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Running title: Plasma IgA neutralization in HIV-2 infection

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ABSTRACT (word count limit: 208)

Human immunodeficiency virus type 2 (HIV-2) is less transmissible and less pathogenic compared to HIV-1 and, when matched for CD4+ T cell count, the plasma viral load in HIV-2 infected individuals is approximately one log lower than in HIV-1 infected individuals. The explanation for these observations is elusive, but differences in virus controlling immunity generated in the two infections may be contributing factors. In the present study, we investigated neutralization by immunoglobulin A (IgA), in parallel with IgG, purified from plasma of HIV-1, HIV-2 and HIV-1/HIV-2 dually (HIV-D)-infected individuals. Neutralization was analyzed against HIV-1 and HIV-2 isolates using a plaque reduction assay. In HIV-2 infection, intratype-specific neutralization by IgA was frequently detected, although at a lesser magnitude then the corresponding IgG neutralizing titers. In contrast, neutralization by IgA could rarely be demonstrated in HIV-1 infection despite similar plasma IgA levels in both infections. In addition, IgA and IgG of HIV-D plasma neutralized the HIV-2 isolate more potently than the HIV-1 isolate, suggesting that the difference between neutralizing activity of plasma IgA and IgG depends on the virus itself. Taken together, these findings suggest that both IgA and IgG adds to the potent intratype neutralizing activity detected in HIV-2 plasma, which may contribute to virus control in HIV-2 infection.

INTRODUCTION

In an effort to understand the correlates of protective immunity to human immunodeficiency virus type 1 (HIV-1) infection, marked attention has been given to the small group of HIV-1 long-term nonprogressors (LTNP) and elite controllers that either do not develop disease or do so with slower pace [1,2]. However, less attention has been given to individuals infected with HIV-2, an infection that is compatible with normal lifespan in most infected individuals and, if disease develops at all, progression rate is lower than in HIV-1 infection [3]. Also vertical and heterosexual transmission of HIV-2 occurs less frequently than with HIV-1 [4-6]. Moreover, a recent study found that HIV-1 disease progression was inhibited by concomitant HIV-2 infection [7]. Although the underlying mechanisms behind differences between HIV-1 and HIV-2 infections remain to be clarified, viral replication and the envelope glycoprotein complex (Env) characteristics could be important. Indeed, similar levels of proviral load but approximately one log lower plasma virus load in HIV-2 infection compared to HIV-1 infection could indicate more effective control of viral replication in HIV-2 infection [8-11]. In addition, the HIV-2 Env seems to have a more open configuration characterized by multiple broadly cross-reactive epitopes and reduced CD4 dependence, which are uncommon characteristics of HIV-1 [12,13]. Moreover, as shown in our earlier work, HIV-2-infected individuals display plasma with potent neutralizing activity (NAc) against HIV-2 isolates (intratype neutralization), whereas NAc of HIV-1 plasma against HIV-1 isolates is of lower magnitude [14].

The magnitude and frequency of virus specific serum immunoglobulin A (IgA) has been studied and found to be low in chronic HIV-1 infections in HIV-1 infected chimpanzees as well as in

SIV-infected rhesus macaques [15-18]. The knowledge on HIV-2 specific serum IgA is limited, and to our knowledge, only one study reports on neutralization by IgA in HIV-2 infection [19].

HIV-1 is mainly a mucosal pathogen targeting CD4+CCR5+ memory T cells abundantly present in gut-associated lymphoid tissue (GALT) [20]. The predominant antibody isotype in GALT is IgA. Interestingly, in humans, including HIV-1 infected cases, it has been shown that part of mucosal IgA is also secreted directly into the blood [21-23]. Furthermore, it is known that a large proportion of the circulating antibody secreting cells (ASC) express IgA and most of them are derived from mucosal immune reactions [16, 24, 25]. IgA may also display anti-inflammatory effects mediated by FcαRI binding [26, 27]. Instead, effector functions beyond binding to antigen and classical neutralization, i.e. complement activation and induction of phagocytosis, has been reported to be a minor feature of IgA [28, 29].

In the present study, we examined, side by side, the neutralizing activity of IgA and IgG in plasma of HIV-1, HIV-2, and HIV-1/HIV-2 dually (HIV-D) infected individuals. Our results show that intratype neutralization by plasma IgA is frequently detected in HIV-2 but not in HIV-1 infection. Furthermore, this study suggests that the magnitude of HIV-2 plasma IgA, as well as IgG, intratype neutralizing activity exceeds the magnitude of HIV-1 plasma IgA and IgG neutralization.

MATERIALS AND METHODS

Study Population

Women attending the Agiubef sexual health and family planning clinic for urogenital problems in Bissau, Guinea-Bissau from November 2006 to January 2011 were invited to participate in the study. After informed consent, HIV test counseling was given. Subsequently, a blood sample was drawn and, general and gynecological examinations were performed. At the clinics, all women were offered free treatment for the diagnosis of other sexually transmitted diseases and condoms. In this screening, including 1287 individuals, women with HIV-1 (n=47), HIV-2 (n=21) and HIV-D (n=22) infections were identified and after one more blood sampling, referred to HIV treatment and support centers. For the current study, 10 HIV-1, 10 HIV-2 and 8 HIV-D infected women were selected on the basis of CD4+ T cell count availability (Table 1). The study participants were further characterized by assessment of age, plasma viral load and plasma total IgG and IgA levels (Table 1). None of the study participants received anti-retroviral treatment at the time of blood sampling. HIV-negative controls (n=16) were selected on the basis of plasma sample availability.

Blood Sampling, HIV-1, HIV-2 Status and CD4+ T cell Determination

Venous blood samples were drawn and collected in vacutainer tubes (BD Biosciences, San Jose, CA) with EDTA as anti-coagulant. Plasma was then kept frozen at -80°C until use in neutralization assays, viral load determinations and other laboratory analyses. Serological testing for HIV-antibodies was done using the Behring Enzygnost HIV-1/HIV-2 ELISA (Behring, Marburg, Germany). Confirmation and differential HIV-1/HIV-2 diagnoses were performed

using Capillus HIV-1/HIV-2 (Cambridge Biotech Limited, Galway, Ireland), and Immunocomb

II HIV-1 and HIV-2 BiSpot RST (Origenics, Yavne, Israel). CD4+ T cell counts were

determined by flow cytometry using the CyFlow instrument (Partec GmbH, Münster, Germany)

and the CD4% antibody kit (CyTecs GmbH, Görlitz, Germany) according to the manufacturer's

instruction.

HIV Plasma Viral Load

Viral RNA was extracted and purified from 200 µl blood plasma samples, using RNeasy Lipid

Tissue Mini Kit (Qiagen, Stockholm, Sweden) according to manufacturer's instructions. RNA

was eluted in 30 µl RNase-free water, treated with DNase I (Fermentas, St Leon-Rot, Germany)

and reverse transcribed using random primers and the Maxima Reverse Transcriptase (Fermentas)

according to manufacturer's instructions. HIV-1 and HIV-2 viral load was determined using an

in-house TaqMan qPCR with primers and probes as described [30, 31]. Briefly, HIV-1 RNA was

quantified using primers LTR HIV-1 U (5'-GCCTCAATAAAGCTTGCCTTGA-3'), LTR HIV-

1 L (5'-GGCGCCACTGCTAGAGATTTT-3') and probe Pr LTR 1 (5'-FAM-

AAGTAGTGTGTGCCCGTCTGTTRTKTGACT-TAMRA-3'). HIV-2 RNA was quantified

using primers 1108 F3 (5'-GCGCGAGAAACTCCGTCTTG-3'), 1234 L140 (5'-

TCCAACAGGCTCTCTGCTAATCC   -3') and probe S65GAG2 (5'-FAM-

TAGGTTACGGCCCGGCGGAAAGA-TAMRA-3').   All primers were synthesized by

Invitrogen (Stockholm, Sweden) and probes by Applied Biosystems. qPCR was done using the

AB StepOnePlus System (Applied Biosystems, Stockholm, Sweden) and AmpliTaq Fast DNA

polymerase (Applied Biosystems) according to manufacturer's instructions. PCR conditions

were as follows: 95°C for 10 min, 40 cycles of 95°C for 20 s, 60°C for 45 s. Stocks of HIV-1

strain IIIB and HIV-2 strain NIH-Z, which had been quantified by electron microscopy (Advanced Biotechnology Incorporated, Columbia MD, USA), were used as standards. Known virus copy numbers were spiked into HIV-negative human plasma and extracted as described above. Both HIV-1 and HIV-2 assays had a limit of quantification of 5 RNA copies/qPCR reaction resulting in a cut-off value for quantification of 281 RNA copies/ml plasma. Both assays had a linear range between 10^6 and 5 RNA copies/reaction. Standards and samples were treated under the same conditions.

Assessment of IgG and IgA Levels

IgG levels in plasma were measured by an in-house ELISA [13, 32]. Briefly, plates were coated overnight with AffiniPure goat anti-human IgG (20 μg/ml) (Jackson Immunotech, Marseille, France). Alkaline phosphatase-conjugated anti-human IgG (diluted to 1:5000) (Jackson Immunotech, Marseille, France) was used as a detection antibody and human IgG (R&D Systems, Minneapolis, MN) was used as a standard. IgA levels were also measured by an in-house ELISA (the protocol was kindly provided by Dr. Carol Ann Fraser from Imperial College London, London, UK). Briefly, plates were coated overnight with goat anti-human IgA (2 μg/ml) (Jackson Immunotech). Alkaline-phosphatase conjugated AffiniPure goat anti-human IgA (diluted to 1:2500) (Jackson Immunotech) was used as a detection antibody and human serum IgA (Jackson Immunotech, Marseille, France) was used as a standard. The ELISA was repeated in triplicate for each sample.

Plasma IgG Purification

IgG was purified from plasma using protein G sepharose HP pre-packed into spin columns (GE Healthcare Life Sciences, Buckinghamshire, UK). Briefly, plasma samples were first inactivated at 56°C for 30 minutes and then clarified by centrifugation at 4000 x g for 20 min. 200 μl plasma and 400 μl binding buffer (20 mM Na phosphate pH 7.0) (GE Healthcare Life Sciences) was added to the equilibrated protein G sepharose HP and the mixture was incubated at room temperature for 4 min on a tube rotator. Thereafter unbound fractions were removed by centrifugation at 100 x g for 30 seconds and kept in the fridge. The columns were then washed 2 times with 600 μl of binding buffer. Neutralization buffer (30 μl 1 M Tris-HCl pH 9.0) (GE Healthcare Life Sciences) was added to the bottom of fresh collection tubes and bound IgG was eluted with elution buffer (400 μl, 0.1 M glycine-HCl, pH 2.7) (GE Healthcare Life Sciences) and centrifuged at 70 x g for 30 s into the neutralization buffer. Elution was repeated with another 400 μl of elution buffer. IgG purification was repeated with unbound fractions in the fridge. The eluted IgG and non-IgG fractions were concentrated using Amicon Ultra-15 centrifugal filter units with a 30.000-Da cutoff (Merck Millipore, Billerica, MA). Purified IgGs were kept at -80°C until use. Non-IgG fractions were kept at 4°C until purification of IgA on the same day. Residual IgA in the IgG fraction was assessed by IgA ELISA, as described above, and found not to exceed 0.1 μg/ml.

IgA Purification from Non-IgG Plasma Fraction

IgA was purified from non-IgG fractions of plasma using peptide M/Agarose gel slurry (InvivoGen, Toulouse, France). Briefly, 400 μl peptide M/Agarose gel slurry (equals to 200 μl resin) was loaded into a Pierce microcentrifuge spin column (Thermo Fisher Scientific, Hampton, NH); then IgG-depleted plasma was loaded onto the column and incubated with rocking at room

temperature for 30 min. After incubation, the column was washed and the flowthrough fractions were collected. The bound antibodies were then eluted with IgG elution buffer (6x200 μl, Thermo Scientific, Rockford, IL) and neutralized immediately with neutralization buffer (6x20 μl 1 M Tris-HCl pH 9.0) (GE Healthcare Life Sciences). The column was subsequently regenerated by washing with regeneration buffer (2x300 μl PBS with 20% ethanol) and used for another round of purification of the flowthrough to increase the yield. Finally, the eluted fractions from the two consecutive purification processes were combined, buffer exchanged with PBS, and concentrated using Amicon Ultra-15 centrifugal filter units with a 30.000-Da cutoff (Merck Millipore). Purified IgAs were kept at 4°C until use. Residual IgG in the IgA fraction was assessed by IgG ELISA, as described above, and found not to exceed 0.1 μg/ml.

Primary HIV-1 and HIV-2 Isolates

For the analysis of neutralizing activity (NAc) of HIV positive plasma IgG and IgA, one HIV-1 and one HIV-2 isolate were used. HIV-1 C Br (92BR025) originated from Brazil and was isolated within the framework of WHO Network for HIV Isolation and Characterization [33]. The HIV-2 A isolate GB/1812 (original name 1812) originated from Guinea Bissau [34, 35]. Both viruses were isolated from peripheral blood mononuclear cells (PBMC) [35, 36] and were found to use CCR5 but not CXCR4 for cell entry [34, 36], which was the rationale for the use of GHOST(3)-CCR5 cells as targets in the neutralization assays. Based on our previous experience, the viruses were chosen such as to ensure neutralization sensitivity with samples collected in Guinea-Bissau [14].

Neutralization Assay Using GHOST(3)-CCR5 Cells

NAc of plasma Ig (IgG or IgA) from HIV-1 and/or HIV-2 infected individuals was determined in a plaque reduction assay using the GHOST(3)-CCR5 cells as targets and plaque reduction, assessed by expression of green-fluorescent protein, as readout [32]. In brief, GHOST(3)-CCR5 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO) (Life Technologies, Paisley, UK) complemented with 7.5% FCS and 10,000 IU/ml penicillin-streptomycin (Invitrogen, Carlsbad, CA) in 25 cm^2 culture flasks and split twice a week at a ratio of 1:15-20. Briefly, virus stocks and plasma Ig were mixed and diluted in DMEM infection medium, i.e. complete DMEM medium supplemented with 2 μg/ml polybrene (Sigma Aldrich, St. Louis, MO), such that the final virus concentration gave 10-40 fluorescent plaque forming units (PFU) per well and final Ig concentrations corresponded to plasma Ig levels (starting at an 1:20 plasma dilution). Prior to infection, the medium in the well was replaced with 50 μl fresh medium. The virus and Ig mixtures were incubated at 37 °C for one hour and subsequently titrated in triplicate (150μl/well). The day after infection, plates were washed once and fresh medium added (200 μl). As positive neutralization controls IgG and IgA from one of the plasma with high neutralizing activity was included throughout the experiments; positive virus controls consisted of wells with cells and virus without Ig; negative controls consisted of wells with virus mixed with a pool of plasma Ig from 6 HIV-uninfected women from the same cohort; and cell controls consisted of cells only. Three days after infection, green-fluorescent expressing plaques were enumerated under the fluorescent microscope. Percentage neutralization was expressed as % plaque reduction in the virus cultures containing Ig relative to virus without sample Ig and calculated by the formula [37]: Plaque reduction (%) = [1-(PFU with sample Ig) x100] / PFU without sample Ig. The cut-off for neutralization in this assay was based on intra-assay variation, determined in three consecutive assays run on the same day. Since the standard deviation (SD) of

intra-assay variation was repeatedly found to be less than 10%, a 30% reduction in fluorescent

PFU, corresponding to >3 SD, was chosen as cut-off [37]. Reduction of PFU by negative control

plasma Ig, tested individually from six HIV uninfected subjects, was repeatedly below 30% (data

not shown).

Magnitude of Plasma IgG and IgA Neutralizing Activity

For analysis of the magnitude of plasma Ig NAc, plasma Ig was titrated against one HIV-1 and

one HIV-2 isolate. The magnitude of NAc in an individual plasma Ig was determined by its

neutralization score defined as log-transformed titers [38]. Log-transformed titers were calculated

by dividing the highest dilution giving neutralization by 100 before applying a log-base 3

transformation and then adding 1 [Y=log3 (dilution/100)+1]. All titers below 1:40 were given a

value of 33 for the sake of calculation of a neutralization score.

HIV-Specific Neutralizing Activity in Relation to Total Plasma IgG and IgA

For determination of proportions of IgG and IgA with specific, i.e. neutralizing activity, the

reciprocal intratype neutralizing titer of plasma IgG and IgA were related to total plasma IgG and

IgA levels. Reciprocal intratype neutralizing titer of plasma IgG and IgA were divided by the

amount of IgG and IgA, respectively, present in 150 μl, corresponding to the volume assessed in

neutralization assay setup (see above).

Statistical Methods

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For comparisons between unrelated categorical variables, Fisher´s exact test was used. Differences between independent subgroups of numerical variables were assessed by Mann Whitney´s U test or Kruskal-Wallis as appropriate. Magnitude of IgG and IgA derived from HIV-D plasma directed against HIV-1 and HIV-2 isolates were compared using Wilcoxon signed rank test. Spearman´s rank test was used to assess correlations. Analyses were performed using SPSS and GraphPad Prism 5 version 5.2.

Ethical Considerations

The study was approved by the Ethics Committee at the Ministry of Health in Guinea-Bissau and the Ethical Committee at the University of Lund, Sweden. Study participants were counseled and provided informed verbal consent. Participants were offered free medical examination with free essential medications. Study participants that tested positive for HIV were referred to HIV treatment and support centers.

RESULTS

Neutralization of HIV-1 and HIV-2 by Plasma IgG and IgA

To study neutralizing activity mediated by plasma IgG and IgA in HIV-1 and HIV-2 infections, neutralization of one HIV-1 and one HIV-2 isolate was assessed in a plaque reduction assay. Purified IgG and IgA were tested in dilution steps starting from the concentration corresponding to a 1:20 plasma dilution. IgG from HIV-1 infected individuals showed moderate level neutralization of the HIV-1 isolate (intratype neutralization), similarly to our previous experience with HIV-1 plasma, median reciprocal titer 50 (Table 2). Neutralization of the HIV-2 isolate with HIV-1 IgG (intertype neutralization) was of similar level, median reciprocal titer 60, with the exception of IgG of two plasma (Table 2). In contrast, intratype neutralization mediated by HIV-2 plasma IgG was found to be stronger, median reciprocal titer 163840 (Table 2). The strong intratype NAc of HIV-2 IgG contrasted the lack of demonstrable intertype neutralization. Plasma IgA neutralization, whether intra- or intertype, was absent in 9/10 HIV-1 infected. In HIV-2 infection, plasma IgA with intratype neutralization was instead found more frequently, 7/10 cases ($p<0.05$). Quantitative comparison of the magnitude of neutralization confirmed that intratype neutralization with plasma IgG as well as IgA differed significantly between HIV-1 and HIV-2 infections (Figure 1A and 1B) ($p<0.001$ and $p<0.01$, respectively). Likewise, HIV-2 IgG intratype neutralization was of significantly higher magnitude than intertype neutralization ($p<0.001$), whereas HIV-1 IgG intertype and intratype neutralization were at similarly low level. Moreover, magnitude of IgG-mediated intratype neutralization was found to be higher than corresponding IgA neutralization in HIV-2 infection ($p<0.001$). In addition, we noted the magnitudes of HIV-2 intratype neutralizing IgG and IgA correlated ($p<0.05$, $r=0.72$). In

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summary, these findings suggest that intratype-specific neutralization by plasma IgA is frequently detected in HIV-2, but not in HIV-1 infection. Moreover, the magnitudes of intratype neutralization mediated by both IgG and IgA of HIV-2 plasma exceeded that of HIV-1 IgG and IgA, respectively.

Neutralization by IgG and IgA Derived From HIV-D Plasma

For the purpose of analyzing HIV-1 and HIV-2-directed neutralizing antibody responses generated within the same host we tested IgG and IgA purified from plasma of HIV-D individuals. Results showed that HIV-D IgG neutralized both the HIV-1 and HIV-2 isolates (Table 2). Furthermore, the magnitude of HIV-2 directed IgG neutralization was found to be higher than that of HIV-1 directed IgG ($p<0.001$) (Figure 1A). In fact, the magnitude of HIV-1 neutralization was similar to that seen with plasma IgG from HIV-1 singly infected. HIV-D IgA did not neutralize the HIV-1 isolate, with the exception of IgA from one plasma, similarly to HIV-1 IgA, but neutralized the HIV-2 isolate as frequently and potently as IgA from HIV-2 singly infected (Table 2 and Figure 1). Taken together, this indicates that type of virus play role in eliciting the frequency and strength of neutralization by both plasma IgG and IgA.

IgG and IgA Intratype Neutralizing Activity in Relation to Total Plasma IgG and IgA

For the analysis of proportions of IgG and IgA with specific activity, i.e. neutralizing capacity, in plasma of HIV-1 and HIV-2 infected individuals the total plasma levels of IgG and IgA were determined. It was noted that plasma IgG levels varied depending on the type of HIV infection.

Plasma of HIV-1 and HIV-D infected displayed significantly higher levels of plasma IgG (p<0.0001 and <0.01, respectively) compared to plasma of HIV-uninfected, while IgG levels in HIV-2 plasma, although somewhat elevated, were not significantly different from HIV-negative individuals (Figure 2A). Thus, the elevated IgG levels in HIV-1-infected individuals may be the sign of hypergammaglobulinemia and reflect chronic immune activation, not as prevalent in HIV-2 infection. Unlike IgG, plasma IgA levels were similar in the four groups examined (Figure 2B).

Next, analysis of the proportion of IgG and IgA with intratype neutralizing capacity in plasma of HIV-1 and HIV-2 infected individuals was assessed by division of reciprocal neutralization titers with total plasma IgG and IgA levels expressed in mg. Results showed that HIV-2 intratype neutralizing titer per mg IgG was elevated, in median 5400-fold, as compared to corresponding HIV-1 IgG titer, p<0.001 (Figure 3). Proportion of HIV-2 IgA with intratype neutralizing capacity was also found to be increased as compared to corresponding HIV-1 IgA, however, the median difference was more moderate, around 10-fold. Thus, despite lower or similar total Ig levels detected in plasma of HIV-2 infected, as compared with plasma of HIV-1 infected, the proportion of antibodies with neutralizing capacity appeared higher in HIV-2 infection. Moreover, the HIV-1 and HIV-2 neutralizing IgG and IgA titers in plasma of dually infected individuals displayed similar proportions in relation to total IgG and IgA as corresponding intratype reactivates in the singly infected individuals (data not shown). Since variation in plasma IgG levels could reflect disease progression, we went on to analyze NAc in relation to clinical parameters. An inverse correlation between CD4 count and magnitude of intratype IgG neutralization was noted in infection with HIV-1 (p<0.05 r=-0.70) but not with HIV-2. Moreover, since all plasma of the HIV-2 infected displayed viral load levels below the level of

quantification (<281 RNA copies/ml) (Table 1), no correlations was noted between magnitudes of HIV-2 IgG or IgA intratype neutralization and viral load. Thus, although the power of the statistical analyzes was limited by the low numbers of participants, these results suggest that magnitude of HIV-1 intratype IgG neutralization is elevated with severity of the disease, while magnitude of HIV-2 IgG and IgA intratype neutralization appears not.

DISCUSSION

In the present work, we compared side-by-side plasma IgA- and IgG-mediated neutralization in HIV-1, HIV-2 and HIV-D infections. We show that plasma IgA-mediated intratype neutralization is more frequent in HIV-2 infection than in HIV-1 infection. Moreover, the magnitudes of both IgG and IgA intratype neutralization in HIV-2 plasma exceeded the magnitude of neutralization by HIV-1 IgG and IgA.

In agreement with recent publications, on different cohorts, by our group and others [14, 39, 40], the current study provides evidence that HIV-2-directed neutralizing antibody responses in HIV-2 infection are of high magnitude. Moreover, we here reveal that both IgG and IgA contribute to this phenomenon, since the magnitude of intratype neutralization by HIV-2 IgG and IgA was higher than that of HIV-1 IgG and IgA, respectively. Furthermore, in HIV-2 infection we found a correlation between intratype neutralizing IgG and IgA titers. This correlation could not be found in HIV-1 infection since only one out of ten plasma displayed intratype neutralizing IgA. The paucity of plasma IgA neutralization in HIV-1 infection is in agreement with another study [41]. In contrast, we noted that plasma IgA from seven out of ten HIV-2 infected displayed intratype neutralizing capacity. In support of this finding, the only previous study that pursued the analysis of HIV-2 neutralizing IgA in HIV-2 infected individuals detected this with similar frequency [19].

There may be several explanations for the differences in magnitude of IgG and IgA intratype neutralization between HIV-1 and HIV-2 infections. Differences seem to be dependent on the viruses themselves, since we here observed, in similar manner to our previous study on plasma neutralizing activity [14] that HIV-D individuals harbored IgG and IgA that neutralized HIV-1 and HIV-2 at magnitudes comparable to the corresponding intratype neutralization by IgG and IgA

from singly infected individuals. Moreover, the common phenotypes of HIV-1 and HIV-2 seem to differ in neutralization sensitivity. Therefore, isolates to be used in the present study were selected based on our previous experience [14]. HIV-1 C Br was in our earlier study found to be the most sensitive HIV-1 isolate in neutralization assays using HIV-1 plasma from Guinea-Bissau. We also avoided use of the most neutralization sensitive HIV-2 isolate, thereby bringing virus sensitivities closer to each other. Interestingly, we noted that intertype neutralization by plasma IgG was significantly stronger in HIV-1 than HIV-2 infection. Also in this case, the overall higher neutralization sensitivity of HIV-2 may provide an explanation [14, 39, 40]. The difference in frequency of neutralization sensitive virus variants in HIV-1 and HIV-2 infections is further revealed by previous studies of ours and others [14, 39, 40] and goes along with studies showing that escape from neutralizing antibodies is rare in HIV-2 infection while common in HIV-1 infection [13, 35, 42].

Interestingly, our findings also revealed that the magnitude of IgG, as well IgA intratype neutralization in relation to the level of plasma IgG and IgA was higher in HIV-2 than HIV-1 infection. Thus, despite the fact that HIV-1 plasma contained elevated levels of IgG the virus specific effector function was not increased, but rather appear to be diluted. It is well known that progressive HIV-1 infection is characterized by chronic immune activation, including aberrant cytokine secretion and unspecific activation of T and B cells [43, 44]. Chronic B cell immune activation, is also characterized by manifestation of hypergammaglobulinemia in parallel with disease progression [45]. In line with this, the present study showed that IgG levels in plasma of HIV-1 infected, either singly or dually infected, were elevated as compared with HIV-negative individuals, while HIV-2 plasma IgG was not. This despite the unexpected observation that four out of 10 HIV-2 infected individuals had CD4+ T cell count <200/µl, while this low CD4 count

was only recorded for one out of the 10 HIV-1 positive participants. A finding that might be explained by the fact the participants in the current study had attended a sexual health and family planning clinic for urogenital problems, some of which could be AIDS defining. Nevertheless, we also noted that none of the HIV-2 infected, where plasma viral load could be assessed, displayed plasma viral RNA above 281 copies/ml. This observation together with observations of others suggests that the low degree of immune activation present in HIV-2 infection is associated with the relatively low level of plasma viral load [46, 47]. Thus, it is even more intriguing that both IgG and IgA, of HIV-2 infected individuals display potent intratype neutralizing activity in the absence of strong antigenic stimulation. However, the magnitude of intratype IgG neutralization appeared elevated with severity of HIV-1 disease, here detected as decreasing CD4+ T cell counts, while this was not the case in HIV-2 infection.

It is known that HIV-1 preferentially infects CD4+CCR5+ memory T cells in GALT, and thereby affects the structure of germinal centers in this tissue. Since GALT hosts ASC that almost exclusively secrete IgA, defects in the ability to generate high-affinity HIV-1 specific IgA may be the result [48, 49]. Although the extent of GALT involvement and lymphocyte depletion in HIV-2 infection has not been explored yet, the frequent HIV-2 specific plasma IgA responses detected indicate better preservation of germinal centers in the GALT of HIV-2 infected. In contrast, aberrant chronic immune activation and augmented replication of HIV-1 may lead to destruction of IgA secreting cells in GALT, depriving the HIV-1 infected host of anti-HIV-1 IgA. Presence of powerful IgA-mediated neutralization in the presence of intact gut mucosa in HIV-1 infected LTNP also supports this hypothesis [50, 51].

Monomeric serum IgA has been described as an antibody isotype with anti-inflammatory properties [27, 52, 53] inasmuch it lacks complement-activating capacity and suppresses chemotaxis, phagocytosis, as well as cytokine release [29, 54-56]. We speculate that blunting of inflammatory processes via anti-HIV-2 plasma IgA may contribute to the lower immune activation in HIV-2 infection.

Taken together, HIV-2 displays features that allow potent intratype neutralization by both IgG and IgA, characteristics that are distinct from those of HIV-1. We believe that continued studies on the interaction between HIV-2 and neutralizing antibodies may reveal important knowledge on the makeup of potent humoral immune responses.

Acknowledgements

Figure legends

Figure 1. Magnitude of intra- and intertype neutralization of HIV-1, HIV-2 and HIV-D plasma IgG and IgA. Median magnitude scores of intra- and intertype neutralization of HIV-1 (n=10), HIV-2 (n=10) and HIV-1/HIV-2 dually (HIV-D) (n=8) plasma IgG (A) and IgA (B) against HIV-1 and HIV-2 isolates. The diameter of circles correspond to the magnitude of neutralization of plasma IgG and IgA. Titer-based magnitude of 0, 0.4, 0.5, 1.1, 2.1, 7.8 and 8.6 correspond to reciprocal titers of <40, 50, 60, 120, 335, 176000 and 423000, respectively. *p<0.05, **p<0.01, ***p<0.001

Figure 2. Plasma IgG and IgA levels The total amount of IgG (A) and IgA (B) in plasma of HIV-1 (n=10), HIV-2 (n=10), HIV-1/HIV-2 dually (HIV-D) (n=8) infected and HIV-negative (HIV-N) (n=16) individuals were quantified by ELISA. **p<0.01, ***p<0.001

Figure 3. Intratype functionality of plasma IgG and IgA in HIV-1 and HIV-2 infections. Immunoglobulin functionality was calculated by dividing the highest reciprocal neutralizing titer of immunoglobulin by corresponding plasma immunoglobulin level (mg) present in the assay. IgG and IgA titres where neutralization was not detected at 1:20 dilution were given a reciprocal titer of 10. Boxes illustrate interquartile range with median indicated and whiskers minimum and maximum. *p<0.05, ***p<0.001

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TABLE 1. Characteristics of study participants

	HIV-1 positive (n=10)	HIV-2 positive (n=10)	HIV-D positive (n=8)	p value [a]
Age: median (IQR[b])	26.5 (22.0-28.0)	32.0 (29.2-39.2)	34.0 (24.0-41.0)	p=0.113
Viral load, RNA copies/ml:				
<282	20%	80%	25%	
282-10000	60%		25%	
>10000	20%	(data missing for 2)	25% (data missing for 2)	p=0.006
CD4+ T cell count/µl:				
median (IQR)	472 (333-633)	493 (184-839)	352 (259-788)	p=0.891
CD4+ T cell count/µl:				
>499	40%	50%	38%	
200-499	50%	10%	50%	
<200	10%	40%	12%	p=0.294
Total IgG, mg/ml:				
median (IQR)	22.3 (16.4-31.2)	14.9 (7.4-19.4)	22.3 (12.7-30.2)	p=0.05
Total IgA, mg/ml:				
median (IQR)	1.0 (0.8-1.3)	1.0 (0.5-1.2)	0.8 (0.6-1.1)	p=0.454

[a] p-values calculated using Kruskal-Wallis, comparing means over the columns or Fisher's exact test when appropriate

[b] IQR: interquartile range

Characteristics of study participants

TABLE 2. Reciprocal titers of IgG and IgA neutralizing activity in individual plasma against HIV-1 and HIV-2 isolates

Plasma	Code	Neutralizing activity of IgG		Neutralizing activity of IgA	
		HIV-1 C Br	HIV-2 A GB/1812	HIV-1 C Br	HIV-2 A GB/1812
HIV-1	1215	80	<20	<20	<20
	1218	20	640	<20	<20
	1239	80	<20	<20	<20
	1242	160	80	<20	<20
	1246	20	40	<20	<20
	1264	<20	80	<20	<20
	1381	20	40	<20	<20
	1388	80	40	<20	80
	1426	20	40960	<20	<20
	1411	640	5120	20	<20
HIV-2	150	<20	327680	<20	160
	313	<20	81920	<20	80
	323	<20	163840	<20	160
	512	<20	1310720	<20	1280
	646	<20	1310720	<20	5120
	914	<20	163840	<20	<20
	1071	20	40960	<20	<20
	1075	<20	81920	<20	<20
	1277	<20	163840	<20	2560
	1397	<20	327680	<20	80
HIV-D	404	160	327680	<20	640
	413	20	327680	20	640
	423	80	5242880	<20	320
	427	160	163840	<20	40
	636	320	163840	<20	80
	637	160	655360	<20	320
	1438	<20	655360	<20	640
	1458	20	163840	<20	160

[a]Given reciprocal titres corresponds to 1/dilution of plasma giving IC30 in the plaque reduction neutralization assay applied and boxes are color coded as follows: white, <20; yellow, 20-640; orange, 1280-81920; red, 163840-5242880.

Reciprocal titers of IgG and IgA neutralizing activity in individual plasma against HIV-1 and HIV-2 isolates

FIGURE LEGENDS

Figure 1. Magnitude of intra- and intertype neutralization of HIV-1, HIV-2 and HIV-D plasma IgG and IgA. Median magnitude scores of intra- and intertype neutralization of HIV-1 (n=10), HIV-2 (n=10) and HIV-1/HIV-2 dually (HIV-D) (n=8) plasma IgG (A) and IgA (B) against HIV-1 and HIV-2 isolates. The diameter of circles correspond to the magnitude of neutralization of plasma IgG and IgA. Titer-based magnitude of 0, 0.4, 0.5, 1.1, 2.1, 7.8 and 8.6 correspond to reciprocal titers of <40, 50, 60, 120, 335, 176000 and 423000, respectively. *p<0.05, **p<0.01, ***p<0.001

Figure 2. Plasma IgG and IgA levels The total amount of IgG (A) and IgA (B) in plasma of HIV-1 (n=10), HIV-2 (n=10), HIV-1/HIV-2 dually (HIV-D) (n=8) infected and HIV-negative (HIV-N) (n=16) individuals were quantified by ELISA. **p<0.01, ***p<0.001
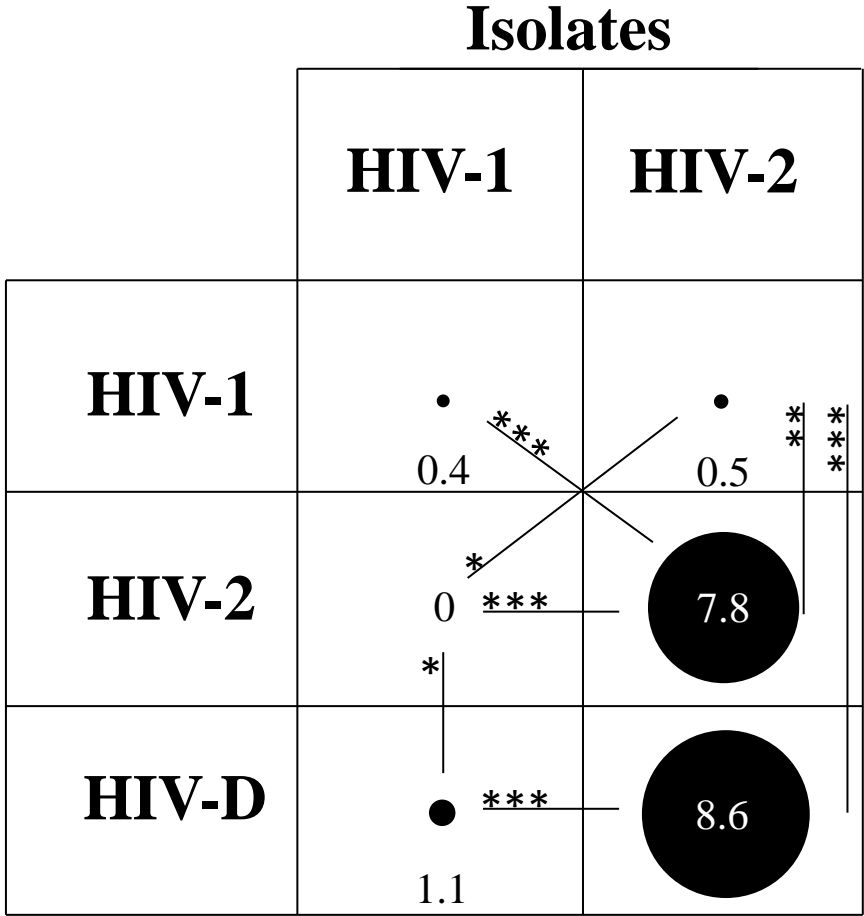
Figure 3. Intratype functionality of plasma IgG and IgA in HIV-1 and HIV-2 infections. Immunoglobulin functionality was calculated by dividing the highest reciprocal neutralizing titer of immunoglobulin by corresponding plasma immunoglobulin level (mg) present in the assay. IgG and IgA titres where neutralization was not detected at 1:20 dilution were given a reciprocal titer of 10. Boxes illustrate interquartile range with median indicated and whiskers minimum and maximum. *p<0.05, ***p<0.001
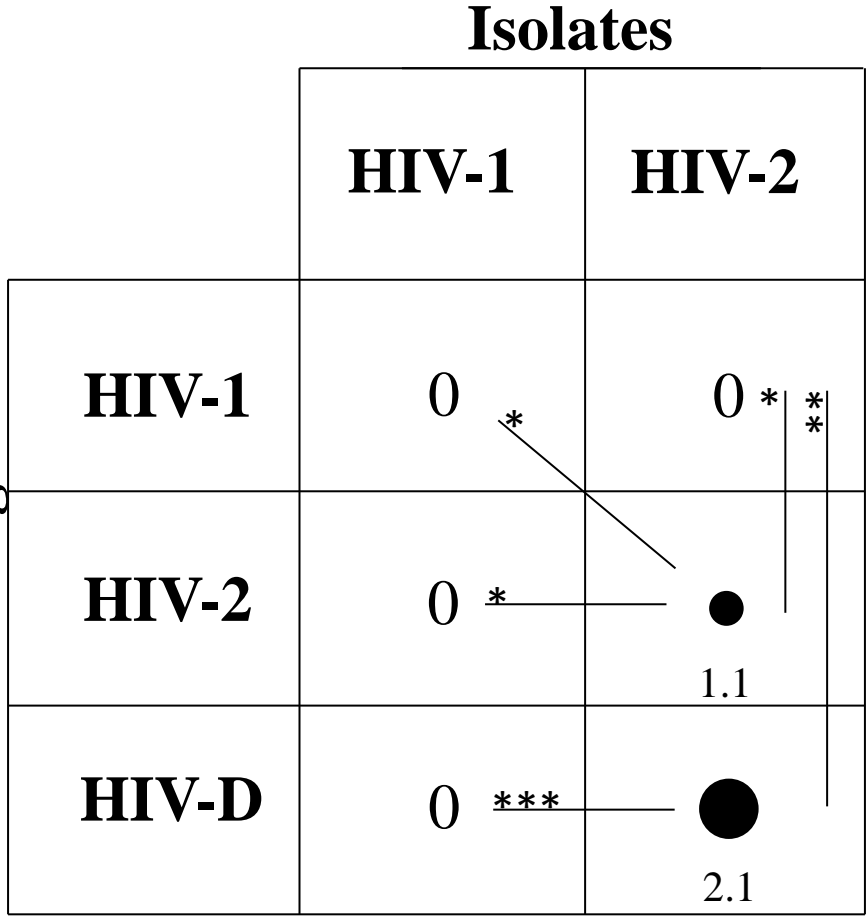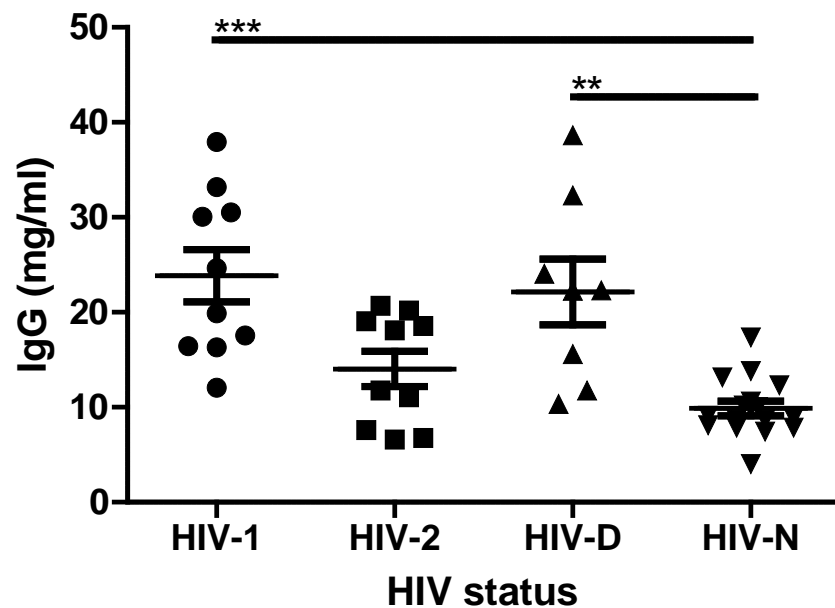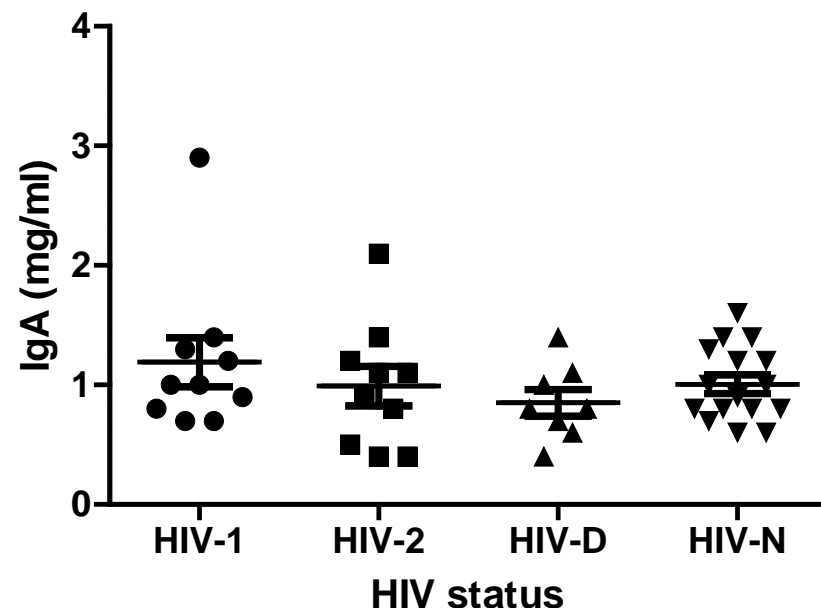
FIG. 1

A

B

FIG. 2

A

B

FIG. 3