the timing of the assay and may prove suitable for viral diagnosis at a distance.

Acknowledgments

This study was supported by a research grant from the National Health and Medical Research Council of Australia.

References

1. Ada, G. L., Yap, K. L.: Matrix protein expressed at the surface of cells infected with influenza viruses. Immunochem. **14**, 643—651 (1977).
2. Askonas, B. A., Webster, R. G.: Monclonal antibodies to haemagglutinin and to H-2 inhibit the cross-reactive cytotoxic T cell populations induced by influenza. Europ. J. Immunol. **10**, 151—156 (1980).
3. Atanasiu, P.: Immunofluorescent and immunoperoxidase techniques for the rapid diagnosis of rabies. Ann. de Microbiol. (Institut Pasteur) **126 B**, 69—75 (1975).
4. Becht, H., Gruschkau, H., Rott, R.: The M protein of influenza viruses has no immunizing effect. Med. Microbiol. Immunol. **167**, 285—288 (1979).
5. Biddison, W. E., Doherty, P. C., Webster, R. G.: Antibody to influenza virus matrix protein detects a common antigen on the surface of cells infected with type A influenza viruses. J. exp. Med. **146**, 690—697 (1977).
6. Braciale, T. J.: Immunologic recognition of influenza virus-infected cells. II. Expression of influenza A matrix protein on the infected cell surface and its role in recognition by cross-reactive cytotoxic T cells. J. exp. Med. **146**, 673—689 (1977).
7. Cambridge, G., Mackenzie, J. S., Keast, D.: Cell-mediated immune response to influenza virus infections in mice. Infect. Immun. **13**, 36—43 (1976).
8. Doherty, P. C., Effros, R. B., Bennink, J.: Heterogeneity of the cytotoxic response of thymus-derived lymphocytes after immunization with influenza viruses. Proc. Natl. Acad. Sci. U.S.A. **74**, 1209—1213 (1977).

9. EFFROS, R. B., DOHERTY, P. C., GERHARD, W., BENNINK, J.: Generation of both cross-reactive and virus-specific T-cell populations after immunization with serologically distinct influenza A viruses. J. exp. Med. **145**, 557—568 (1977).
10. ENGVALL, E., PERLMANN, P.: Enzyme-linked immunosorbent assay. III. Quantitation of specific antibodies by enzyme-labelled anti-immunoglobulin in antigen-coated tubes. J. Immunol. **109**, 129—135 (1972).
11. ENNIS, F. A., MARTIN, W. J., VERBONITZ, M. W.: Haemagglutinin-specific cytotoxic T-cell response during influenza infection. J. exp. Med. **146**, 893—898 (1977).
12. FAZEKAS DE ST. GROTH, S., WEBSTER, R. G.: Disquisitions on original antigenic sin. I. Evidence in man. J. exp. Med. **124**, 331—345 (1966).
13. GARDNER, P. S., GRANDIEN, M., MCQUILLIN, J.: Comparison of immunofluorescence and immunoperoxidase methods for viral diagnosis at a distance: a W.H.O. collaborative study. Bull. Wld. Hlth. Org. **56**, 105—110 (1978).
14. GAUSH, C. R., SMITH, T. F.: Replication and plaque assay of influenza virus in an established line of canine kidney cells. Appl. Microbiol. **16**, 588—594 (1968).
15. GERHARD, W., WEBSTER, R. G.: Antigenic drift in influenza A viruses. I. Selection and characterization of antigenic variants of A/PR/8/34 (H0N1) influenza virus with monoclonal antibodies. J. exp. Med. **148**, 383—392 (1978).
16. HACKETT, C. J., ASKONAS, B. A., WEBSTER, R. G., VAN WYKE, K.: Monoclonal antibodies to influenza matrix protein: detection of low levels of matrix protein on abortively infected cells. J. gen. Virol. **47**, 497—501 (1980).
17. HACKETT, C. J., ASKONAS, B. A., WEBSTER, R. G., VAN WYKE, K.: Quantitation of influenza virus antigens on infected target cells and their recognition by cross-reactive cytotoxic T-cells. J. exp. Med. **151**, 1014—1025 (1980).
18. KOSZINOWSKI, U. H., ALLEN, A., GETHING, M.-J., WATERFIELD, M. D., KLENK, H.-D.: Recognition of viral glycoproteins by influenza A-specific cross-reactive cytolytic T lymphocytes. J. exp. Med. **151**, 945—958 (1980).
19. KURSTAK, E., KURSTAK, C.: Application of the immunoperoxidase technique in virology and cancer immunology. In: KURSTAK, E., MORRISET, R. (eds.), Viral Immunodiagnosis, 3—30. New York: Academic Press 1974.
20. LAMBRE, C., KASTURI, K. N.: A microplate immunoenzyme assay for anti-influenza antibodies. J. immunol. Meth. **26**, 61—67 (1979).
21. LAVER, W. G., DOWNIE, J. C., WEBSTER, R. G.: Studies on antigenic variation in influenza virus. Evidence for multiple antigenic determinants on the haemagglutinin subunits of A/Hong Kong/68 (H3N2) virus and the A/England/72 strain. Virology **59**, 230—244 (1974).
22. LOG, T., CHANG, K. S. S.: Enzyme immunoassay for feline oncornavirus-associated cell membrane antigen (FOCMA) and detection of FOCMA in cell extract by enzyme immunoassay inhibition test. J. immunol. Meth. **26**, 291—303 (1979).
23. MACKENZIE, J. S.: Studies with temperature-sensitive mutants of influenza virus. In: BARRY, R. D., MAHY, B. W. J. (eds.), The biology of large RNA viruses, 507 to 534. New York: Academic Press 1970.
24. MORGAN, C., RIFKIND, R. A., ROSE, H. M.: The use of ferritin-conjugated antibodies in electron microscopic studies of influenza and vaccinia viruses. Cold Spring Harbor Symp. Quant. Biol. **27**, 57—65 (1962).
25. OXFORD, J. S., SCHILD, G. C.: Immunological and physicochemical studies of influenza matrix (M) polypeptides. Virology **74**, 394—402 (1976).
26. POTTER, C. W., OXFORD, J. S.: Determinants of immunity to influenza infection in man. Brit. med. Bull. **35** (1), 69—75 (1979).
27. REGINSTER, M., JOASSIN, L., FONTAINE-DELACOMBE, P.: Ligands for antibody to M protein are exposed on the surface of influenza virions: effect of proteolytic treatment on their activity. J. gen. Virol. **45**, 283—289 (1979).
28. VIRELIZIER, J. L., ALLISON, A. C., OXFORD, J. S., SCHILD, G. C.: Early presence of ribonucleoprotein antigen on the surface of influenza virus-infected cells. Nature **266**, 52—54 (1977).
29. VOGEL, J., SHELEKOV, A.: Adsorption-haemagglutination test for influenza virus in monkey kidney tissue culture. Science (U.S.A.) **126**, 358—359 (1957).

30. WATANABE, H., POLLETT, M., MACKENZIE, J. S.: Purification of antibodies to influenza A virus structural proteins by affinity chromatography, and their participation in haemagglutination-inhibition, neutralization and enzyme-linked immunosorbant assay. Aust. J. exp. Biol. med. Sci. **57**, 303—312 (1979).
31. WATANABE, H., POLLETT, M., MACKENZIE, J. S.: The use of enzyme-linked immunosorbent assay to determine the appearance, specificity and immunoglobulin class of antibodies to the structural proteins of influenza A virus in mice. Aust. J. exp. Biol. med. Sci. **58**, 189—199 (1980).
32. WEBSTER, R. G., HINSHAW, V. S.: Matrix protein from influenza A virus and its role in cross-protection in mice. Infect. Immun. **17**, 561—566 (1977).
33. WRIGLEY, N. G.: Electron microscopy of influenza virus. Brit. med. Bull. **35** (1), 35—38 (1979).

Authors' address: Dr. J. S. MACKENZIE, University Department of Microbiology, Queen Elizabeth II Medical Centre, Nedlands, Western Australia 600 9.

Received June 24, 1980