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Serum immunoglobulin A (IgA)-mediated immunity in human immunodeficiency virus type 2 (HIV-2) infection

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Abstract

In the present study, we sought to define the importance of serum IgA (sIgA)-mediated immunity in HIV-2 infection. Serum samples from a total of 29 HIV-2-infected patients from Guinea-Bissau (n = 20) and Portugal (n = 9) were studied. Samples from seronegative individuals were used as controls. Antibody reactivity to native and recombinant envelope glycoproteins as well as peptides representing various regions of the envelope glycoproteins was investigated. Furthermore, the capacity of purified IgA to neutralize the HIV-$2_{SBL6669}$ strain was tested. All serum samples showed IgA reactivity against whole HIV-2 antigen. Twenty-eight out of 29 IgA samples (96%) reacted with native HIV-2 gp125, 26/29 (90%) with recombinant gp105, and 29/29 (100%) with recombinant gp36. When using peptides, the most prominent IgA reactivity was seen against the peptide representing aa 644–658 of the transmembranous protein gp36, to which 72% of the sera reacted. Purified sIgA antibodies showed neutralizing effects against HIV-$2_{SBL6669}$ in 17/29 cases (59%). This work decribes the HIV-2-specific sIgA antigenic response. Moreover, our findings show, for the first time, that sIgA may play a role in the in vitro neutralization of HIV-2.
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Introduction

HIV-2, the second type of lentivirus discovered to cause immunodeficiency in humans, was first isolated from a West African patient (Albert et al., 1987; Clavel et al., 1986; Kanki et al., 1987). HIV-2 is confined mainly to West Africa where the highest prevalence rates have been reported from Guinea-Bissau. Fairly high numbers have also been detected in Portugal and in India.

The envelope proteins play, as with other enveloped viruses, an important role in the interaction with the receptor, in fusion, induction of cytopathic effect, and development of humoral and cellular immune responses. The two glycoproteins are known to be highly antigenic and immunogenic. HIV-2 infections, like HIV-1 infections, result in the production of neutralizing antibodies predominantly directed against regions in the envelope glycoprotein (Ranki et al., 1987; Weiss et al., 1985) and there is also in vitro evidence of HIV-2-specific antibodies with capacity to neutralize HIV-1 (Böttiger et al., 1989, 1990; Robert-Guroff et al., 1992; Weiss et al., 1988). The primary neutralizing anti-HIV-2 immunoglobulin G (IgG) antibody response is strain specific and directed against the third variable region (V3) (Björling et al., 1991, 1994; Matsushita et al., 1995; McKnight et al., 1996; Mörner et al., 1999). Also other regions within HIV-2 gp125 have epitopes important for IgG binding (Björling et al., 1991; Skott et al., 1999).

At a later phase a more broadly reactive virus-neutralizing activity in the sera of HIV-1-infected individuals can be found. Antibodies responsible for HIV-2-neutralizing activity have been characterized to predominantly identify dis-

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continuous sites and of particular importance in this context may be the discontinuous structures responsible for the CD4 binding to the native gp125 (for review, see Björling et al., 1996, 1999). All these studies have been focused on the biological activity mediated by HIV-2-specific IgG.

The demonstration of HIV-1-specific IgA directed against envelope (Env) glycoproteins suggests a potential role for IgA in viral neutralization, as functional IgA serves in a defensive capacity against other human viral pathogens (Dimmock, 1993). Kozlowski and co-workers (1994) showed an increased production of HIV-1-specific polymeric serum IgA (sIgA) due to the rise of both subclasses IgA1 and IgA2 during early HIV-1 infection. Serum IgA1 (sIgA1) antibodies were directed predominantly against the envelope glycoproteins, and for patients with AIDS and symptomatic HIV-1 infection a declining titer of sIgA antibodies against these antigens could be seen. We and other groups have earlier shown IgA-mediated neutralization of HIV-1 (Devito et al., 2000a, b; Kozlowski et al., 1994; Skott et al., submitted) and a role for IgA-mediated HIV-1 neutralization in vivo has also been suggested (Burrer et al., 2001). We have also quantitatively compared the biological function of HIV-1-specific serum IgA (sIgA)/secretory IgA (S-IgA) versus immunoglobulin G (IgG) antibodies per milligram both against laboratory-adapted virus and primary isolates and found that IgA antibodies seem to be more effective in neutralizing HIV-1 as opposed to IgG (Skott et al., submitted). We could also correlate a strong sIgA-mediated HIV-1-neutralizing activity with defined epitope specificity with preference for the V4 region of gp120 (Skott et al., 1999), in contrast to sera derived from highly exposed HIV-1 individuals with preference for an epitope, LQAR, within the coil-coiled pocket in the aminoterminal part of gp 41 (Pastori et al., 2000).

This is the first study describing an HIV-2-specific humoral sIgA antigenic and neutralizing response, and furthermore we show the epitope specificity of HIV-2-specific sIgA derived from HIV-2 serum.

Results

Purification of IgA1

IgA1 could be purified from all serum samples with an average purification efficiency of 85%, with purity and possible residual contaminating IgG evaluated by Commassie-stained electrophoresis gels (Fig. 1). No high-mass molecular weight proteins or residual IgG in the serum samples could be detected. To confirm these results an ELISA was performed using the same samples to determine that the amount of residual IgG did not exceed 0.1 μg/ml. A similar procedure was performed for purified IgG to eliminate IgA contamination. OD values ranged from 0.14 to 0.169 for purified IgG samples and from 0.051 to 0.210 for purified IgA samples, respectively.

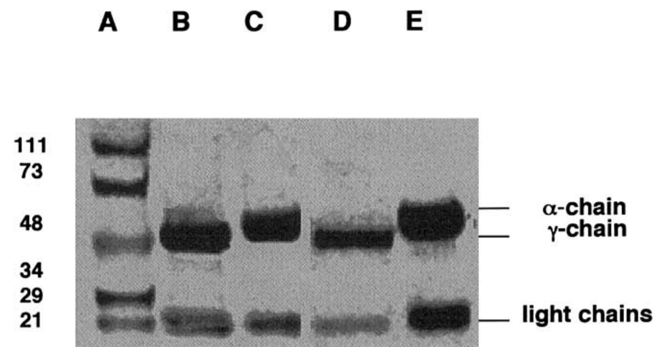

Fig. 1. SDS-PAGE separated and Coomassie blue-stained purified immunoglobulin G (IgG) and immunoglobulin A (IgA) serum fractions from two HIV-2-positive patients. Lanes B and D represent purified IgG from serum and lanes C and E purified IgA from serum. The migration of molecular weight standards is indicated in kDa to the left (A).

Quantification of total sIgA and IgG

sIgA could be detected in all serum samples at individual values of 0.3–7.1 mg/ml. The values of the control group ranged from 0.1 to 4.2 mg/ml. The mean values of total sIgA derived from HIV-2 antibody-positive patients did not differ significantly compared to the control group.

Total serum IgG concentrations ranged between 0.2 and 7.5 mg/ml. The values for the control group ranged between 0.3 and 5.5 mg/ml. Total serum IgG levels in the African controls were not done.

Antibody reactivity against whole HIV-2 antigen, native and recombinant envelope proteins of HIV-2

All serum samples 29/29 (100%) showed IgA reactivity against the whole HIV-2 antigen. For native gp125 a similar pattern could be seen. Twenty-eight out of 29 samples (96%) were positive in IgA ELISA, for rgp105 26/29 (90%) and rgp36 29/29 (100%), respectively. None of the controls showed sIgA reactivity to whole HIV-2 antigen, native gp125, gp105, or gp36 (Table 1).

All serum samples showed IgG reactivity against whole HIV-2 antigen, native gp125, and rgp105 in ELISA. For rgp36, 25 out of 29 sera showed reactivity. None of the controls showed any seroreactivity to either protein (data not shown).

Quantification of IgA in relation to ELISA reactivity against HIV-2 proteins

To compare reactivity between the HIV-2 serum samples used in ELISA against native gp125, rgp105, and rgp36, the IgA OD values were also expressed as OD value/mg total purified IgA to the different proteins (Fig. 2). All of the proteins were coated onto the plate in the same concentration (0.1 μg/well), and when comparing between the different proteins used, all sera displayed the highest reactivity in OD value/mg IgA against rgp36.

Table 1
Amino acid sequences of synthetic peptides corresponding to selected linear sites in the envelope glycoproteins of HIV-2

Peptide	Residues	Region	Amino acid sequence	Serum IgA reactivity (%)
S1–2	160–186/ROD	V2	CQFNMTGLERDKKKQYNETWYSKDVVC[a]	14
S1–19	189–205/ROD	V2/C2	CETNNSTNQTQCYMNHC	—[c]
S11–11	283–297/ROD	V3	SGLVFHSHYQPINKR	28
A43–29	311–330/ISY	V3	SGRRFHSQKIINKKPRQAWC	10
A43–36	318–337/ISY	V3	QKIINKKPRQAWCRFKGEWR	24
S1–18	399–419/ROD	V4	CNMTWFLNWIENKTHRNYAPC	7
S12–37	582–603/ISY	gp36	RLNSWGCAFRQVCHTTVPWVND	38
A43–9	615–634/ISY	gp36	WQEWEHKIRFLEANISESLE(C)[b]	45
A43–10	634–648/ISY	gp36	EQAQIQQEKNMYELQ(C)	45
A43–12	644–658/ISY	gp36	MYELQKLNSWDVFGN(C)	72
Whole viral lysate				100
Native gp125				96
rgp105				90
rgp36				100

Reactivity mediated by IgA derived from a panel (n = 29) of human anti-HIV-2 sera against whole viral lysate, native gp125, rgp105, rgp36, and peptides are also shown.

[a] Amino acid sequences were derived from the published sequences of the HIV-2 ROD and the ISY molecular clone of the SBL6669 HIV-2 strain [33].

[b] (C), a C-terminal cysteine was added to the peptide to allow coupling to keyhole limpet hemocyanin.

[c] No IgA antibody reactivity was seen.

IgA antibody reactivity in serum to synthetic peptides corresponding to the envelope proteins of HIV-2

The external glycoprotein of the envelope of HIV-2 was mimicked by six different peptides corresponding to the second variable region (V2, aa 160–186), two different parts of the third variable region (V3, aa 283–297 and aa 311–337), and the fourth variable region (V4, aa 399–419) (Table 1).

The peptide representing the V2 region was recognized by IgA antibodies in 4 out of 29 patients (14%), and no serum IgA reactivity could be seen against the peptide representing the V2/C2 region (189–205). The IgA serore-activity to the peptides representing the V3-loop was strongest to the peptide corresponding to the aminoterminal part of the loop (aa 283–297), with 8/29 (28%) positive samples, compared to the peptides mimicking a more C-terminal part of the V3 region (aa 311–337) which showed lower reactivity, 3/29 (10%) and 7/29 (24%), respectively (Table 1). Only 2 out of 29 samples (7%) recognized the peptide representing the V4 region aa 399–419 (Table 1). The transmembraneous protein, gp36, was represented by four

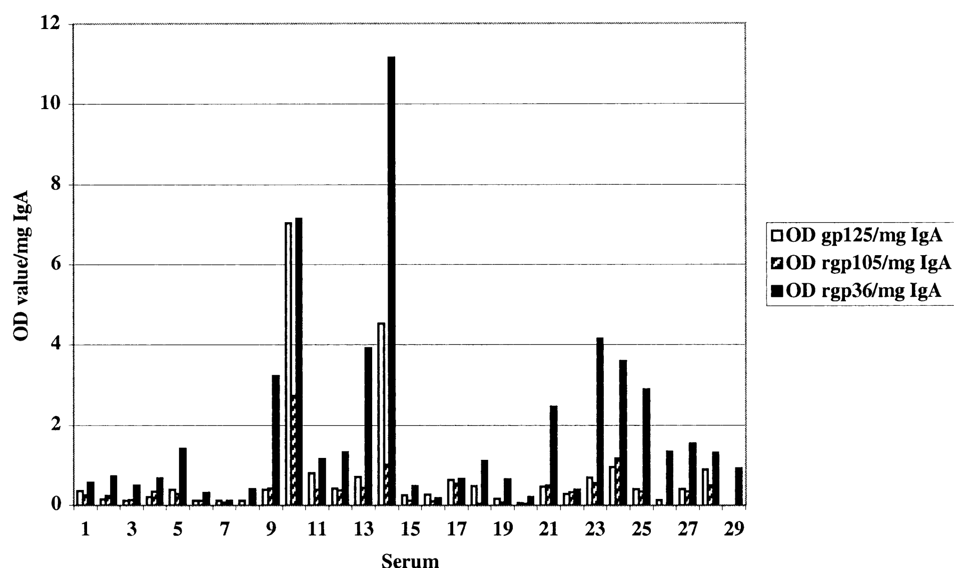

Fig. 2. Antibody reactivity in ELISA mediated by IgA derived from a panel (n = 29) of human anti-HIV-2 sera against HIV-2 native gp125, rgp105, and rgp36. All proteins were coated in a concentration of 0.1 μg/well. OD values/mg Ig are expressed on the y axis and categories on the x axis represents the individual sera.

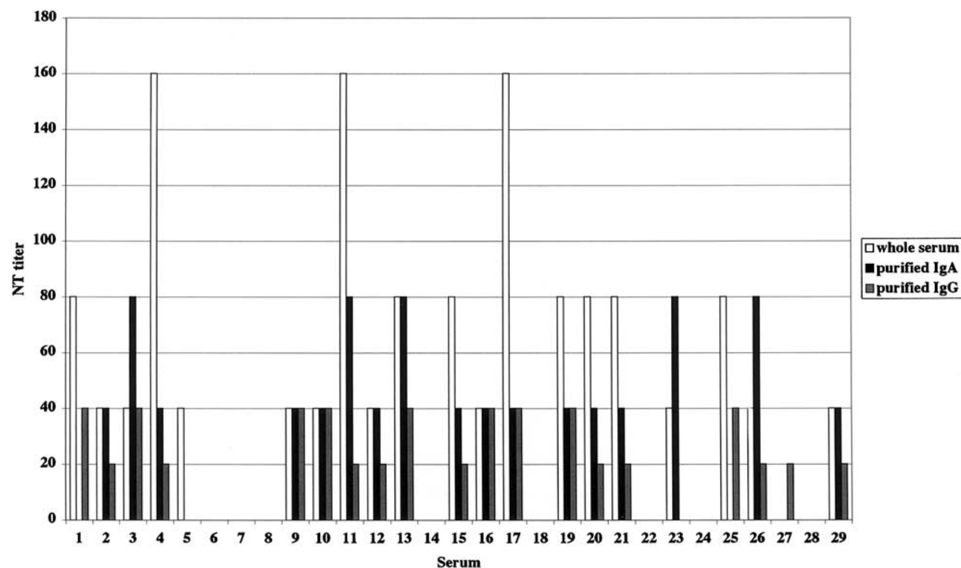

Fig. 3. Neutralizing activity against the T-cell-line-adapted strain HIV-$2_{SBL6669}$ mediated by whole serum in correlation to purified antibodies (IgA, black bars; IgG, gray bars; and whole serum, white bars). Neutralization titer (NT) is defined by the last dilution resulting in a >80% reduction of infectivity of the virus in peripheral blood mononuclear cells. NTs are expressed on the *y* axis and categories on the *x* axis represents the individual sera.

peptides corresponding to aa 582–603 and from aa 615 to aa 658. The peptide representing the amino terminal part of gp36 was recognized by 11/29 (38%) serum IgA samples. Peptides A43–9, 10, and 12 were recognized by 45, 45, and 72% of the sera, respectively. The most prominent IgA activity could be seen against aa 644–658 (Table 1).

Neutralizing activity of individual serum samples against HIV-$2_{SBL6669}$

Purified sIgA antibodies showed a neutralizing effect against HIV-$2_{SBL6669}$ in 17/29 cases with titers between 40 and 80 (Fig. 3). The neutralizing activity mediated by sIgA antibodies could not be correlated to the total amount of IgA in serum. Furthermore, neither a high sIgA reactivity to rgp36 nor a high gp125/gp105 reactivity (OD/mg) was indicative of antibodies with virus-neutralizing capacity. This was exemplified by samples 5, 24, and 28 that exhibited high rgp36 reactivity and sample 14 that exhibited high gp125 reactivity yet showed no detectable neutralizing activity.

Neutralization mediated by purified IgG from serum against HIV-$2_{SBL6669}$ could be observed in 19 out of 29 cases with titers ranging from 20 to 80 (Fig. 3), with a somewhat lower activity compared to the same samples of purified IgA. Whole serum-mediated neutralization of HIV-$2_{SBL6669}$ could be confirmed in 20 out of 29 cases with titers ranging from 40 to 160 (Fig. 3). Neutralizing activity was observed both in sera derived from HIV-2-positive patients from Guinea-Bissau and Portugal. No neutralizing activity was detected in any of the control serum samples (whole serum, purified sIgA or IgG).

Quantification of IgA in relation to neutralizing activity

To compensate for the variation in IgA and IgG concentration and subsequently in the amount of IgA and IgG obtained during purification, HIV-2-neutralizing IgA and IgG titers were also expressed as neutralizing IgA (or IgG) titer/mg total purified IgA (or IgG). When comparing Igs per milligram, IgG was shown to be more potent than IgA in neutralization. No correlation could be seen between the amount of purified IgA or IgG and the neutralizing titer against HIV-$2_{SBL6669}$ (data not shown).

Discussion

Besides immune exclusion, additional roles for IgA have been recognized, such as blocking of the adherence for viral antigens to the epithelial cells and inhibition of virus assembly, internal neutralization (Mazanec et al., 1993). Human serum IgA can initiate phagocytosis by Kuppfer cells (liver cells) in vivo (probably are S-IgA too large to be important) shown by phagocytosis of bacteria. This suggests a second line of defense in mucosal immunity, by eliminating invasive bacteria entering through the portal circulation and thus preventing disease (van Egmond et al., 2000).

IgA-mediated neutralization has been shown for many viral pathogens. Among these are poliovirus, cytomegalovirus, type A influenza virus (Dimmock, 1993), Puumala hanta virus (de Carvalho et al., 2000), rotaviruses (Greenberg et al., 1988; Johansen and Svensson, 1994), and Epstein Barr virus (Yao et al., 1991). We and other groups

have earlier shown this phenomenon also for HIV-1 (Devito et al., 2000a, b; Moja et al., 2000; Skott et al., submitted).

In another study describing uninfected HIV-exposed individuals, Mazzoli and co-workers (1999) reported that the sIgA protecting against infection recognized HIV epitopes located at the transmembrane protein while the HIV-positive individuals recognized mainly gp120 and gp160.

In this study we analyzed the epitopes of HIV-2 recognized by serum HIV-2-specific IgA in HIV-2-infected patients. The neutralizing activity mediated by sIgA antibodies could not be correlated to the total amount of sIgA in serum. A high sIgA reactivity to rgp36 or gp125/rgp105 was not found to be directly indicative of neutralizing activity. When using peptides corresponding to the external envelope protein gp125, we could only find reactivity with purified IgA in a small portion of the serum samples, at most 28% to the V3 region. These results differ from earlier findings reported by us for HIV-1 (Skott et al., 1999). However, we could observe a strong sIgA-mediated neutralizing activity in the same serum with distinct reactivity against peptides corresponding to the central extramembrane region of gp36. This is in line with earlier reported results for HIV-1-specific IgA binding to two different neutralizing immunodominant epitopes within the extramembrane portion of gp41 (Muster et al., 1994; Pastori et al., 2000; Skott et al., 1999; Vanini et al., 1993).

In three of our patient samples containing purified sIgA or IgG antibodies we could see a strong neutralizing capacity compared to a somewhat less efficient activity for homologous whole serum. Although this was a rare event in the present study, this phenomenon has earlier been discussed in a study concerning influenza virus neutralization: a low amount of purified IgG was more efficient in neutralization as compared to whole serum, due to the axial rotation and necessary mobility for the antibody to bind and inhibit the virus (Dimmock, 1993).

In conclusion, we have reported, for the first time, that human sIgA antibodies derived from whole serum in natural HIV-2 infection have the capacity to neutralize HIV-2. Our data in this study demonstrate, without excluding possible roles of other Ig subclasses, that in addition to IgG, sIgA plays an important role in the in vitro neutralization of HIV-2.

The identification of viral immunodominant epitopes important for protection via an IgA-mediated response against HIV could be one tool for the development of new therapies against HIV infection. Further studies will be required to define the in vivo protective role of both sIgA and mucosally derived secretory IgA in HIV-2 infection.

Materials and methods

Serum samples

The study group consisted of 20 HIV-2 antibody-positive sera derived from patients from a cohort in Guinea-Bissau and 9 sera derived from HIV-2-positive patients from Portugal. Furthermore, 12 HIV-2 seronegative sera derived from the same cohort in Guinea-Bissau and 7 healthy seronegative samples derived from individuals living in Sweden were included as controls.

Blood samples were drawn from the subjects, whole blood was centrifuged at 1200 rpm, and serum was separated and stored immediately at −20°C until analyzed. The serum samples were inactivated at 56°C for 30 min before use.

Peptide synthesis

Ten peptides representing previously known IgG-specific antigenic sites in the envelope proteins of HIV-2 were synthesized as earlier described by Björling and co-workers (1991). The peptide amino acid sequences were derived from the published sequences of HIV-2_{ISY} and HIV-2_{ROD} (Table 1). All peptides were analyzed using high-performance liquid chromatography (HPLC) to confirm their grade of purity, exceeding 65% before use.

Whole HIV-2 antigen, native gp125, and recombinant glycoproteins

As whole HIV-2 antigen, we used purified virions of SBL6669 grown on U937:2 cells, kindly provided by Professor G. Biberfeld, Swedish Institute for Infectious Disease Control. Native gp125, derived from HIV-$2_{SBL6669}$, was purified by affinity chromatography using *Galanthus nivalis* agglutinin (Gilljam, 1993). Native gp125 was kindly provided by the department of Virology at the Swedish Institute of Infectious Disease Control. Recombinant gp105 (rgp105), derived from HIV-2_{ROD}, was produced in baculovirus expression system. Recombinant gp105 and gp36 (rgp36) were commercially obtained from Intracel (London, UK) and ViroGen Corp. (Watertown, MA), respectively.

Purification of IgA and IgG antibodies

IgA was purified from serum on *Artocarpus integrifolia* agglutinin (jacalin). Briefly, 200 μl of jacalin/agarose beads (Vector, Burlingame, CA) was added to 20 μl of serum diluted 1:10 in RIPA buffer (2% v/v Triton X-100, 150 mM NaCl, 600 mM KCl, 5 mM dinatrium EDTA, pH 7.8). The mixture was incubated on an end-to-end rotator at room temperature for 2 h. After binding, the mixture was centrifuged for 3 min (10,000*g*) and washed three times with low-salt extraction buffer (LSEB, 2% v/v Triton X-100, 150 mM NaCl, 600 mM KCl, 5 mM disodium EDTA, 3 mM PMSF, 1 μg aprotinin/ml in 10 mM Tris-HCl, pH 7.8), and then eluted overnight at 4°C with 50 μl 200 mM methyl-α-D-galactopyranoside. The following day, the supernatant was harvested after centrifugation for 3 min (3000*g*). An equal volume of protein G-Sepharose (Pharmacia, Uppsala, Sweden) was added and incubated on an end-to-end rotator

for 2 h to exclude remaining IgG from the first purification step. After centrifugation for 3 min (3000*g*), the supernatant containing IgA was harvested. The protein G-Sepharose was washed with start buffer (20 mM Na_2HPO_4, pH 7.0), and IgG was eluted with elution buffer (0.1 M glycine-HCl, pH 2.2) for 10 min, followed by centrifugation for 1 min (13,000 rpm). After harvest the eluted IgG was immediately adjusted to pH 7 using neutralization buffer (2.5 M Tris-Base, pH 9.0).

SDS electrophoresis gels

Separating gel was performed by mixing 3.9 ml acrylamide 40%, 2.1 ml bis-acrylamide 2%, 3.1 ml Tris-HCl 1.5 M, (pH 8.8), and 2.0 ml ddH_2O and polymerization ingredients 56 μl, 20% SDS, 75 μl ammonium persulfate, and 10% 5.6 μl Temed and polymerized for 1 h at room temperature.

The stacking gel was done by mixing 0.5 ml acrylamide 20%, 265 μl bis-acrylamide 2%, 25 μl SDS 20%, 665 μl Tris-HCl 0.5 M (pH 6.8), and 3.5 ml ddH_2O and polymerized with 100 μl ammonium persulfate, and 10%, 3.5 μl TEMED for 1 h. The gel was thereafter kept at +4°C until use.

The purified IgA and IgG samples (5 μl) were mixed with 15 μl of sample buffer in Eppendorf tubes and heated to 100°C for 3 min; thereafter the samples were loaded (20 μl) onto the gel. Five microliters molecular weight markers were included in each run as reference (Amersham Laboratories, Buckinghamshire, Great Britain). The electrophoresis procedure was performed with running buffer (Tris 15.0 g, SDS 5.0 g, glycine 72.0 g, dest H_2O up to 1000 ml) at 100 V for 45 min. The next step was to stain the gel into a staining buffer (0.25 g of Coomassie brilliant blue R-250 in 90 ml of methanol:H_2O (1:1 v/v) and 10 ml of glacetic acid) for 4 h onto a slow rocking platform. Destaining of the gel was done overnight onto a slow rocking platform with destaining buffer containing methanol/acetic acid solution (20% methanol and 10% acetic acid). Thereafter the gel was read and then put onto a filter paper with a thin plastic layer on top and dried overnight in a vacuum gel dryer.

Quantification of immunoglobulins

An ELISA was used for quantification of total and purified IgA and IgG, respectively, and also as a control for residual sIgA and IgG together with SDS electrophoresis gels. Human IgA (Jackson Laboratories, WestGrove, PA) and IgG (Calbiochem, San Diego, CA) were used to generate standards. The OD values were analyzed using DS3-1.46B software (DeltaSoft 3, Biometallic Inc) on a computer to calculate the concentrations of IgA and IgG, respectively.

Enzyme-linked immunosorbent assay (ELISA)

Peptide, whole HIV-2 antigen, native gp125, rgp36 and gp105 ELISAs were performed as follows. Briefly, microtiter plates (Nunc, Denmark) were coated overnight at room temperature with in-house-made peptides (1 μg/well), gp125 (0.1 μg/well), gp105 (0.1 μg/well, Intracel), rgp36 (0.1 μg/well, Virogen), or whole HIV-2 antigen (0.1 μg/well), respectively, dissolved in 0.05 M carbonate buffer, pH 9.6. The following day the plates were washed with ELISA-washing buffer (0.05% Tween 20 in PBS) and blocking was performed with 5% bovine serum albumin at 37°C for 2 h. After washing, serum was added at a 1:100 dilution and incubated for 1 h at 37°C. After another wash, biotinylated goat anti-human IgA conjugate (Southern Biotechnology, Birmingham, AL) diluted 1:10,000 in conjugate buffer (20% goat serum, 20% fetal calf serum, 0.5% bovine serum albumin, and 0.05% Tween 20), 100 μl/well, was added for 1 h at 37°C. After washing, streptavidin-HRP (Southern Biotechnology) was added as a second conjugate diluted 1:5000 with conjugate buffer for 1 h at 37°C. After a final washing step, *o*-phenylenediamine dihydrochloride (OPD)-substrate was added. The reaction was allowed to continue for 30 min and was then stopped by adding 2.5 M H_2SO_4. Optical density values were read at 490 nm.

Neutralization assay

For the neutralization assay, the HIV-2 strain SBL-6669 was used. Peripheral blood mononuclear cells (PBMC) from two healthy donors were prestimulated with phytohemagglutinin (PHA) for 3 days and then used in a neutralization assay as previously described (Björling et al., 1993). Briefly, diluted tissue culture supernatant of virus-infected peripheral blood mononuclear cells (50 $TCID_{50}$, 100 μl) was incubated for 1 h at 37°C with 100 μl antibody dilution. PBMC (1×10^5) in 50 μl was added to the virus-antibody reaction mixture and incubated overnight. All dilutions were performed in RPMI 1640 medium (GIBCO, Life Technologies Ltd. Paisly, Scotland) supplemented with 10% fetal calf serum, 3 mM glutamine, IL-2, and antibiotics. This medium mixture was also used for cell culture during the experiment. Medium change was performed at Days 1 and 4. Seven days after infection, supernatants were collected and analyzed by a capture ELISA (Thorstensson et al., 1991). The neutralization titer was defined as the last dilution step that showed an 80% reduction or more of optical density at 490 nm in the culture supernatant compared to the HIV antibody-negative serum. Titers equal to or above 20 were considered to represent positive neutralization and assays were repeated at least on two occasions. The purified IgA samples were not concentrated after purification. The purified samples used in the neutralization assay constituted a 1/2.5 dilution since 20 μl serum was used initially and the final volume after elution was 50 μl. The

neutralization assay was performed in six steps at twofold dilutions.

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