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Cross-reactivity of Malaysian rat cytomegalovirus strains with its human counterpart

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Abstract. This study probes into the prospect of cross-reactivity of HCMV with RCMV which has not been acknowledged to date. We describe the uncovering of a protein with an estimated size of between 61-68 kDa from local RCMV strains which reacted with HCMV positive sera. Our findings are a first disclosure of a plausible immunological cross-reactivity between RCMV with its human counterpart which grounds substantial interest implying existence of conserved determinants between rat and human CMV polypeptides. The cross-reactive protein most likely represents an enveloped glycoprotein, though the precise identification and its degree of similarity needs to be evidently defined and further elucidated in forthcoming experiments.

INTRODUCTION

It is generally acknowledged that herpesvirus can infect a wide-ranging cell range (Sinzger *et al.*, 2008) yet exhibits strict host specificity and cytomegalovirus (CMV) is of no exception. Nevertheless, previous literature have also described cross-species infectivity and protein counterparts in human cytomegalovirus (HCMV) which are conserved, immunologically cross-reactive or structurally related with other CMV proteins of other species (Pande *et al.*, 1991, Perot *et al.*, 1992, Degré *et al.*, 2001, Davison *et al.*, 2003, Tang & Maul, 2006, Whitteker *et al.*, 2008, Millard *et al.*, 2010). Similarly antigenic cross-reactions have been described among proteins from a number of viruses (Balachandran *et al.*, 1987; Tsai *et al.*, 1990) suggestive of the existence and sharing of common immunogenic epitopes between different species of CMV and within different viruses.

Animal models resembling that of humans are in constant demand to aid studies on HCMV infection (Bravo *et al.*, 2003, Barry *et al.*, 2006, Kern, 2006; Schleiss, 2006). Maastricht and English rat cytomegalovirus (RCMV) strains are well characterized RCMVs at both the molecular and the biological level (Bruggeman *et al.*, 1982, Priscott & Tyrell, 1982, Vink *et al.*, 2000) and have thus been exploited as surrogate systems to provide us improved understanding on CMV infection. In Malaysia, novel strains of local RCMV; namely UPM/Sg and RCMVALL-03 were isolated from *Rattus argentiventer* (Lai *et al.*, 1999) and *Rattus rattus diardii* (Loh *et al.*, 2003) respectively. The latter is of considerable interest as its isolation from the uterus and placenta offers advantages over other models since it can

be transmitted to the fetus transplacentally, causing infection *in utero* (Loh *et al.*, 2006). For this reason the use of RCMV ALL-03 may hypothetically provide greater insights on CMV targets for future development of new antiviral therapies designed to impede vertical virus transmission.

RCMV shares many biological properties and bears resemblances with HCMV in countless aspects, including the pathogenesis of infection which has been predicted to be very similar (Stals *et al.*, 1990). Such comparable characteristics connote the existence of conserved determinants between proteins of these two species of CMV. Nevertheless, in spite of the promising potential of the two Malaysian strains of RCMV as animal models to imitate infections in human, scarce information is available at the protein and molecular level. Little is known about the similarity that exists between the two species. The proteins of the Malaysian RCMV strains are undisclosed and to this date, there has been no existing study on the correlation between RCMV with its human counterpart. The potential exploitation of RCMV ALL-03 as an animal model necessitates the identification of immunogenic viral proteins that are conserved between the two species. Hence, the primary goal of this preliminary study is to identify potential cross-reactive targets or common complementary regions in HCMV and RCMV that could give rise to future innovations. The present study was undertaken to identify the protein compositions of different strains of RCMV by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and to explore the possibility of immunological cross-reactions between RCMV with its human counterpart.

MATERIALS AND METHODS

Cells and Viruses

Monolayers of rat embryonic fibroblast (REF), Vero and *Crandell-Reese feline kidney* cells (CrFK) were grown in Dulbecco modified Eagle medium (DMEM; Gibco Laboratories, Grand Island, N.Y., USA) RPMI-1640 medium (Gibco Laboratories, Grand Island N.Y., USA) and Minimum Essential Medium (MEM; ATCC, Virginia, USA) respectively, supplemented with antibiotics and 10% (v/v) heat-inactivated fetal bovine serum (Biowest, South America Origin, Nuaille, France). Three RCMV isolates were used in this study namely local RCMVALL-03, local UPM/Sg and RCMV-E (ATCC VR-991). In addition, three Pseudorabies virus (PRV; Aujeszky's disease virus) isolates (PRV-CD, PRV-IUDR5, PRV-AIP) and a GPE$^-$ Japanese vaccine strain of Classical Swine Fever Virus (CSFV) were kindly obtained from Dr. Zeenathul Nazariah Allaudin from the Virology Laboratory, Faculty of Veterinary Medicine, UPM, Malaysia.

Virus propagation and purification

In general, monolayer cultures of appropriate cells were infected with the respective viruses. Infected cell cultures exhibiting advanced cytopathic effects (CPE) were harvested from tissue culture supernatant, clarified from cell debris by mild centrifugation, concentrated by 8% (w/v) polyethylene glycol (PEG 6000; Calbiochem, Darmstadt, Germany) precipitation and purified by sucrose density gradient centrifugation method at 40,000 rpm for 16 h in a Sorvall® *WX Ultra-100* centrifuge (TH-641 rotor, *Thermo Electron Corp.*, *Massachusetts*, USA). Visible bands containing purified virus were collected and pelleted at 40,000 rpm for 2 h at 4ºC before being re-suspended in a small volume of TNE [10 mM Tris, 200 mM NaCl, 1 mM EDTA] and stored at -20ºC until further use.

Sera

Residual blood samples that had been submitted for routine serological testing were separated by centrifugation and serologically screened for CMV antibodies using ENZYWELL CMV IgG kit (Diesse; Monteriggioni, Italy) according to the manufacturer's instructions. Anti-CMV IgG antibody content was determined spectrophotometrically (SPECTRAmax; Molecular Devices, California, USA) at 405 nm and only HCMV seropositive sera were used in this study.

SDS-PAGE

The Laemmli (1970) method of the SDS-PAGE was used to fractionate the RCMV, PRV and CSFV viral proteins. Cells and viruses were lysed in sample solubilization buffer and denatured proteins were resolved on a 10% (w/v) SDS-PAGE gel under reducing conditions. Proteins were visualized by staining the gels with Coomassie brilliant blue and molecular weights (M_r) of protein were estimated by comparing with co-migrating pre-stained Spectra™ BR protein marker (Fermentas Inc., Maryland, USA). The estimate sizes of the proteins were determined by the AlphaImager™ gel documentation system (Alpha Innotech Corp.; San Leandro, CA, USA) based on comparisons with co-migrating protein markers.

Western Blotting

The electrophoretic transfer of the polyacrylamide gel resolved proteins and its immunological identification were carried out as described by Towbin *et al.* (1979) with some modifications. A Trans-Blot® Semi-Dry Electrophoretic Transfer Cell (Bio-Rad Laboratories Inc., California, USA) was used for this purpose according to the manufacturer's instructions. 0.45 µm-pore-size PVDF membranes (Osmonics Inc., Minessota, USA) were exposed to 1:100 dilutions of HCMV seropositive human sera for 1 h and washed with PBS-T [Phosphate buffered saline (pH 7.4) with 0.05% Tween-20]. Bound antibody was detected with 1:2000 dilution of horseradish peroxidase-conjugated rabbit anti-human IgG (Abcam; Cambridge, UK) for 1 h followed by washing with PBS-T. The immunoreative bands were developed by ABTS® peroxidase substrate (KPL; Maryland, USA) rinsed with water, air dried and photographed.

RESULTS

Cytopathogenicity

RCMV exhibited CPE-like features including alterations in size, shape and morphology in contrast to control REF cells which remained intact. Focal rounding and accumulation of enlarged cells were apparent creating plaques that increased in number and size following infection with the viral agent. CPE progressed slowly over a two week period to involve 80 to 90% of the entire cell monolayer as depicted in Figure 1.

Virus polypeptides

The protein profile of the Malaysian strains of RCMV, alongside CSFV, PRV and cell lysates is shown in Figure 2. The number and distribution of polypeptides of our local RCMV were comparable to that of the reference strain with nominal differences. Common extracellular virions of RCMV (Lanes 2-4) were composed of eight common

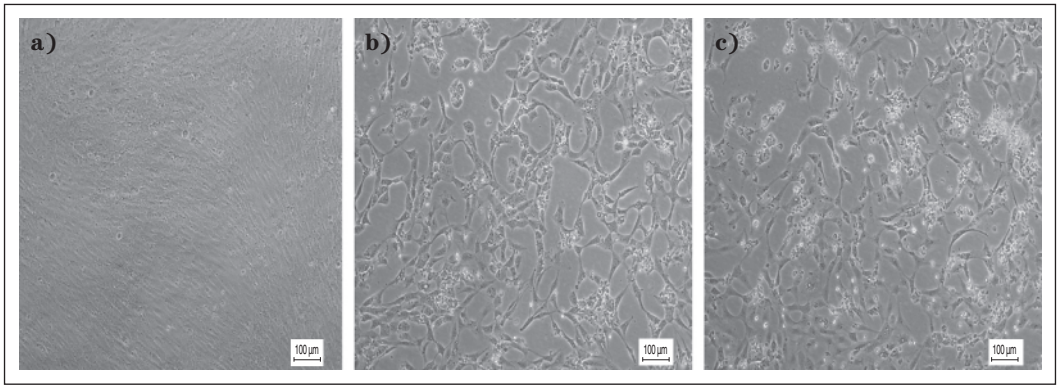

Figure 1. Typical cytopathic developments in REF cells following infection with RCMV ALL-03
Panels a: Mock-infected REF cells showing intact fibroblastic monolayer. Panel b: Numerous small foci surrounded by enlarged rounded cells were evident at days 8-10 p.i. Panel c: Infected REF cells demonstrating extensive plaque formation, cell degeneration and detachment throughout the cell monolayer at 10-14 days p.i indicating the spread of the viral agent on the monolayer (Magnification x 20, Bar = 100µm)

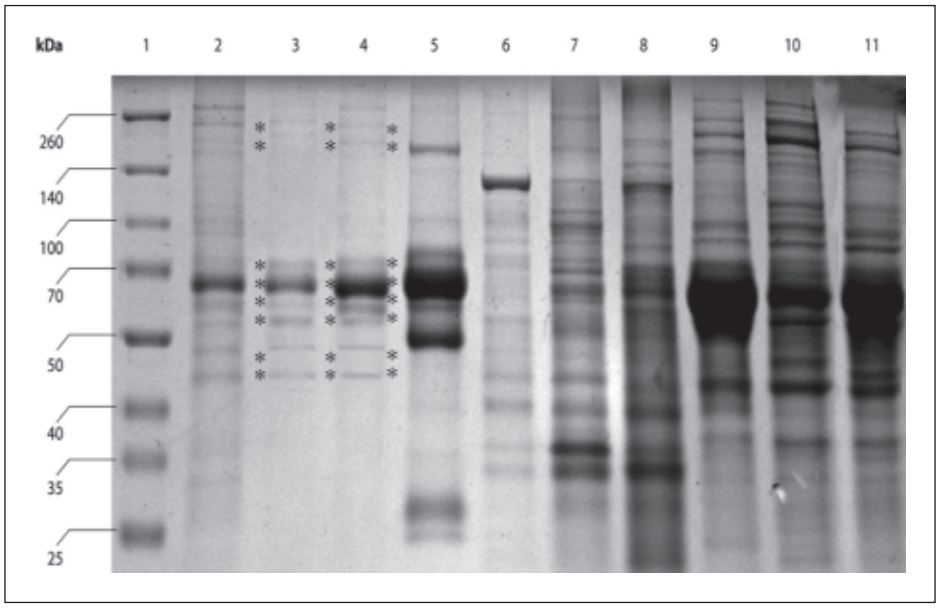

Figure 2. Electrophoretic mobility of virus and control cell proteins separated under reducing conditions and stained with Coomasie Brilliant Blue
Lane 1: Spectra^{TM} BR protein marker, Lane 2: RCMVALL-03, Lane 3: UPM/Sg, Lane 4: RCMV-E, Lane 5: stock CSFV, Lane 6: PRV-CD, Lane 7: PRV-IUDR5; Lane 8: PRV-AIP, Lane 9: Vero cell, Lane 10: CrFK cell and Lane 11: REF cell. The protein profiles of RCMV were analogous to each other (* represents common bands) with some minor differences in band intensities and molecular weights

protein bands, the M_r estimated to be at 44 kDa, 48 kDa, 53 kDa, 57 kDa, 61kDa, 76 kDa, 197 kDa and 231 kDa. However, slight variations exists, as the relative intensities of protein bands with M_r between 70 and 140 kDa were reduced in the UPM/Sg and RCMV-E strain compared to RCMVALL-03 and for this reason, some bands were only apparent in RCMV ALL-03 and lacking in the other two strains. It is highly anticipated that the proteins detected are all of viral origin, nonetheless the possibility of cellular contaminants could not be completely excluded, especially bands of higher M_r. The SDS-PAGE also revealed variations in the protein fingerprint patterns showing evidence in diversity among RCMV, CSFV, and PRV isolates. For CSFV (lane 5), there are a number of bands that were exclusively confined to CSFV (M_r between 23-30 kDa, 35 kDa, 40kDa and 200 kDa) which were absent in RCMV. Similarly, the three protein profiles of PRV isolates (lanes 6-8) were generally analogous to each other with the common polypeptide bands similar to those described previously. [Supporting evidence is provided at the end of this manuscript].

Detection of a 61-68 kDa band by Western blot

RCMV proteins with an estimated molecular weight (M_r) of between 61–68 kDa was found to react strongly with the HCMV positive serum and produced a noticeable immunostaining signal on Western blot in RCMVALL-03, UPM/Sg and RCMV-E strains as seen in Figure 3. The reactivity however was only observed within RCMV but was absent in CSFV, PRV isolates and in uninfected control cells.

DISCUSSION

The analysis of viral SDS-PAGE protein patterns is an effective means of differentiating virus strains at species and intra species level and was thus used in this study to compare polypeptide patterns of virions. In general, our results as depicted

in Figure 2 revealed superficial resemblance of RCMV at the species level, yet the patterns were remarkably different to that of PRV and CSFV isolates. To our knowledge, this is the first report to date with regards to protein profiles of the local RCMV ALL-03. Lai *et al.*, 1999 analyzed UPM/Sg and RCMV-E polypeptide banding patterns and documented that the common protein bands were estimated to have M_r of 32 kDa, 40 kDa, 44 kDa, 50 kDa, 51 kDa, 55 kDa, 85 kDa and 96 kDa (unpublished findings). The slight discrepancy in the molecular weight of the proteins observed in our profiles and the ones made earlier may be explained by the different concentrations of starting material, sensitivities in the technique and incongruities in the technical parameters used which could alter the migration rates of proteins. Furthermore, the existence of thick bands may represent mobility overlaps of several proteins that failed to separate, making the determination of an exact M_r intricate giving rise to dissimilar findings.

We identified in this study the cross-reactivity of HCMV with RCMV. Intriguingly, despite the fact that polyclonal sera were used, only a single RCMV protein was immunoreactive with HCMV as shown in Figure 3, which was not found in other viral and control cell proteins. This single display of cross reactivity implies the presence of common antigenic determinants confined exclusively to the CMV group. We crudely determined the size of the expressed protein, which had an apparent M_r of between 61-68 kDa. Observations from our analysis were reinforced and validated by an additional study utilizing monoclonal antibody (mAB) with target specificity for human CMV glycoprotein B (gB) (unpublished observations). Indistinguishable results were obtained, whereby RCMV proteins of the same molecular weight were expressed, strengthening the possibility of a cross reaction between RCMV and its human counterpart.

Although the precise identity of the protein cannot be clearly defined, based on the predictable size, comparative information using specific CMV gB mAb and the absence of this band in the negative controls, it is highly anticipated that the identified band seen in Figure 3 probably represents, or is at least antigenically related to gB (gp55-116). Moreover, this assumption is further corroborated by the fact that most of the neutralizing antibodies detected in human immune sera react with gB (Weber *et al.*, 1993). This glycoprotein is copious, representing greater than 50% of the protein mass envelope, which can be readily identified following SDS-PAGE without the need of additional immunological techniques (Britt & Mach, 1996). gB is essential for HCMV infectivity, inoculations of which have been known to produce neutralizing antibodies and induce cellular responses (Britt *et al.*, 1990, Marshall 1992), thus can be considered as immunological targets and probable candidates for subunit vaccines.

Our data described in this report are first demonstration of cross-reactivity between novel Malaysian RCMV strains with its human counterpart. The protein detected in this study may be a potential target, but its discovery at the present time is based on unspecific estimates of molecular weights and its exact role in host immunity is yet to

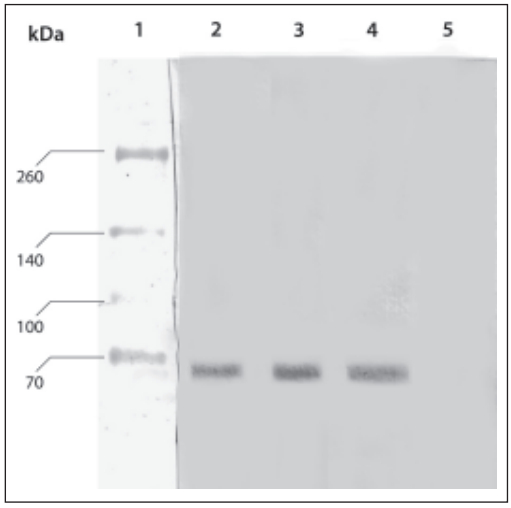

Figure 3. Immunological reactivity of RCMV with HCMV. Protein bands were probed with HCMV positive human sera, followed with horseradish peroxidase-conjugated rabbit anti-human IgG and visualized by ABTS
Lane 1: Spectra™ BR protein marker, Lane 2: RCMVALL-03, Lane 3: UPM/Sg, Lane 4; RCMV-E, Lane 5; REF control cells. The black arrow indicates the position of RCMV protein (M_r between 61-68 kDa) which reacted with HCMV positive sera

be determined. Further studies must be performed as the true relevance of these findings can only be established following improved efforts directed towards the determination and the characterization of the nature and specificity of this cross-reactive protein. Additional studies are eagerly awaited towards the uncovering of this promising protein which could help define targets for future development of antiviral compounds or improvements in diagnostic testing of CMV infection.

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