4. Botzler C, Li G, Issels RD, Multhoff G (1998) Definition of extracellular localized epitopes of Hsp70 involved in an NK immune response. Cell Stress Chaperones 3: 6–11
5. Botzler C, Schmidt J, Luz A, Jennen L, Issels R, Multhoff G (1998) Differential Hsp70 plasma-membrane expression on primary human tumors and metastases in mice with severe combined immunodeficiency. Int J Cancer 77: 942–948
6. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254
7. Burke DS, Nisalak A, Johnson DE, Scott RM (1988) A prospective study of dengue infections in Bangkok. Am J Trop Med Hyg 38: 172–180
8. Chen Y, Maguire T, Hileman RE, Fromm JR, Esko JD, Linhardt RJ, Marks RM (1997) Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate. Nat Med 3: 866–871
9. Chen YC, Wang SY, King CC (1999) Bacterial lipopolysaccharide inhibits dengue virus infection of primary human monocytes/macrophages by blockade of virus entry via a CD14-dependent mechanism. J Virol 73: 2650–2657
10. Chevalier M, Rhee H, Elguindi EC, Blond SY (2000) Interaction of murine BiP/GRP78 with the DnaJ homologue MTJ1. J Biol Chem 275: 19620–19627
11. Crill WD, Roehrig JT (2001) Monoclonal antibodies that bind to domain III of dengue virus E glycoprotein are the most efficient blockers of virus adsorption to Vero cells. J Virol 75: 7769–7773
12. Daughaday CC, Brandt WE, McCown JM, Russell PK (1981) Evidence for two mechanisms of dengue virus infection of adherent human monocytes: trypsin-sensitive virus receptors and trypsin-resistant immune complex receptors. Infect Immun 32: 469–473
13. Delpino A, Castelli M (2002) The 78 kDa glucose-regulated protein (GRP78/BiP) is expressed on the cell membrane, is released into cell culture medium and is also present in human peripheral circulation. Biosci Rep 22: 407–420
14. Di Cesare S, Poccia F, Mastino A, Colizzi V (1992) Surface expressed heat-shock proteins by stressed or human immunodeficiency virus (HIV)-infected lymphoid cells represent the target for antibody-dependent cellular cytotoxicity. Immunology 76: 341–343
15. Freiden PJ, Gaut JR, Hendershot LM (1992) Interconversion of three differentially modified and assembled forms of BiP. EMBO J 11: 63–70
16. Haas IG (1994) BiP (GRP78), an essential hsp70 resident protein in the endoplasmic reticulum. Experientia 50: 1012–1020
17. Haas IG, Wabl M (1983) Immunoglobulin heavy chain binding protein. Nature 306: 387–389
18. Halstead SB (1989) Antibody, macrophages, dengue virus infection, shock, and hemorrhage: a pathogenetic cascade. Rev Infect Dis 11: S830–S839
19. Hung SL, Lee PL, Chen HW, Chen LK, Kao CL, King CC (1999) Analysis of the steps involved in Dengue virus entry into host cells. Virology 257: 156–167
20. Ishiyama T, Koike M, Akimoto Y, Fukuchi K, Watanabe K, Yoshida M, Wakabayashi Y, Tsuruoka N (1996) Heat shock-enhanced T cell apoptosis with heat shock protein 70 on T cell surface in multicentric Castleman's disease. Clin Exp Immunol 106: 351–356
21. Kahn-Perles B, Salamero J, Jouans O (1994) Biogenesis of MHC class I antigens: involvement of multiple chaperone molecules. Eur J Cell Biol 64: 176–185
22. Martinez-Barragan JJ, del Angel RM (2001) Identification of a putative coreceptor on Vero cells that participates in dengue 4 virus infection. J Virol 75: 7818–7827

23. Moreno-Altamirano MM, Sanchez-Garcia FJ, Munoz ML (2002) Non Fc receptor-mediated infection of human macrophages by dengue virus serotype 2. J Gen Virol 83: 1123–1130
24. Nguyen TL, Nguyen TH, Tieu NT (1997) The impact of dengue haemorrhagic fever on liver function. Res Virol 148: 273–277
25. Rosen L, Khin MM, UT (1989) Recovery of virus from the liver of children with fatal dengue: reflections on the pathogenesis of the disease and its possible analogy with that of yellow fever. Res Virol 140: 351–360
26. Shin BK, Wang H, Yim AM, Le Naour F, Brichory F, Jang JH, Zhao R, Puravs E, Tra J, Michael CW, Misek DE, Hanash SM (2003) Global profiling of the cell surface proteome of cancer cells uncovers an abundance of proteins with chaperone function. J Biol Chem 278: 7607–7616
27. Tassaneetrithep B, Burgess TH, Granelli-Piperno A, Trumpfheller C, Finke J, Sun W, Eller MA, Pattanapanyasat K, Sarasombath S, Birx DL, Steinman RM, Schlesinger S, Marovich MA (2003) DC-SIGN (CD209) mediates dengue virus infection of human dendritic cells. J Exp Med 197: 823–829
28. Triantafilou M, Fradelizi D, Triantafilou K (2001) Major histocompatibility class one molecule associates with glucose regulated protein (GRP) 78 on the cell surface. Hum Immunol 62: 764–770
29. Triantafilou K, Fradelizi D, Wilson K, Triantafilou M (2001) GRP78, a coreceptor for coxsackievirus A9, interacts with major histocompatibility complex class I molecules which mediate virus internalization. J Virol 76: 633–643
30. Xiao G, Chung TF, Pyun HY, Fine RE, Johnson RJ (1999) KDEL proteins are found on the surface of NG108-15 cells. Brain Res Mol Brain Res 72: 121–128

Author's address: Dr. Duncan R. Smith, Molecular Pathology Laboratory, Institute of Molecular Biology and Genetics, Mahidol University, Salaya Campus, 25/25 Phuttamonthol Sai 4, Nakorn Pathom, 73170, Thailand; e-mail: duncan_r_smith@hotmail.com

Intervirology

International Journal of Basic and Medical Virology

itor-in-Chief Prof Dr med UWE GERD LIEBERT

torial Office S Karger AG
Editorial Department
INTERVIROLOGY
P O Box
CH-4009 Basel (Switzerland)
Tel +41 61 306 13 44
Fax +41 61 306 14 34
E-Mail s aeschbach@karger ch

April 20, 2004 /aes

Prof. Duncan R. Smith, PhD
Molecular Pathology Laboratory
Institute of Molecular Biology
and Genetics
Mahidol University
Salaya Campus
25/25 Phutthamonthol Sai 4
Nakorn Pathom
73170
Thailand

by fax no. 00662 441 9906

VERSION 1

Manuscript no. 47414, entitled
Virus overlay protein binding assay (VOPBA) reveals serotype specific heterogeneity of dengue virus binding proteins on hepg2 human liver cells

Dear Professor Smith,

Although we think it would be better if you commented whether there are any difference of infectious rate to HepG2 cell among serotyopes we have, nevertheless, decided to accepted your manuscript for publication in INTERVIROLOGY. Now, so that the typesetting can proceed, we need to have a copy of the paper on a disk. Please send it to Ms. Nadine Schwald in our Production Department 6 at the above address. After that, the galley proofs will be sent to you for correction as soon as possible.

Sincerely yours,
S. Karger AG, Basel
Editorial Office
INTERVIROLOGY

Susan Aeschbach-Jagger
Secretary to Prof. O. Hino

VIRUS OVERLAY PROTEIN BINDING ASSAY (VOPBA) REVEALS SEROTYPE SPECIFIC HETEROGENEITY OF DENGUE VIRUS BINDING PROTEINS ON HepG2 HUMAN LIVER CELLS.

Sumalee Jindadamrongwech and Duncan R. Smith*

Molecular Pathology Laboratory

Institute of Molecular Biology and Genetics

Mahidol University

*To whom all correspondence should be addressed at:

Molecular Pathology Laboratory

Institute of Molecular Biology and Genetics

Mahidol University, Salaya Campus

25/25 Phuttamonthol Sai 4

Nakorn Pathom, 73170

Thailand

Tel: 662 – 441 - 9003

Fax: 662 – 441 – 9906

Email: duncan_r_smith@hotmail.com

Key Words: dengue, flavivirus, HepG2, liver, receptor

Running title: Dengue virus binding proteins

ABSTRACT

Objective: This study sought to investigate the presence of dengue virus binding proteins expressed on the surface of HepG2 cells and to determine if there were serotype specific differences in binding.

Methods: HepG2 cell membrane proteins were extracted and separated by SDS-PAGE, transferred to nitrocellulose membranes and incubated with dengue virus serotypes 2, 3 and 4 under varying hybridization conditions. The positions of dengue virus binding proteins were established with a pan specific anti-dengue virus monoclonal antibody.

Results: Dengue virus binding proteins were seen at approximately 78-80, 90, 98, and 102 kDa for dengue serotype 2, 90, 130 and 182 kDa for dengue serotype 3 and 90 and 130 kDa for dengue serotype 4. Binding of the serotypes 3 and 4 was significantly altered by the hybridization conditions, while serotype 2 was affected to a lesser extent.

Conclusions: The virus overlay assay used here provides further evidence that there is a serotype specific component regulating the entry of the dengue virus into cells. Given that several virus binding proteins are seen for each serotype, multiple proteins may be required to facilitate the entry of the virus into some cell types.

INTRODUCTION

The tissue tropism of a virus is a key determinant of viral pathogenicity, and this is often modulated by the presence or absence of appropriate molecules on the surface of a cell that can be used by the virus to gain entry into that cell. The dengue virus consists of four closely related but antigenically distinct serotypes termed dengue 1 to dengue 4 and is known to infect and replicate in several cell types, however the nature of the viral receptors is still unclear, although evidence suggests that the entry of the virus may be modulated by both cell and serotype specific factors [1].

While immune cells such as monocytes-macrophages and T lymphocytes have been proposed to be the main targets of the dengue virus [2], non-immune cells such as hepatocytes, endothelial cells, and brain cells have also been reported as potential targets [3 – 6]. Liver specimens from fatal cases of dengue fever have been shown to contain dengue virus antigens [7], and the virus itself has been isolated from the liver of fatal cases of dengue [8]. Additionally, changes in liver function have been associated with severe cases of dengue infection [9] suggesting that hepatocytes may be a target for the virus especially in the severe, fatal cases [10]. In a recent report we used the virus overlay protein binding assay to identify and characterize a dengue virus serotype 2 binding molecule expressed on the surface of HepG2 cells (a human liver cell line) and showed that antibodies to GRP78 inhibited infection, although only by approximately 2 fold [11]. Given that the dengue virus comprises of four closely related, but antigenically distinct serotypes we sought to further evaluate the binding of two additional serotypes of the dengue virus to HepG2 membrane proteins to determine the similarity of binding of these additional serotypes.

MATERIALS AND METHODS

Virus and cells

Dengue virus serotype 2 (strain 16681), serotype 3 (strain 16562), and serotype 4 (strain 1036) were kindly provided by Dr Siritorn Butrapet (Center for Vaccine Development, Mahidol University, Thailand). Each serotype was propagated in Vero cells at 37 °C, 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM; HyClone, Logan, Utah, USA) supplemented with 5% heat inactivated fetal bovine serum (FBS; Gibco BRL, Gaithersburg, MD, USA) and 100 units of penicillin and 100µg streptomycin/ml. Virus was purified by sucrose gradient ultracentrifugation at 4 °C and resuspended in TNE buffer (10 mM Tris-HCl, pH 7.5, 140mM NaCl, 1mM EDTA) containing 1% BSA. The human hepatoma cell line (HepG2) was cultured at 37°C, 10% CO_2 in 10% FBS-DMEM supplemented with 100 units of penicillin and 100µg streptomycin/ml.

Cell membrane Preparation

HepG2 cells were scraped from culture plates and washed in TBS (50mM Tris HCl, pH 7.6, 150mM NaCl). Cells were lysed in ice cold buffer M (100mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 1mM EDTA, 0.2% Triton X-100, 1 mM PMSF) by vortexing and centrifuged at 600g for 3 minutes to remove nuclei and cell debris. The supernatant was collected and centrifuged at 6,000g for 5 minutes to remove membranous organelles and at 20,000g to pellet membrane proteins. The pellet was resuspended in buffer M and keep at -80°C until required. Protein concentrations were determined by the Bradford method [12].

Viral overlay protein binding assay (VOPBA)

A total of 80μg HepG2 membrane proteins per lane were separated on an 8% SDS-PAGE gel and transferred overnight to nitrocellulose membranes by the WetBlot technique (Bio-Rad, Richmond, CA, USA). The membranes were blocked with 5% skim milk in TBS at room temperature (RT) for 1 hour with constant agitation. A total of $1x10^7$ pfu purified dengue virus in 1% skim milk in TBS was incubated with the membranes for 2 hours at 4°C, RT, or 37 °C as indicated in the results. After three washes with TBS, the membranes were incubated with a pan specific anti-dengue virus monoclonal antibody (HB114), a gift from Dr. Siritorn Butrapet, at a dilution of 1:100 in 5% skim milk in TBS for 2 hours at RT. The membranes were washed and then incubated with HRP-conjugated rabbit anti-mouse IgG (Sigma Chemical Co., St Louis, MO, USA) at a dilution of 1:3000 in 5% skim milk in TBS for 1 hour at RT. The signal was generated by the ECL Plus Western Blotting Analysis kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). A reaction with no virus was used as the negative control in each experiment.

RESULTS

To characterize the binding of the dengue virus serotypes 2, 3 and 4 to extracellular membrane proteins from HepG2 cells, membrane proteins were extracted and separated by electrophoresis through 8% SDS-PAGE gels and proteins transferred to nitrocellulose membranes by Wetblot electroblotting. Membranes were incubated with purified virus and the position of virus binding detected using a pan-specific anti-dengue monoclonal antibody. The initial virus overlay was undertaken at 4°C for 2 hours (Figure 1). The pattern of virus binding for serotype 2 was as reported previously [11], with a major virus binding band of 78-80 kDa, and three weaker bands of 90, 95 and 102 kDa, several bands between 130 and 150 kDa and 180 and 250 kDa. At an overlay temperature of 4°C, neither dengue serotype 3 nor serotype 4 bound efficiently to the membrane proteins. No signal was seen in the negative control lane (no virus).

Increasing the temperature of overlay to R/T (25°C) resulted in the loss of the 95kDa band for dengue serotype 2 as well as loss of some of the higher molecular weight weaker bands (Figure 1). Dengue virus serotype 3 produced a significant banding pattern at R/T with predominant bands of approximately 90 kDa, 130 kDa and 180 kDa and weaker bands of approximately 80 kDa and 100 kDa. Weak virus binding was observed for dengue serotype 4 at 78-80, 90 and approximately 130 kDa. Increasing the temperature further to 37°C for dengue serotypes 3 and 4 resulted in a further enhancement of the dengue binding signal. For serotype 3, predominant bands were noted at 90, 130 and 180 kDa, while for dengue serotype 4, predominant bands were observed at 90 and 130 kDa.

DISCUSSION

Previous studies on dengue virus binding proteins using the virus overlay protein binding assay (VOPBA) have revealed variable sizes of protein bands [13-17]. However, these studies have used a range of different cells and virus serotypes, and as such comparisons between them are difficult. This is compounded by the fact that both dengue serotype and cell type may influence which protein receptors are used to effect cell entry [1, 18]. Additionally it has been reported that the dengue virus may have differences in cell tropism as determined by previous cell passage history [19-20]. Hence, it is difficult to compare between different studies, making identification of the dengue virus receptor complex. The variations due to cell type were excluded in this study by using the human hepatoma cell line, HepG2, as a target cell for dengue virus binding. We found that DEN-2 (strain 16681) predominantly bound a protein band of approximately 78-80 kDa from HepG2 membrane, while DEN-3 (strain 16562) and DEN-4 (strain 1036) bound the same two protein bands of approximately 90 and 130 kDa, as well as an additional 182 kDa in DEN-3. The absence of the 78-80 kDa band found in dengue 2 and previously characterized as GRP78 [11] from VOPBA of serotypes 3 and 4 would suggest that GRP78 may well be a serotype specific receptor element. Interestingly, while the hybridization of dengue serotype 2 to HepG2 membrane proteins is relatively unaffected by the hybridization temperature, serotypes 3 and 4 are sensitive to the temperature used. Previous VOPBA studies using C6/36 cells (derived from Aedes albopictus) and dengue serotype 4 have also noted different binding patterns at different temperatures with enhanced binding at higher hybridization temperatures [14, 17] suggesting that protein labiality may play a role in binding of the dengue virus to its cognate receptor proteins.

ACKNOWLEDGEMENTS

This work was supported by the Thailand Research Fund. SJ is Royal Golden Jubilee Fund Scholar. The authors thank Dr. Siritorn Butrapet for kindly providing some materials used in this study

REFERENCES

1. Bielefeldt-Ohmann H, Meyer M, Fitzpatrick DR, and Mackenzie JS. Dengue virus binding to human leukocyte cell lines: receptor usage differs between cell types and virus strains. Virus Res 2001;73:81-89.

2. Halstead SB. Antibody, macrophages, dengue virus infection, shock, and hemorrhage: a pathogenetic cascade. Rev Infect Dis 1989; Suppl 4:S830-9.

3. Kurane I, Kontny U, Janus J, and Ennis FA. Dengue-2 virus infection of human mononuclear cell lines and establishment of persistent infections. Arch Virol 1990;110:91-101.

4. Marianneau P, Cardona A, Edelman L, Deubel V, and Despres P. Dengue virus replication in human hepatoma cells activates NFκB which in tern induces apoptotic cell death. J Virol 1997;7:3244-3249.

5. Avirutnan P, Malasit P, Seliger B, Bhakdi S, and Husmann M. Dengue virus infection of human endothelial cells leads to chemokine production, complement activation, and apoptosis. J Immunol 1998; 161:6338-6346.

6. Leitmeyer KC, Vaughn DW, Watts DM, Salas R, Villalobos de Chacon I, Ramos C, and Rico-Hesse R. Dengue virus structural differences that correlate with pathogenesis. J Virol 1999; 73:4738-4747.

7. Couvelard A, Marianneau P, Bedel C, Drouet MT, Vachon F, Henin D, Deubel V. Report of a fatal case of dengue infection with hepatitis: demonstration of dengue antigens in hepatocytes and liver apoptosis. Hum Pathol. 1999; 30:1106-1110.

8. Rosen L, Khin MM, U T. Recovery of virus from the liver of children with fatal dengue: reflections on the pathogenesis of the disease and its possible analogy with that of yellow fever. Res Virol 1989;140:351-360.

9. Kuo CH, Tai DI, Chang-Chien CS, Lan CK, Chiou SS, Liaw YF. Liver biochemical tests and dengue fever. Am J Trop Med Hyg 1992;47:265-270.

10. Huerre MR et al. Liver histopathology and biological correlates in five cases of fatal dengue fever in Vietnamese children. Virchows Arch 2001;438:107-115.

11. Jindadamrongwech, S., Thepparit, C. and Smith, D.R. Identification of GRP 78 (BiP) as a liver cell expressed receptor for dengue virus serotype 2. Arch Virol DOI: 10.1007/s00705-003-0263-x

12. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248-254.

13. Moreno-Altamirano MMB, Sanchez-Garcia JS, and Munoz ML. Non Fc receptor-mediated infection of human macrophages by dengue virus serotype 2. J Gen Virol 2002; 83:1123-1130.

14. Martinez-Barragan JJ and del Angel RM. Identification of a putative coreceptor on vero cells that participates in dengue 4 virus infection. J Virol 2001;75:7818-7827.

15. Munoz ML Cisneros A, Cruz J, Das P, Tovar R, and Ortega A. Putative dengue virus receptors from mosquito cells. FEMS Microbiol Letters 1998; 168:251-258.

16. Bielefeldt-Ohmann H. Analysis of antibody-independent binding of dengue viruses and dengue virus envelope protein to human myelomonocytic cells and B lymphocytes. Virus Res 1998;57: 63-79.

17. Salas-Benito JS and del Angel RM. Identification of two surface proteins from C6/36 cells that bind dengue type 4 virus. J Virol 1997;71:7246-7252.

18. Marianneau P, Megret F, Olivier R, Morens DM, and Deubel V. Dengue 1 virus binding to human hepatoma HepG2 and simian vero cells surface differs. J Gen Virol 1996;77:2547-2554.

19. Brandt WE, McCown JM, Top FH, Bancroft WH, and Russell PK. Effect of passage history on dengue-2 virus replication in subpopulations of human leukocytes. Infect Immun 1979; 26:534-541.

20. Sung JS, Diwan AR, Falkler WA, Yang HY, and Halstead SB. Dengue carrier culture and antigen production in human lymphoblastoid lines. Intervirology 1975;5:137-149.

FIGURE LEGEND

Figure 1 Dengue virus binding to HepG2 cell membrane proteins at different temperatures by virus overlay protein binding assay (VOPBA). M represents the protein markers in kilodaltons