Rapid detection of anti-hepatitis A virus neutralizing antibodies in a microplate enzyme immunoassay

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The slow growth of hepatitis A virus (HAV) in cell culture is one of the primary pitfalls in the development of sensitive and rapid methods for the detection and quantification of HAV and associated neutralizing antibodies. Currently, *in vitro* assays frequently require 8 days or more to detect and quantify the presence of HAV neutralizing antibodies. This study describes a rapid immunoassay that allowed the detection of anti-HAV neutralizing antibodies in only 3 days. This microplate-based enzymic assay may be applicable in virological diagnostics, in evaluating the immunogenicity of HAV vaccines and in quantifying neutralizing antibodies during the course of HAV infection.

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INTRODUCTION

Hepatitis A virus (HAV), a member of the family *Picornaviridae*, is a non-enveloped, positive-sense RNA virus with a pervasive worldwide transmission (Brown, 1989). HAV causes acute liver infection with a sudden onset of symptoms such as fever and nausea (Jelic *et al.*, 1990; Nainan *et al.*, 2006). The virus is transmitted via the faecal–oral route and infects approximately 1.4 million people every year (Chen & Cantrell, 2006). The HAV genome encodes a single polyprotein of approximately 200 kDa, which is subsequently processed into the structural proteins VP1–VP4 encoded by the viral region P1, and non-structural polypeptides encoded by viral regions P2 and P3 (Endo *et al.*, 2007). HAV grows extremely slowly in cell culture and often replicates without any visible cytopathic effect, unlike other members of the family *Picornaviridae* such as poliovirus and human rhinovirus (Gauss-Muller *et al.*, 1986; Stapleton *et al.*, 1993; Zahn *et al.*, 1984). Various *in vitro* assays have been developed to assess the activity of anti-HAV neutralizing antibodies; however, these assays are often lengthy (requiring 8 days–3 weeks) (Beales *et al.*, 1996; Cao *et al.*, 2008; Kim *et al.*, 2004; Konduru *et al.*, 2008) and may be more labour-intensive than microplate-based assays. Thus, a simpler and more rapid method for detecting and quantifying HAV neutralizing antibodies, perhaps amenable to a higher-throughput format, would be beneficial.

Over the past few years, cytopathic variants of HAV have been generated (Brack *et al.*, 1998; Emerson *et al.*, 1993). These HAV strains cause acute rather than persistent infection and produce a much higher viral yield than non-cytopathic variants. Using a cytopathic variant, we have developed a sensitive, specific and reproducible microplate-based assay for evaluating HAV neutralizing antibody responses. In this immunoassay, only one replication cycle of HAV appeared to be required in order to detect infectivity. To our knowledge, this is the first report that describes a colorimetric assay capable of detecting HAV infectivity and HAV neutralizing antibodies in only 3 days.

METHODS

Cells and virus. Fetal rhesus monkey kidney (FRhK-4) cells and HM175/18f, a cell-culture-adapted, cytopathic variant of the HM175 strain of HAV (Lemon *et al.*, 1991), were obtained from Dr Syed Sattar (University of Ottawa, Canada). FRhK-4 cells were grown in Iscove's modified Dulbecco's medium (IMDM; Hyclone, Thermo Fisher Scientific) supplemented with 4 mM L-glutamine, 0.4 % HEPES, 10 % heat-inactivated fetal bovine serum (FBS) (Hyclone, Thermo Fisher Scientific) and 1 % penicillin/streptomycin (Cellgro) at 37 °C and 5 % CO₂. The infection medium contained 2 % FBS. Confluent cell monolayers were washed with PBS (Fisher) and trypsinized with 0.2 % Trypsin/EDTA solution (Sigma). The HM175/18f virus strain was propagated in FRhK-4 cells and virus titre was quantified by plaque assay.

Serum samples. Serum samples from rhesus monkeys immunized with commercial HAV vaccine (Havrix 1440; GlaxoSmithKline) were

Abbreviation: FBS, fetal bovine serum.

collected approximately 2 weeks after the second of two vaccinations. Animal procedures were carried out at Frontier Biosciences (Chengdu, PR China). Human serum was collected from HAV-vaccinated healthy individuals. All serum samples were heat-inactivated for 30 min at 56 °C prior to use.

Neutralization assay. FRhK-4 cells were seeded in 96-well flat-bottomed plates (Corning) at a density of 2.5×10^3 cells per well with IMDM supplemented with 10% FBS and 1% penicillin/streptomycin. The following day, the cells were washed with PBS and the medium was replaced with 100 µl 2% FBS/IMDM and 1% penicillin/streptomycin. Sera and control samples were serially diluted in 2% FBS/IMDM in a 96-well round-bottomed plate (Corning) in a 100 µl final volume. Wells used as the virus control (virus alone) received 200 µl HAV diluted in 2% FBS/IMDM. Wells used as the cell control (cells alone) received 20 µl 2% FCS/IMDM. Diluted HAV (100 µl; 1×10^6 p.f.u. ml⁻¹) was then added to all wells except the cell control wells. Sera and virus were incubated together for 2 h at 37 °C. After incubation, 100 µl of the sera and virus mixture was added to the plates seeded with FRhK-4 cells. After 3 days, the cell medium was decanted off and plates were washed once in 200 µl PBS. The PBS was then decanted off and the cells were fixed with 100 µl cold 80% acetone in PBS for 10 min at room temperature. The acetone was then decanted off and the plates were air dried for 20 min by inverting over the front air grill of a biosafety cabinet to ensure evaporation of the fixative. Plates were then washed five times in $1 \times$ PBS (Fisher) with 0.05% Tween 20 (Sigma) and incubated for 1 h in the dark with 100 µl mouse anti-HAV VP3 primary antibody (1 µg ml⁻¹; Accurate Chemical) in 5% FBS/PBS. Plates were washed five times in 0.05% Tween 20/PBS and incubated for 1 h in the dark with 100 µl horseradish peroxidase-conjugated goat anti-mouse IgG Fc secondary antibody (Bethyl) diluted 1:10 000 in 5% FBS/PBS. Plates were then washed a further five times in 0.05% Tween 20/PBS and developed with 100 µl TMB substrate (BioFX Laboratories) for 12 min. TMB-Stop solution (100 µl; BioFX Laboratories) was added to stop the reaction. The A_{450} was then read on a microplate reader (EMax; Molecular Devices). Neutralization was defined as the ability of serum to neutralize HAV by >50% compared with the virus control. The percentage neutralization was calculated by the following formula, using mean absorbance values from virus control (VC), cell control (CC) and serum sample wells: % neutralization = $(VC - \text{absorbance obtained for a dilution of serum}) / (VC - CC) \times 100$.

Statistical analysis. A *t*-test was applied for the statistical analysis of the data and was conducted with a Mann-Whitney test using Prism software.

RESULTS AND DISCUSSION

HAV grows exceptionally slowly in cell culture, and this has been problematic for the rapid detection of the virus by various assays (Bishop & Anderson, 1997; Gosert *et al.*, 2000; Pinto *et al.*, 2007). Methods for quantification of this virus have been limited mainly to complex assays (Sanchez *et al.*, 2006; Siegl *et al.*, 1984; Yeh *et al.*, 2008). Although anti-HAV antibodies can be detected by ELISA, this assay cannot exclusively measure neutralizing antibodies (Lemon *et al.*, 1992; Shouval *et al.*, 1993). The present study describes a novel application of an existing neutralization assay that relies principally on a cytopathic variant of HAV (HM175/18f), HAV-permissive FRhK-4 cells and a monoclonal antibody to the HAV VP3 structural protein. We first measured HAV infectivity by flow cytometry and

subsequently by a microplate-based enzymic assay. As newly formed HAV virions tend to remain trapped within cells, permeabilization of the cells at the time of fixation and during their preparation for flow cytometry was necessary to maximize the reactivity of the anti-VP3 antibody with cell-associated virus. This method has been used previously for the detection and quantification of rabies neutralizing antibodies (Bordignon *et al.*, 2002a, b). However, we were not able to differentiate between HAV-infected and uninfected FRhK-4 cells over 1–5 days (data not shown), regardless of the amount of virus or concentration of antibody used. Thereafter, the infectivity of FRhK-4 cells was measured by a microplate-based enzymic method, based on quantifying the amount of HAV VP3 antigen present in the infected cells. FRhK-4 cells were inoculated with different amounts of HM175/18f HAV and incubated for 3 days. At daily intervals, cells were fixed and HAV VP3 antigen was quantified by indirect ELISA of cell-associated viral VP3 antigen. At a viral concentration of 0.5×10^5 p.f.u. per well, viral antigen became detectable within 48 h after HAV inoculation, and after 72 h the signal:noise ratio between infected and uninfected cells was easily discernible and approached 3:1 (Fig. 1). Additional incubation time (days 4 and 5) increased the background and did not show any improvement in the assay (data not shown). These results indicated that HAV infection may be detected significantly sooner than in previously described assays. Based on these results, we examined the ability of sera from humans and monkeys vaccinated with the Havrix 1440 vaccine to neutralize HAV. Various conditions, such as virus titre, fixative, cell density and antibody concentration, were optimized. Fourfold dilutions of four human sera were set up in 96-well plates and incubated with HM175/18f HAV. After a 2 h incubation at 37 °C, virus and serum mixtures were added to the pre-seeded cells and the plates were incubated at 37 °C for 3 days. Cells were fixed and stained with

Fig. 1. Microplate-based enzymic method for measuring HAV in infected and uninfected FRhK-4 cells over 1–3 days. Infected and uninfected FRhK-4 cells were fixed and HAV VP3 antigen was quantified by direct ELISA.

anti-HAV VP3 primary antibody and horseradish peroxidase-conjugated goat anti-mouse secondary antibody. After the addition of the chromogenic substrate, the colorimetric reaction was stopped and the A_{450} was read on a microplate reader. All serum dilutions tested (up to 1:100 000) were able to neutralize the virus by >50 % compared with the virus control (Fig. 2). Control sera (non-vaccinated) were not able to neutralize HAV beyond 10–15 %, even at a 1:40 dilution. The fundamental difference between this method and previously described methods is that, unlike this method, previous methods require a longer time and higher levels of HAV replication. In addition, most methods require cell destruction to release cell-associated virus, whereas in this assay a 10 min fixation by acetone appeared to suffice. Additionally, we evaluated a different fixative for use in our microplate-based assay, namely BD FixPerm reagent (BD Biosciences). However, this reagent was shown to be inappropriate for use in a microplate assay due to the excessive foaming produced in the plate wells during the washing and developing steps. We attributed this to the presence of saponins in the BD Perm/Wash buffer.

In summary, we have shown that it is possible to detect HAV infection in microplate-based cultures of FRhK-4 cells after only 3 days, instead of the typical 8–21 days required in other assays. Additionally, we have demonstrated that it is also possible to detect the presence of

functional HAV neutralizing antibodies within this brief time period. The implications of these findings are important for the rapid measurement of the immunogenicity of HAV vaccines, and for virological studies and diagnostic purposes.

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Fig. 2. Neutralization of HAV in vaccinated human and rhesus macaque sera. Serum samples from humans and monkeys ($n=3$) immunized with the Havrix 1440 vaccine were collected 2 weeks after the last vaccination. Diluted serum samples were incubated with HM175/18f virus for 2 h and then added to pre-seeded FRhK-4 cells. The plates were developed after 3 days as described in Methods, and the percentage neutralization was calculated. Data are shown as the group mean $\pm$ SEM. Sera from non-vaccinated animals did not neutralize virus beyond 10–15 %, even at a 1:40 dilution.

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