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Evaluating Levels of Cell-Mediated Nk Antibody Dependent Cellular Cytotoxicity in Hcv Infection of Iraqi Patients

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ABSTRACT

HCV -specific binding antibody reactions, together with those mediating antibody-dependent cellular cytotoxicity (ADCC), delivered the best useful correlate of inferior risk of infection in the HCV vaccine clinical test. The aim of this study was to match two high-throughput current cytometry created methods to measure HCV -specific ADCC reactions, the GranToxilux and PanToxilux tests.

Plasma from nine HCV seropositive individuals was separated for binding antibody titers against HCV subtype C gp120 by ELISA and western blot. Plasma from six HCV -negative individuals was involved as controls. Both ADCC analyses used subtype C gp120-coated CEM.NKRCCR5 cells as targets. The PanToxilux test (which measured together granzyme B and caspase reactive) measured complex levels of direct natural killer (NK) cell killing of K562 tumor cells than the GranToxilux test (granzyme B alone; p b 0.05). In ADCC examines in which NK cell killing was directed compared to gp120-coated CEM.NKRCCR5 cells in an antibody-dependent method, plasma from HCV -positive personalities yielded significantly higher levels of ADCC activity than the HCV-negative controls. In contrast to straight killing, the GranToxilux assay measured comparable levels of ADCC killing as the PanToxilux examine but had significantly lesser background cytotoxicity against target cells coated with HCV negative serum.

In conclusion, the PanToxilux test was added sensitive for distinguishing direct NK cell killing of K562 cells than the GranToxilux test, while the GranToxilux test implemented enhanced at detecting HCV -specific ADCC activity, for the reason that of minor background cytotoxicity from HCV negative serum.

Key words: ADCC, Gran Toxilux test, HCV, Pan Toxilux test.

1. INTRODUCTION

PanToxiLux™ is OncoImmunin, Inc.'s most recent live cell-based kit for measuring the successful delivery of a lethal hit by cytotoxic lymphocytes to individual target cells. This kit is formulated for 80 assays with dual laser (488 and 633 nm) cytometry capability. Antibody responses following HCV infection include HCV -specific antibodies that can neutralize HCV, binding antibodies that facilitate complement binding or phagocytosis, and antibodies that mediate ADCC whereby NK cells lyse antibody-coated HCV -infected target cells (reviewed by Lewis, 2014; Over Baugh& Morris, 2012). Most HCV -specific antibodies that mediate ADCC are directed against Env (Alsmadi et al., 1997; Baum et al., 1996), although antibodies directed against Nef, Tat and Pol have also been reported as ADCC targets(Florese et al., 2010; Yamada et al., 2004). ADCC involves components of both the innate (macrophages, eosinophils, monocytes and NK cells) and acquired immune systems (B cells) producing antibodies (Lanier et al., 1986; Haynes et al., 2011; Perez-Shibayama et al., 2014).Different factors may influence the magnitude of ADCC responses during HCV infection

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(reviewed by Wren et al., 2013), including this type of the antibodies involved, their subclass and glycosylation of the Fc component on these antibodies (Ackerman et al., 2013; Peipp et al., 2008). IgG subclasses IgG1 and IgG3 are the most potent inducers of anti-HCV ADCC (Koene et al., 1997; Ljunggren et al., 1988). The potency of antibodies involved in ADCC are influenced by glycosylation of their Fc, with potency being reduced or increased in the absence or presence of major sugar residues (including fucose, galactose and siliac acid)(Boyd et al., 1995; Scallon et al., 2007; Shinkawa et al., 2003). The removal of focuses residue has been shown to increase ADCC up to a100-fold, while the addition of siliac acid decreases ADCC activity (Niwa et al., 2006; Raju, 2008). Several approaches have been used to measure ADCC activity. ADCC involves the interaction of at least three components of the immune system: (1) HCV-infected or coated target cells that express HCV antigens on their cell surface; (2) a source of HCV-specific antibodies to target these HCV antigens; and (3) cytolytic effector cells (such as NK cells) to recognize the Fc region of these antibodies. Typically, ADCC assays have either measured killing of the HCV -coated or HCV -infected target cells(Ahmad and Menezes, 1996; Brenner et al., 1989; Gupta et al., 2005;Sconocchia et al., 1999) or measured effector cell functions (Aicheler and Stanton, 2013; Alter et al., 2004; Mahajan et al., 2003; Zaritskaya et al., 2011). Fluorescence-based assays have been broadly adopted to measure ADCC (Gómez-Román et al., 2006; Chung et al., 2008). The rapid and fluorometric ADCC assay relies on double staining target cells with a membrane dye (PKH-26, released when targets are killed and a viability dye (CSFE) prior to addition of effector cells. A refinement of this flow cytometry-based approach to measuring ADCC are the GranToxilux® and PanToxilux™ high-throughput ADCC assays (Pollara et al., 2011). The GranToxilux® assay is based on the hydrolysis of a cell-permeable fluorogenic peptide substrate containing a sequence recognized by the serine protease, Granzyme B (GzB) (Pollara et al., .2011).GzB is delivered to target cells during ADCC, and this assay picks up the delivered GzB using flow cytometry. The PanToxilux™assay differs from the GranToxilux assay in that it detects both GzB and upstream caspase activities (Packard et al., 2007). The aim of this study was to compare the PanToxilux™ and GranToxilux® assays to measure HCV -specific ADCC.PanToxiLux™ is similar to GranToxiLux® can be used for selection of antibodies operating via an antibody dependent cellular cytotoxicity (ADCC) mechanism in both low and high throughput screening (HTS) modes (Packard et al., 2007).

2. MATERIALS AND METHODS

.2.1 Cell lines

K562 target cells were used to measure direct killing by NK cells. In addition, CEM.NKRCCR5 cells were used to measure HCV-specific ADCC activity. CEM.NKRCCR5 cells have been modified to express CD4 and CCR5 receptor on their cell surface, and are not directly sensitive to NK cell killing. They become sensitive to ADCC killing when they are coated with HCV gp120 in the presence of HCV -specific antibodies(Trkola et al., 1999).

.2.2 Study participants, plasma and PBMC isolation

Blood was obtained from 8 HCV negative individuals for isolation of peripheral blood mononuclear cells (PBMCs), as a source of NK cell effector cells for this study. In addition, whole blood (40ml)was collected by venipuncture from 9 chronically HCV -infected individuals who were naïve to HAART. Plasma from these HCV -infected donors was used as the source of HCV -specific antibodies for the ADCC assays. Whole blood was collected into vacutainer tubes containing acid citrate dextrose as an anti-coagulant (ACD; BD Bioscience, San Diego, CA). The University of Cape Town's Faculty of Health Sciences Research Ethics committee REC REF 258/2006) approved all aspects of this study, and written informed consent was obtained from all the participants.

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2.3.Measurement of plasma HCV gp120 ELISA IgG concentrations

To measure HCV gp120 IgG titers in plasma from HCV positive individuals, a high binding ELISA plate (Corning®, Sigma-Aldrich®) was coated with purified envelope gp120 consensus C [4 μg/ml] diluted in bicarbonate buffer (Sigma-Aldrich®) at 100 μl per well. The plate was covered with film and incubated in the fridge at 4 °C overnight. Wells were washed four times with 0.05%Tween-20 in PBS (Sigma-Aldrich®) blocked with 100 μl of 5% non-fat dry milk in PBS/0.05% Tween-20 (dilution buffer), and incubated at 37 °C for 1 h. Excess blocking solution was discarded. Next, a 3-fold serial dilution of HCV positive plasma, starting with 1:10, was carried out using the dilution buffer. Serially diluted plasma (50 μl) was then added to the wells and plate was incubated for 1 h at 37 °C. Wells were washed as previously and 50 μl of anti human IgG-HRP (1:1000 dilution in dilution buffer) was added and incubated for 1 h at 37 °C. The wells were washed and 100 μl of 1-StepUltra TMB-ELISA substrate (Thermo Fisher Scientific, Waltham, USA(was added for 3–5 min (min). Development of color was stopped by addition of 25 μl of 1MH2SO4. The plate was read on the Versa Max®micro plate (Molecular Devices) reader using the SOFTmax PRO 4.3.1set at a wavelength of 450 nm. In addition, western blots were used to confirm specificity of plasma samples from HCV-infected individuals using the Genetic Systems HCV Western Blot kit (Bio-Rad, California, USA), according to the manufacturer's instructions. For this experiment10μl of plasma was added to 1000 μl of wash buffer.

.2.4 Measurement of NK cell direct killing of K562 cells

PBMCs from the HCV negative donors (source of NK cells) were thawed, counted, adjusted to 1×10^6 cells/ml, and rested overnight in R10 at 37 °C and 5% CO2. For the direct NK killing assays, 1×10^5 K562cells were labeled with 1 μl TFL4 and 1 μl NFL1 in the dark at RT for15 min and then washed. Labeled K562 cells were combined with PBMCs adjusted to viable effector to viable target ratio of 5:1. GranToxilux substrate (75 μl) or PanToxilux substrate (75 μl) was added to each tube. In addition, three control tubes were included:

(1)K562 target cells alone; (2) K562 cells alone with GranToxilux substrate;and (3) K562 cells alone with PanToxilux substrate. The mixture of effector : target cells were incubated at 37 °C and 5% CO2 for 60 min, washed twice using wash buffer and re-suspended in 200 μl of wash buffer for acquisition. Approximately 100,000 events were acquired for K562 cells alone controls, and all of the sample was acquired of theK562 and PBMC mixture (~500,000 events) using the BD LSRFortessa.The data obtained was then analyzed using Flow Jo software (version Tree star; Ashland, OR). Each experiment was performed at least twice.

.2.5 Measurement of ADCC killing of CEM.NKRCCR5 cells

PBMCs from healthy donors were used as the source of the NK cells. Recombinant gp120 from subtype C HCV was used to coatCEM.NKRCCR5 target cells in preparation for the ADCC assay. The amount of recombinant gp12 0 (rgp120) to coat the target cells was determined by competitive binding of the CD4-FITC conjugated antibody (Bioscience, San Jose, USA), to the CD4 receptor expressed on the surface of the CEM.NKRCCR5 cells (supplementary Pollara et al., 2011). The first concentration of rgp120 to reduce the mean fluorescence intensity MFI) by N50% of the uncoated CEM.NKRCCR5 cells was chosen to coat the cells in the final assay (5 μg/ml, supplementary Fig. 1A). Gp120-coated CEM.NKRCCR5 cells (~2 × 105 cells coated with 5 μg/ml rgp120) were labeled with 1 μl TFL4, 1 μl NFL1 for 15 min in a 37 °C water bath. After two washes, cells were counted and mixed with PBMCs ata final viable effector to viable target ratio of 30:1, and 75 μl of either the GranToxilux or PanToxilux substrate. After incubation for 5 min at RT, HIV+ plasma (25 μl of 1:1000 dilution), as a source of ADCC antibodies, was added to the CEM.NKRCCR5 cell suspension and incubated for 15 min at RT. HCV negative plasma was included as a negative

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control. All samples were used at a 1:1000 dilution in the final assays. The following tubes were used in each experiment: (1) CEM.NKRCCR5 cells alone; (2) and CEM.NKRCCR5 cells alone with GranToxilux substrate(3);CEM.NKRCCR5 cells alone with PanToxilux substrate; (4) PBM (NK) effectors alone with GranToxilux substrate; (5) PBMC (NK) effectors alone with PanToxilux substrate; and finally (6) the mixture of PBMC (NK) effectors together with CEM.NKRCCR5 cells and GranToxilux substrate; and (7) the mixture of PBMC (NK) effectors together withCEM.NKRCCR5 cells with PanToxilux substrate. The plates were centrifuged for 1 min at 1200 rpm, and incubated for 1 h at 37 °C and 5%CO2. After washing, cells were re-suspended in 200 µl of wash buffer and transferred to 5 ml FACS tubes. The cells were acquired using the BD LSRFortessa™ and 500,000 events were acquired. A minimum of events representing viable gp120-coated CEM.NKRCCR5 cells was acquired for each condition. Each experiment was performed at least twice. Analysis was performed using Flow Jo 9.7.5 software.

3.RESULTS

3.1. Specificity and titer of HCV-specific IgG in plasma from HCV-infected individuals

Plasma from a panel of HCV -infected individuals were compared in their ability to mediate ADCC activity in this study. The HCV infected individuals were healthy and naïve to HAART with CD4+ T cell counts of N300 cells/µl (viral loads of N50 and b54.000 RNA copies/ml). HCV positive individuals had significantly higher HCV gp120-specific plasma antibodies than HCV negative individuals used as negative controls (at 1:100 plasma dilution, $p = 0.0004$; at 1:1000 dilution, $p = 0.0007$). In addition to gp120-specific antibodies, all HCV positive plasma samples had antibodies binding to gp160, p65, p55/51, p41, p40, p31, p24 and p18 .

3.2. Comparison of GranToxilux and PanToxilux assays for measuring direct NK cell killing

To select a universal effector NK cell donor for this study, eight HCV seronegative samples were evaluated for direct NK cell activity against K562 targets. The results can be summarizing the gating strategy used to measure direct killing of K562 target cells by NK cells in the GranToxilux and PanToxilux tests. In the GranToxilux test, GzB activity ranged from 2.0–8.4%. In the PanToxilux assay, the frequency of GzB and caspase activity detected in K562 target cells ranged from (6.9–35.2%). The NK cell donor with the highest frequencies of killing in the GranToxilux and PanToxilux assay was selected for further experiments. All of the PBMC donors showed higher direct cytolytic activity in the PanToxilux assay than the GranToxilux assay against K562 cells ($p = 0.007$). A Bland-Altman statistical test was performed to evaluate whether the two assays were in agreement. This test calculates the mean difference between two assays of measurement the “bias” and 95% limits of agreement as the mean difference (Bland and Altman, 1999). If the bias is close to zero, then the assays are in agreement. This analysis showed that the two assays were not in agreement, with a bias of −11 and 95% limits of agreement from −29.82 to 6.69. (S. Migueles, Osborne. Royce et al., 2009)

.3.3 Comparison between GranToxilux and PanToxilux assays for measuring ADCC

The level of gp120-specific ADCC activity in HCV -infected samples measured by (GzB+ CEM.NKRCCR5 target cells) detected in the GranToxilux test ranged from 3.5–42%.

In contrast, the frequency of GzB+ target cells resulting from coating with HCV negative plasma samples ranged between 0.1 and 1.4% ($p = 0.0028$). The frequency of target cells staining positive for GzB and caspase activity using HCV + plasma in the PanToxilux assay ranged from (7.5–66.5%).The amount of GzB and caspase activity induced by plasma from HCV negative individuals ranged from(1 to 6%) ($p = 0.0004$). While the magnitude of ADCC activity measured in plasma

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from HCV -infected individuals was similar between the GranToxilux and PanToxilux assays, the background level of ADCC detected in plasma from HCV negative individuals was significantly higher in the PanToxilux than the GranToxilux assay (p=0.0043), suggesting that the GranToxilux assay was superior to the PanToxilux assay by virtue of low background.The average bias between the two assays was−2 using the Bland–Altman test, indicating that these two assays were largely in agreement.

4. DISCUSSION

In our study we try to improve a practical method for measuring the capacity of antibodies to direct ADCC against cells infected with HCV. The usage of immortalized NK cell lines in arrangement with luciferase as an indicator of cytotoxicity enables the titration of many samples in parallel on a routine basis. The dose-dependent elimination of infected target cells by ADCC over serial antibody dilutions generates a curve, which facilitates quantitative comparisons over a large dynamic range. Thus, the potential for antibodies to direct ADCC against physiologically relevant targets can be quantified in a format analogous to state-of-the-art methods for measuring neutralizing antibodies (Gerald and Douglas 2000; Haider and Al-Maliki 2015; Mohamad 2010). The use of this approach indicates that there is incomplete overlap between the subsets of antibodies that direct ADCC against virus-infected cells and the antibodies measured in neutralization and gp120-binding assays.

The result in this study demonstrated that is a significant difference between the healthy and the patient groups by measuring the level IgG of plasma during viral loads of HCV -infected and HCV -expected. would result in on-going maturation of these antibodies to further increase ADCC activity, CD4 blocking, or addition of neutralizing activity remains to be determined.

IgG antibodies capable of binding to the V1V2 region of gp120 correlated with protection and Env-specific IgA antibodies correlated with increased risk of infection (Lavanchy, 2011). There was a no significant trend toward a lower risk of HCV- infection among the vaccinated study subjects with higher levels of ADCC activity measured using the assay described here. Moreover, among vaccinated study subjects with low or undetectable Env-specific IgA responses, there was a borderline significant relationship between ADCC activity and lower risk of infection. Thus, although ADCC was not identified as a correlate of protection, these results point to a role for Env-specific antibody responses in the modest protection observed in the RV144 trial.

On other hand our study aimed to comparison between GranToxilux and PanToxilux test for measuring ADCC activity this study speculated that cryopreservation may induce this negative regulatory pathway, since treatment of cells with (2.0–8.4%). In the Pan Toxilux assay, the frequency of GzB and caspase activity detected in K562 target cells ranged from (6.9–35.2%) respectively. Indeed, the addition of freezing medium was sufficient to induce a loss of ADCC activity that could be prevented with CsA or FK-506. Consistent with our model, CsA and FK-506 also prevented the induction of unresponsiveness after treating the NK cell line with ionomycin to mobilize an influx of calcium into the cytoplasm. Since these calcineurin inhibitors are used as immunosuppressant drugs in clinical settings (Gerald and Douglas 2000), it is perhaps counterintuitive that their use would promote cytotoxic effector function. However, these observations are consistent with a model in which treatment with freezing medium or ionomycin stimulates the NK cells to prompt a calcineurin-dependent negative regulatory pathway, which can be blocked by CsA or FK-506. This solution to maintaining the cytolytic activity of the NK cell line through cryopreservation enabled the routine and reproducible measurement of ADCC activity.

We have shown that the antibodies that direct ADCC are correlated with the antibodies measured by other types of tests but that these relationships are not absolute. Therefore, the best practice going forward may be to measure both virus-specific neutralization and ADCC titers. Since neutralization

assays using primary HCV - isolates do not currently detect vaccine-elicited antibody responses, the evaluate described here may be particularly useful for evaluating the ability of antibody responses to recognize physiologically relevant forms of Env.

The Pan Toxilux test (which measured together granzyme B and caspase reactive) measured complex levels of direct natural killer (NK) cell killing of K562 tumor cells than the Gran Toxilux test (granzyme B alone; p b 0.05). In ADCC examines in which NK cell killing was directed compared to gp120-coated CEM.NKRCCR5 cells in an antibody-dependent method, plasma from HCV -positive personalities yielded significantly higher levels of ADCC activity than the HCV-negative controls. In contrast to straight killing, the Gran Toxilux assay measured comparable levels of ADCC killing as the Pan Toxilux examine but had significantly lesser background cytotoxicity against target cells coated with HCV negative serum. In conclusion, the Pan Toxilux test was added sensitive for distinguishing direct NK cell killing of K562 cells than the GranToxilux test, while the GranToxilux test implemented enhanced at detecting HCV -specific ADCC activity, for the reason that of minor background cytotoxicity from HCV negative serum.

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