HIV-neutralizing immunoglobulin A and HIV-specific proliferation are independently associated with reduced HIV acquisition in Kenyan sex workers

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Objectives: HIV-neutralizing immunoglobulin A (IgA) and HIV-specific cellular immunity have been described in highly exposed, persistently seronegative (HEPS) individuals, but well controlled studies have not been performed. We performed a prospective, nested case–control study to examine the association of genital IgA and systemic cellular immune responses with subsequent HIV acquisition in high-risk Kenyan female sex workers (FSWs).

Design and methods: A randomized trial of monthly antibiotic prophylaxis to prevent sexually transmitted disease/HIV infection was performed from 1998 to 2002 in HIV-uninfected Kenyan FSWs. After the completion of trial, FSWs who had acquired HIV (cases) were matched 1 : 4 with persistently uninfected controls based on study arm, duration of HIV-seronegative follow-up, and time of cohort enrolment. Blinded investigators assayed the ability at enrolment of genital IgA to neutralize primary HIV isolates as well as systemic HIV-specific cellular IFNγ-modified enzyme-linked immunospot and proliferative responses.

Results: The study cohort comprised 113 FSWs: 24 cases who acquired HIV and 89 matched controls. Genital HIV-neutralizing IgA was associated with reduced HIV acquisition ($P = 0.003$), as was HIV-specific proliferation ($P = 0.002$), and these associations were additive. HIV-specific IFNγ production did not differ between case and control groups. In multivariable analysis, HIV-neutralizing IgA and HIV-specific proliferation each remained independently associated with lack of HIV acquisition. Genital herpes (HSV2) was associated with increased HIV risk and with reduced detection of HIV-neutralizing IgA.

Conclusion: Genital HIV-neutralizing IgA and systemic HIV-specific proliferative responses, assayed by blinded investigators, were prospectively associated with HIV nonacquisition. The induction of these immune responses may be an important goal for HIV vaccines.

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Introduction

The development of preventive HIV-1 (HIV) vaccines or microbicides as tools in the global pandemic has been hampered by a lack of established immune correlates of protection. The passive transfer of potent HIV-neutralizing antibodies protected against mucosal viral challenge in macaques [1–3], and passive transfer to HIV-infected individuals delayed viral rebound in some patients interrupting antiretroviral therapy [4]. Furthermore, higher levels of neutralizing antibodies have been described in long-term nonprogressive infection [5–7] and in mothers who did not transmit HIV to their children [8–10]. For HIV-neutralizing antibodies to protect against sexual HIV acquisition, such antibodies would likely need to be present at the level of the genital tract mucosa. In addition, HIV-specific cellular immune responses are critical in the host control of HIV after infection and may also be an important HIV vaccine goal. In particular, intact HIV-specific CD8+ proliferative capacity has been associated with long-term nonprogression [11].

Sexually transmitted infections (STIs) may disturb the genital tract immune milieu and enhance HIV susceptibility [12], and so it will be important to understand the effect of genital infections on the levels and/or function of HIV-neutralizing antibodies. This may be particularly important for gonorrhea, which can produce an immunoglobulin A (IgA) protease [13] and may impair systemic HIV-specific cellular immune responses [14].

A minority of individuals exposed to HIV appears to resist infection and are defined as highly exposed, persistently seronegative (HEPS) [5]. Such individuals may provide clues to the immune correlates of HIV protection [15]. Both mucosal (genital tract) and systemic HIV-specific immune responses have been described in HEPS groups, including HIV-specific CD4+ and CD8+ T cells, and HIV-neutralizing IgA antibodies [16–18]. Possible explanations for the induction and maintenance of these adaptive immune responses include low level continuous virus replication in mucosal tissue below the level of clinical detection [19]; single rounds of HIV replication followed by viral clearance; immune processing of nonreplication competent viruses or viral fragments [20,21]. It, however, remains unclear whether such immune responses are responsible for protection or are a paraphenomenon of nonproductive HIV exposure [22]. Furthermore, not all studies have found immune responses in HEPS subjects and those that have are generally cross-sectional and unblinded.

We hypothesized that a prospective, blinded study of HIV-specific immunity in HIV-uninfected individuals would demonstrate which, if any, of these immune responses were independently associated with reduced HIV acquisition and performed such a study within a cohort of high-risk Kenyan female sex workers (FSWs) participating in a prospective, randomized clinical trial of HIV prevention [23].

Material and methods

Study population and procedures

A double-blind, randomized trial of monthly antibiotic prophylaxis to prevent STIs and HIV infection was performed from 1998 to 2002 in Kenyan FSWs from Kibera, Nairobi [23]. Inclusion criteria were negative HIV serology, current engagement in sex work, age 18 years at least, expected residence in Nairobi for 2 years at least, and no prior adverse reaction to macrolides. In brief, 466 participants were randomized to oral azithromycin 1 g monthly or an identical placebo. All women underwent a full physical examination and STI testing and therapy at enrolment and every 6 months. HIV serology was performed every 3 months. A behavioral questionnaire was administered at enrolment and every 3 months. Condom provision and risk reduction counseling (both clinic-based and peer-based) were provided.

Cervicovaginal secretions (CVS) were collected from all FSWs at trial enrolment. One cotton-tipped swab was rotated 360° in the cervical os, and a second swab collected secretions from the posterior vaginal fornix. Both swabs were transferred into a vial containing 5 ml of phosphate-buffered saline (PBS), transferred to the laboratory within 2 h on ice, spun down to remove cellular debris, and cryopreserved at −80°C. Peripheral blood mononuclear cells (PBMCs) were isolated from enrolment blood samples by density centrifugation and cryopreserved in liquid nitrogen.

Immunoglobulin A purification

Enrolment IgA1 (IgA) was purified from thawed CVS with jacalin/agarose as previously described [24] with minor modifications. Eight hundred microliters CVS was added to 400 μl jacalin/agarose beads and mixed for 2 h at 4°C followed by centrifugation and collection of the IgA-depleted fraction. Jacalin/agarose beads were washed with PBS pH 7.4, and bound IgA was eluted overnight at room temperature in 1 ml 0.8 mol/l D-galactose pH 7.5. Purified IgA was collected and all fractions were stored at −80°C. An in-house enzyme-linked immunosorbent assay was used to quantify IgA. Plates were coated with monoclonal mouse-antihuman-IgA (Nordic Immunology, Tilburg, the Netherlands); alkaline phosphate-conjugated mouse-antihuman-IgA (Becton Dickinson, Stockholm, Sweden) was the secondary antibody. The standard was pooled normal human IgA (Nordic Immunology).

HIV-neutralization assay

HIV-1 neutralization assays used a predefined protocol and neutralization cutoff [17] and were performed by investigators blinded to clinical outcome. Two HIV

primary isolates previously used in neutralization assays [25–27], RW009 (a dual tropic clade A virus, the most common clade in Kenya) and ZA003 (a R5 tropic clade C virus, the most common clade globally; both from the NIH AIDS Research & Reference Reagent Program; Rockville, Maryland, USA) [28], were collected from phytohemagglutinin (PHA) and interleukin 2 (IL-2) stimulated PBMCs. Median tissue culture infective dose ($TCID_{50}$) values were determined and aliquots were stored at −80°C. As $TCID_{50}$ values may change depending upon PBMCs donor, three viral dilutions were used in each assay. The purified IgA was not further diluted or concentrated when tested for neutralizing capacity. Duplicate wells of each virus dilution and of each sample fraction were incubated for 1 h at 37°C, followed by addition of a mixture of 1×10^5 PHA-stimulated PBMCs from two different donors. After 16–18 h incubation at 37°C, unbound virus was washed away and fresh medium (RMPI 1640; GIBCO, Life Technologies, Gaithersburg, Maryland, USA) supplemented with fetal bovine serum and IL-2 was added. Medium was replaced on day 3, and on day 6 the supernatant was collected and analyzed in a p24 antigen enzyme-linked immunosorbent assay (Murex HIV Antigen Mab kit; Abbott Diagnostics, Abbott Park, Illinois, USA). As previously described [17], neutralization was defined as an at least 67% reduction of p24 antigen in the supernatant as compared with p24 antigen content when the virus isolate was incubated in the presence of low-risk HIV-seronegative sample. Positive control samples (HIV IgG-positive serum) were included in each assay.

HIV-specific, IFNγ-modified enzyme-linked immunospot assays

Modified enzyme-linked immunospot (ELISPOT) assays were performed by research personnel blinded to clinical outcome, as previously described [29] with minor modifications. Fifteen-mer peptides overlapping by 11 spanning the HIV-1 clade A consensus genome (NIH AIDS Research & Reference Reagent Program) were divided into 12 pools of 60–70 peptides. In addition, a panel of 50 predefined, optimized HIV-1 epitopes [29] (Sigma Genesis, The Woodlands, Texas, USA) was tested in 5 pools of 10 peptides. Microtiter plates (96-well; Multiscreen PVDF, Millipore, Bedford, Massachusetts, USA) were coated with anti-IFNγ monoclonal 1-D1K (Mabtech Inc., Mariemont, Ohio, USA) overnight at 4°C. Plates were washed 4× with PBS and blocked with 10% FBS, 100 IU/ml penicillin, 100 μg/ml of streptomycin, 2 mM glutamax for 2 h at 37°C. $1–2 \times 10^5$ PBMCs, thawed and kept at 37°C–5% CO_2 overnight, were incubated for 18 h with either defined epitope pools at 10 μg/ml/peptide (100 μg/ml total peptide concentration), 15-mer pools with each peptide at 1 μg/ml (maximum 100 μg/ml total peptide concentration), or with a positive control (PHA 10 μg/ml or staphylococcal enterotoxin B [SEB] 0.2 μg/ml). Assays were performed in duplicate. Four wells of cells in culture medium alone served as a negative control. Plates were developed and responses counted using an automated ELISPOT reader (CTL, Cleveland, Ohio, USA). HIV-specific IFNγ responses were reported as spot-forming cells (SFU)/10^6 PBMCs after subtraction of background. A positive HIV-specific IFNγ response was defined previously [29] as IFNγ production in PHA-positive or SEB-positive control wells, an HIV-specific response more than 20 SFU/10^6 PBMCs in duplicate wells of overlapping peptide or defined epitope pools, and HIV-specific response at least 2× background.

HIV-specific proliferation assays

HIV-specific cellular proliferation was assayed by ^{3}H-thymidine incorporation after the stimulation of PBMCs with peptide pools. Due to limited cell numbers, PBMCs from corresponding wells of ELISPOT plate were removed, transferred into a sterile 96-well tissue culture plate, and stimulated with the same antigen for 6 further days. Wells were pulsed with 1 mCi/well ^{3}H-thymidine for 6 h, harvested using a 96-well cell harvester (Tomtec Inc., Hamden, Connecticut, USA), and uptake measured with a scintillation counter. A stimulation index was calculated as the mean count per minute from cells stimulated with antigen or mitogen, divided by the mean count per minute from unstimulated cells. An assay was considered positive if the stimulation index in positive control wells was greater than 5 and the HIV-specific stimulation index was greater than 5.

Statistical analysis

Analyses were performed with SPSS version 11.0 software (Chicago, Illinois, USA). FSWs acquiring HIV during the trial (cases) were matched by an external biostatistician to FSWs who had remained HIV uninfected for at least the time to case seroconversion (controls). Matching was performed blind to immune assay results and was based on time of study enrolment, study arm (azithromycin vs. placebo), and duration of HIV-seronegative follow-up. IgA assays were performed for all participants, but PBMCs availability varied. For 15 controls, neither ELISPOT nor proliferation data were available. As all clusters were essentially complete when these records were omitted (they had both cases and controls), these records were omitted from the conditional logistic regression analysis. For a further 10 records (three cases, seven controls) ELISPOT data were available, but proliferation data were not. Single imputation was used to maintain these clusters in the multivariable model and was performed by replacing the missing value of 'proliferation' by its expected value (i.e., the probability that it equals 1), estimated using stepwise logistic regression on all other variables associated with proliferation in univariate analysis. Study outcome (HIV acquisition) was not used to predict the proliferation value.

Univariate associations of HIV acquisition were assessed in a matched case–control format, using Mantel–Haenszel

analysis (dichotomous variables) or conditional logistic regression (continuous variables). Stratified multivariable analyses of the associations of HIV acquisition were performed by conditional logistic regression using a Cox proportional hazards model, with the inclusion of all variables associated with HIV acquisition in univariate analysis ($P \leq 0.05$). SPSS does not offer conditional logistic regression *per se*, and so these analyses were performed using an established adaptation of Cox regression to obtain equivalent results [30,31]. Models were compared using the likelihood ratio (LR) test.

Ethical approval

The study protocol was approved by institutional review boards at the Kenyatta National Hospital (Nairobi, Kenya), the University of Toronto (Toronto, Canada), the University of Manitoba (Winnipeg, Manitoba, Canada), and Karolinska Institutet (Stockholm, Sweden).

Results

Description of study population

Despite a successful HIV risk reduction program and a substantially reduced incidence of STIs, the addition of monthly azithromycin prophylaxis did not reduce the incidence of HIV and 35 participants acquired HIV between May 1998 and November 2002 [32]. Each case was matched with a mean of four FSW controls (women who remained HIV-seronegative). One shipment of study samples was thawed (destroyed) in transit, and so samples from 24 cases and 89 controls were available for analysis (mean, 3.7 controls matched per case). Case and control groups were generally similar, but cases had a higher rate of herpes simplex virus type 2 (HSV2) infection and a higher alcohol intake (Table 1). Sexual risk taking declined after enrolment, as has been described in the larger cohort [32], but at the time of HIV acquisition by the case there was no difference in the estimated HIV exposures over the past year from matched controls (median 15.2 vs. 17.3; $P = 0.9$).

Table 1. Enrolment characteristics of Kenyan sex workers who acquired HIV during follow-up (cases) or remained HIV uninfected (controls).

	Cases ($n = 24$)[a]	Controls ($n = 89$)[a]
Age (years)	27 (19–47)	29 (18–51)
Age at first sex (years)	16 (14–20)	16 (11–25)
Duration of prostitution (years)	4.5 (1–13)	4 (0–24)
Estimated HIV exposures (past year)[b]	186 (3–468)	154 (1–975)
Charge for sex (Ksh)[c]	50 (10–400)	100 (10–500)
Women with regular partner	10 (42%)	47 (53%)
Work from own home	15 (63%)	54 (61%)
Practice vaginal douching	19 (79%)	60 (67%)
Hormonal contraception	7 (29%)	33 (37%)
Ever practice sex during menses	6 (25%)	18 (23%)
Ever practice anal intercourse	3 (13%)	14 (16%)
HSV2 seropositive	24/24 (100%)	51/81 (63%)**
Neisseria gonorrhoeae	3 (13%)	9 (10%)
Syphilis	4 (17%)	4 (4%)
Alcohol intake[d]	2.7 (0–4)	1.6 (0–4)**

HSV2, herpes simplex virus type 2.
[a]Median or number (range or %).
[b]Calculated from self-reported condom use [reported on a semiquantitative scale ranging from 0 (never use) to 5 (always use)] and weekly client numbers, assuming 30% HIV prevalence in male clients. In cases, this number was calculated for the year prior to the first seropositive date; in controls, for the year prior to the date most closely matching the cases' duration of follow-up.
[c]Ksh: Kenyan shilling (1 USD ≈ 70 Ksh).
[d]Alcohol use was reported on a semiquantitative scale ranging from 0 (none) to 4 (> 2 drinks/day).
**$P \leq 0.01$ for comparison between groups (Mantel–Haenszel cluster analysis).

HIV-neutralizing genital tract immunoglobulin A and HIV acquisition

IgA purified from enrolment CVS neutralized the primary clade A isolate in 31/113 participants (27%); 29/89 (33%) controls and 2/24 (8%) cases [Mantel–Haenszel common odds ratio (MHOR) estimate, 0.08; 95% confidence interval (CI), 0.01–0.62; $P = 0.02$; Fig. 1]. Neutralizing IgA activity against the clade C isolate was detected in 70/113 participants (62%); 60/89 (67%) controls and 10/24 (42%) cases (MHOR estimate, 0.31; 95% CI, 0.11–0.85; $P = 0.02$; Fig. 1). Overall, IgA with neutralizing activity against either clade A or clade C was present in the genital tract of 83/113 (74%) FSWs and was strongly associated with reduced HIV acquisition [72/89 controls (81%) vs. 11/24 cases (46%); MHOR estimate, 0.19, 95% CI, 0.06–0.57; $P = 0.003$; Fig. 1]. As each of clade A and clade C neutralizing activity was independently associated with reduced HIV acquisition,

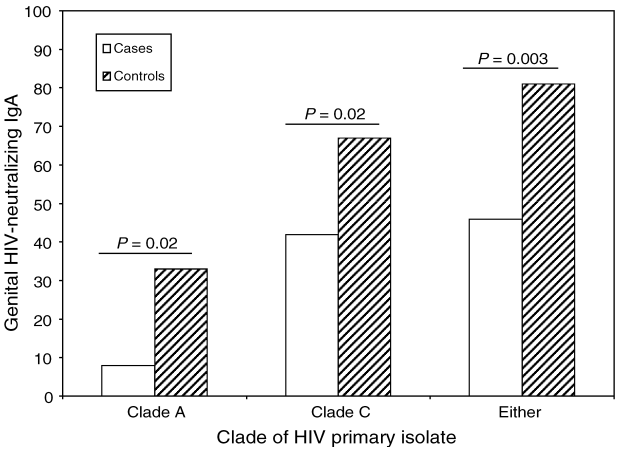

Fig. 1. Frequency of HIV-neutralizing immunoglobulin A (IgA) in the genital tract of female sex worker (FSW) cases and controls. The *y*-axis indicates the percentage of cases (hollow bar) and controls (hatched bar) with genital tract IgA-neutralizing capacity against a primary HIV-1 clade A isolate (left), a primary HIV-1 clade C isolate (center), or either the clade A or clade C isolate (right).

Table 2. Enrolment characteristics of Kenyan sex workers with or without HIV-specific immune responses.

	Neutralizing IgA ($n=83$)[a]	No neutralizing IgA ($n=30$)[a]
(a) Associations of HIV-neutralizing IgA (either clade A or clade C)		
Age (years)	29 (18–48)	27 (19–51)
Age at first sex (years)	16 (12–23)	16 (11–27)
Duration of prostitution (years)	4 (0–24)	4 (1–14)
Estimated HIV exposures (past year)[b]	99.5 (1–975)	127 (4–468)
Charge for sex (Ksh)[c]	100 (10–500)	50 (10–500)
Women with regular partner	42 (51%)	14 (47%)
Work from own home	49 (59%)	20 (67%)
Practice vaginal douching	57 (69%)	22 (73%)
Hormonal contraception	28 (34%)	12 (40%)
Ever practice sex during menses	15 (18%)	9 (30%)
Ever practice anal intercourse	14 (17%)	3 (10%)
HSV2 seropositive	50/76 (66%)	25/29 (86%)**
Neisseria gonorrhoeae	7 (8%)	5 (17%)
Syphilis	4 (5%)	4 (13%)
Alcohol intake[d]	1 (0–4)	2 (0–4)
	ELISPOT positive ($n=39$)[a]	**ELISPOT negative ($n=59$)[a]**
(b) Associations of HIV-specific IFNγ ELISPOT		
Age (years)	32 (20–51)	27 (18–45)**
Age at first sex (years)	16 (12–25)	16 (11–27)
Duration of prostitution (years)	5 (1–24)	3 (0–22)**
Estimated HIV exposures (past year)[b]	97.5 (1–764)	117 (3–975)
Charge for sex (Ksh)[c]	50 (10–400)	100 (10–500)
Women with regular partner	21 (54%)	26 (44%)
Work from own home	34 (87%)	46 (78%)
Practice vaginal douching	10 (26%)	18 (31%)
Hormonal contraception	15 (38%)	20 (34%)
Ever practice sex during menses	8 (21%)	14 (24%)
Ever practice anal intercourse	8 (21%)	7 (12%)
HSV2 seropositive	24/36 (67%)	43/54 (80%)
Neisseria gonorrhoeae	4 (10%)	6 (10%)
Syphilis	4 (10%)	4 (7%)
Alcohol intake[d]	2 (0–4)	2 (0–4)
	Proliferation positive ($n=29$)[a]	**Proliferation negative ($n=58$)[a]**
(c) Associations of HIV-specific proliferation		
Age (years)	31 (18–40)	29 (18–51)
Age at first sex (years)	17 (12–27)	16 (11–25)
Duration of prostitution (years)	3 (1–20)	4.5 (0–24)
Estimated HIV exposures (past year)[b]	78 (1–780)	117 (3–975)
Charge for sex (Ksh)[c]	100 (20–300)	100 (10–500)
Women with regular partner	19 (66%)	25 (43%)
Work from own home	25 (86%)	44 (76%)
Practice vaginal douching	20 (69%)	40 (69%)
Hormonal contraception	12 (41%)	19 (33%)
Ever practice sex during menses	6 (21%)	12 (21%)
Ever practice anal intercourse	5 (17%)	9 (16%)
HSV2 seropositive	19/27 (70%)	39/53 (74%)
Neisseria gonorrhoeae	4 (14%)	5 (9%)
Syphilis	2 (7%)	3 (5%)
Alcohol intake[d]	0 (0–4)	3 (0–4)

HSV2, herpes simplex virus type 2; IgA, immunoglobulin A.
[a]Median or number (range or %)

subsequent analyses defined HIV-neutralizing IgA as the neutralization of either clade A, clade C, or both.

HIV-neutralizing IgA was not associated with level of recent HIV exposure or several other demographic factors (Table 2a). HSV2 infection was associated with reduced HIV-neutralizing IgA (25/29 FSWs without neutralizing IgA were HSV2 infected vs. 50/76 with neutralizing IgA; LR = 4.7; $P=0.03$). When, however, analysis was restricted to HSV2-infected FSWs, HIV-neutralizing IgA remained strongly associated with reduced HIV acquisition (MHOR estimate, 0.25, 95% CI, 0.08–0.79; $P=0.02$). There were no differences between case and control groups in the total level of CVS IgA [median 10.3 vs. 9.1 μg/ml, respectively (range 1–54 vs. 1–58, respectively)], and the lowest concentration able to neutralize either clade A or clade C virus was 1 μg/ml (data not shown).

HIV-specific cellular immune responses

HIV-specific IFNγ ELISPOT assays were performed when enrolment PBMC samples were available ($N=98/113$, 87%) and proliferation assays when sufficient cells remained after the ELISPOT ($N=87/113$, 77%). HIV-specific IFNγ responses were detected in 39/98 participants (40%), but the frequency did not differ between cases and controls (7/24, 43% vs. 32/74, 29%; MHOR estimate, 0.51; 95% CI, 0.17–1.58; $P=0.2$; Fig. 2). Adjusting the cutoff for a positive ELISPOT assay to 50 SFU/million or 100 SFU/million PBMCs reduced the frequency of positive assays, but there remained no association with HIV acquisition status (data not shown). HIV-specific proliferation was detected in 29/87 participants (33%) at enrolment and was more common in controls [27/71, 38% vs. 2/16, 12.5% for observed data only; $P=0.002$ (Fig. 2); 34.0 vs. 13.2% for observed and imputed data; $P=0.005$ (conditional logistic regression); OR, 5.65; 95% CI, 1.37–33.33]. An increased age and prior duration of sex work were associated with HIV-specific IFNγ ELISPOT but not proliferative responses (Table 2b and c). Neither HIV-specific ELISPOT nor proliferation responses were associated with HIV-neutralizing IgA. The detection of both HIV-neutralizing IgA and HIV-specific proliferation within the same participant was associated with reductions in HIV acquisition that were at least additive: HIV was acquired by 8/17 (47%) of FSWs with neither response, 7/46

Table 2. (*Continued*).
[b]Calculated from self-reported condom use [reported on a semiquantitative scale ranging from 0 (never use) to 5 (always use)] and weekly client numbers, assuming 30% HIV prevalence in male clients. In cases, this number was calculated for the year prior to the first seropositive date; in controls, for the year prior to the date most closely matching the cases' duration of follow-up.
[c]Ksh: Kenyan shilling (1 USD ≈ 70 Ksh).
[d]Alcohol use was reported on a semiquantitative scale ranging from 0 (none) to 4 (> 2 drinks/day).
**$P \leq 0.01$ for comparison between groups (Mantel–Haenszel cluster analysis).

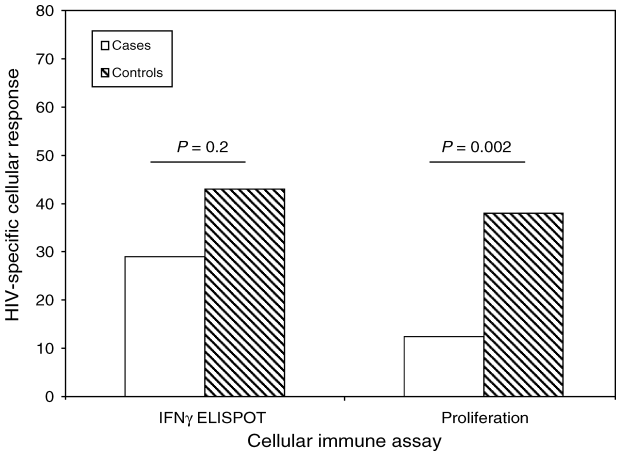

Fig. 2. Frequency of HIV-specific cellular immune responses in the blood of female sex worker (FSW) cases and controls. The *y*-axis indicates the percentage of cases (hollow bar) and controls (hatched bar) with a positive HIV-specific IFNγ ELISPOT assay (left) or HIV-specific proliferative response (right).

(15%) of FSWs with either response, and 1/24 (4%) of FSWs with both responses present at enrolment (LR = 12.0; $P = 0.002$; Fig. 3).

Multivariable associations of HIV acquisition

Matched conditional logistic regression was performed incorporating all variables associated with HIV acquisition in univariate analysis ($P \leq 0.05$): higher alcohol intake, HIV-neutralizing IgA activity, and HIV-specific cellular proliferation. HIV acquisition remained independently associated with HIV-neutralizing genital IgA (adjusted OR, 0.22; $P = 0.01$), HIV-specific proliferation (adjusted OR 0.17; $P = 0.05$), and alcohol intake (adjusted OR per unit, 1.77; $P = 0.03$).

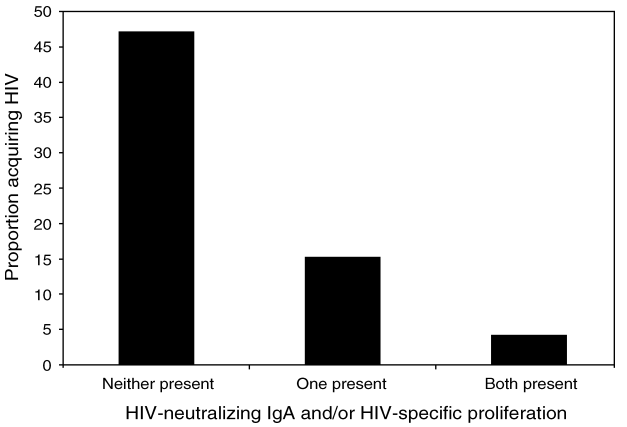

Fig. 3. Association of combined immunoglobulin A (IgA) and proliferative immune responses with HIV acquisition in cases and controls. The *y*-axis indicates the proportion of female sex workers (FSWs) acquiring HIV with neither a neutralizing IgA nor an HIV-specific proliferative response (left), either a neutralizing IgA or an HIV-specific proliferative response (centre), both a neutralizing IgA and an HIV-specific proliferative response (right).

Multivariable analysis was complicated by the fact that all HIV seroconverters were HSV2 infected, making HSV2 serostatus the strongest predictor of HIV acquisition. We, therefore, constructed a similar stepwise multivariable conditional logistic regression analysis model, with analysis restricted to HSV2 seropositive participants. Restriction of the multivariable analysis to HSV2-infected participants did not change the strength of the associations (i.e., the OR remained constant), but statistical significance was reduced (HIV-neutralizing genital IgA, adjusted OR, 0.30, $P = 0.1$; HIV-specific proliferation, adjusted OR, 0.1, $P = 0.1$; alcohol intake, adjusted OR per unit, 2.35, $P = 0.1$), likely due to reduced numbers.

Discussion

The correlates of natural immune protection against HIV – if such immunity exists – remain poorly understood. Several potential immune defenses have been described in HEPS individuals, including HIV-specific CD8+ and CD4+ T cells [16], CD8+ antiviral factor(s) [33], and IgA that can neutralize HIV or block epithelial transcytosis [17,18]. We have now demonstrated an independent association between reduced HIV acquisition and the prior detection of both genital HIV-neutralizing IgA and HIV-specific cellular proliferation. Interestingly, HIV acquisition was least common in those participants with both immune responses present at baseline, suggesting a possible additive effect of humoral and cellular immunity. HIV-specific IFNγ ELISPOT responses, demonstrated in prior studies to be mediated by CD8+ T cells [16,29], were also common but not associated with differences in HIV acquisition. Importantly, this study was nested within a large prospective clinical trial [23], and all immune assays were performed in a blinded fashion. To avoid allocation bias, predefined assay protocols and positive cutoffs were used for the IgA [24] and ELISPOT [29] assays. The proliferation assays were a variation of prior methods [34] adapted to permit the use of the same cells from the ELISPOT assays. These data are, therefore, substantially more robust than those generated in earlier, less rigorously controlled studies.

HIV-neutralizing activity was more common against the clade C than clade A primary isolate in our assay system, although the latter clade predominates in Kenya [28,35,36]. Different primary isolates, however, vary considerably in their neutralization sensitivity. Thus, a much broader panel of clade A and clade C isolates would need to be tested before firm conclusions could be drawn regarding clade specificity. Neutralization of both clades A and C was seen in genital IgA fractions from several participants, in keeping with our previous findings [17]. Further work is needed to demonstrate whether this

activity was mediated by a single cross-reactive IgA antibody clone or the presence of various antibodies, each preferentially recognizing different clades. Mapping the epitope specificity of these genital HIV-neutralizing IgAs will also be important, because such monoclonal antibodies could hold promise as vaccine and/or microbicide candidates. The concentration of total IgA in the mucosal samples was low and the HIV-specific IgA is most likely only a small fraction of this total IgA content. The HIV-neutralizing assay may thus be more sensitive than HIV-specific IgA-binding assays. This may partly explain the controversies regarding the lack of detectable mucosal IgA in binding assays as opposed to detection of HIV-neutralizing IgA in exposed uninfected individuals (reviewed in [37]).

Although rigorous, there are limitations of our study format that merit discussion. First, IgA and cellular immune assays were only performed at a single baseline time point, as CVS was only collected at enrolment. This is relevant, because several FSWs with HIV-neutralizing IgA and/or HIV-specific cellular immune responses did go on to acquire HIV; conversely, several high-risk FSWs without these responses did not acquire HIV. We could not elucidate whether infection occurred despite persistent immune responses in the former group or whether HIV-specific immunity waned prior to HIV acquisition [38]. The latter would imply a need for regular immune 'boosting' of HIV-specific responses or perhaps that responses were only protective against certain HIV strains; although the former would suggest that infection may still occur, despite such responses, perhaps if the challenge dose of HIV exceeds an undefined infection threshold. Likewise, IgA or cellular immune responses could have developed later in participants without responses at baseline, but who did not go on to acquire HIV. Despite suggestions that the maintenance of HEPS HIV-specific IgA and CD8+ T-cell responses require boosting by ongoing HIV exposure [22,38–40], we did not see any association with levels of recent HIV exposure.

It must be emphasized that we can only demonstrate an association between immune responses and HIV protection in an observational cohort study and cannot prove causation. Although IgA and/or cellular immune responses may be protective, it is also possible that HIV exposure without infection secondarily induces HIV-specific immunity, and that protection is mediated by (an)other factor(s). The only way to demonstrate causality would be to randomly induce the putative protective response, and only it, within a randomized, controlled experimental system and to examine the effect on HIV incidence.

As sexual HIV transmission involves two or more sexual partners, one of whom is HIV infected, it is a weakness that the infected partner – that is, the male FSW client responsible for transmission or exposure without transmission – was not available for study. It is possible that unknown factors within this partner, either viral or host, may have promoted the induction of immunity in their female partner(s) rather than HIV transmission. Such factors could include HLA mismatching, genital tract viral load and coinfection status, host immune responses, and many other factors [41]. The fact that FSWs had so many sexual partners, however, may also be seen as an advantage, as this means that such partner-specific factors were 'averaged out' across clients.

STIs, particularly HSV2, but also gonorrhea and others, have been implicated in HIV susceptibility. HSV2 was most strongly associated with HIV acquisition in this study and also with a reduction in the frequency of genital-neutralizing IgA (but not systemic cellular responses). No differences in IgA concentration, however, were seen between the HSV2-positive group as compared with the HSV2-negative group (data not shown). HSV2 infection might have interfered with the induction of HIV-neutralizing IgA through alterations in the genital immune milieu or host factors impairing IgA responses might have enhanced susceptibility to both HIV and HSV2; alternatively, other factors may have both impaired IgA responses and increased HIV susceptibility. Regardless, the strength of the associations between neutralizing IgA and reduced HIV acquisition remained unchanged in the subgroup of HSV2-infected FSWs, although statistical significance was lost due to reduced statistical power.

In summary, IgA with an ability to neutralize either clade A and clade C HIV was frequently detected in the genital tract of high-risk FSWs, as were T cells producing IFNγ and/or proliferating in response to HIV peptides. Of these, neutralizing-IgA and HIV-specific proliferation were independently, prospectively associated with reduced sexual acquisition of HIV. The induction of these responses may be an important intermediate goal for HIV vaccines. The effect of HSV2 infection on the efficacy of IgA-based vaccines will need to be studied carefully.

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References

1. Baba TW, Liska V, Hofmann-Lehmann R, Vlasak J, Xu W, Ayehunie S, *et al*. **Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal simian-human immunodeficiency virus infection.** *Nat Med* 2000; **6**:200–206.
2. Mascola JR, Stiegler G, VanCott TC, Katinger H, Carpenter CB, Hanson CE, *et al*. **Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies.** *Nat Med* 2000; **6**:207–210.
3. Veazey RS, Shattock RJ, Pope M, Kirijan JC, Jones J, Hu Q, *et al*. **Prevention of virus transmission to macaque monkeys by a vaginally applied monoclonal antibody to HIV-1 gp120.** *Nat Med* 2003; **9**:343–346.
4. Trkola A, Kuster H, Rusert P, Joos B, Fischer M, Leemann C, *et al*. **Delay of HIV-1 rebound after cessation of antiretroviral therapy through passive transfer of human neutralizing antibodies.** *Nat Med* 2005; **11**:615–622.
5. Pantaleo G, Menzo S, Vaccarezza M, Graziosi C, Cohen OJ, Demarest JF, *et al*. **Studies in subjects with long-term nonprogressive human immunodeficiency virus infection.** *N Engl J Med* 1995; **332**:209–216.
6. Pilgrim AK, Pantaleo G, Cohen OJ, Fink LM, Zhou JY, Zhou JT, *et al*. **Neutralizing antibody responses to human immunodeficiency virus type 1 in primary infection and long-term-nonprogressive infection.** *J Infect Dis* 1997; **176**:924–932.
7. Cao Y, Qin L, Zhang L, Safrit J, Ho DD. **Virologic and immunologic characterization of long-term survivors of human immunodeficiency virus type 1 infection.** *N Engl J Med* 1995; **332**:201–208.
8. Guevara H, Casseb J, Zijenah LS, Mbizvo M, Oceguera LF 3rd, Hanson CV, *et al*. **Maternal HIV-1 antibody and vertical transmission in subtype C virus infection.** *J Acquir Immune Defic Syndr* 2002; **29**:435–440.
9. Lathey JL, Tsou J, Brinker K, Hsia K, Meyer WA 3rd, Spector SA. **Lack of autologous neutralizing antibody to human immunodeficiency virus type 1 (HIV-1) and macrophage tropism are associated with mother-to-infant transmission.** *J Infect Dis* 1999; **180**:344–350.
10. Scarlatti G, Albert J, Rossi P, *et al*. **Mother-to-child transmission of human immunodeficiency virus type 1: correlation with neutralizing antibodies against primary isolates.** *J Infect Dis* 1993; **168**:207–210.
11. Betts MR, Nason MC, West SM, *et al*. **HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells.** *Blood* 2006; **107**:4781–4789.
12. Kaul R, Pettengell C, Sheth PM, Sunderji S, Biringer A, Macdonald K, *et al*. **The genital tract immune milieu: an important determinant of HIV susceptibility and secondary transmission.** *J Reprod Immunol* 2007; March 27 [Epub ahead of print].
13. Mulks MH, Plaut AG. **IgA protease production as a characteristic distinguishing pathogenic from harmless neisseriaceae.** *N Engl J Med* 1978; **299**:973–976.
14. Kaul R, Rowland-Jones SL, Gillespie G, Kimani J, Dong T, Kiama P, *et al*. **Gonococcal cervicitis is associated with reduced systemic CD8+ T cell responses in human immunodeficiency virus type 1-infected and exposed, uninfected sex workers.** *J Infect Dis* 2002; **185**:1525–1529.
15. Kulkarni PS, Butera ST, Duerr AC. **Resistance to HIV-1 infection: lessons learned from studies of highly exposed persistently seronegative (HEPS) individuals.** *AIDS Rev* 2003; **5**:87–103.
16. Kaul R, Plummer FA, Kimani J, Dong T, Kiama P, Rostron T, *et al*. **HIV-1-specific mucosal CD8+ lymphocyte responses in the cervix of HIV-1-resistant prostitutes in Nairobi.** *J Immunol* 2000; **164**:1602–1611.
17. Devito C, Hinkula J, Kaul R, *et al*. **Cross-clade HIV-1-specific neutralizing IgA in mucosal and systemic compartments of HIV-1-exposed, persistently seronegative subjects.** *J Acquir Immune Defic Syndr* 2002; **30**:413–420.
18. Devito C, Broliden K, Kaul R, *et al*. **Mucosal and plasma IgA from HIV-1-exposed uninfected individuals inhibit HIV-1 transcytosis across human epithelial cells.** *J Immunol* 2000; **165**:5170–5176.
19. Zhu T, Corey L, Hwangbo Y, *et al*. **Persistence of extraordinarily low levels of genetically homogeneous human immunodeficiency virus type 1 in exposed seronegative individuals.** *J Virol* 2003; **77**:6108–6116.
20. Parren PW, Gauduin MC, Koup RA, *et al*. **Relevance of the antibody response against human immunodeficiency virus type 1 envelope to vaccine design.** *Immunol Lett* 1997; **57**:105–112.
21. Larsson M, Fonteneau JF, Lirvall M, Haslett P, Lifson JD, Bhardwaj N. **Activation of HIV-1 specific CD4 and CD8 T cells by human dendritic cells: roles for cross-presentation and non-infectious HIV-1 virus.** *AIDS* 2002; **16**:1319–1329.
22. Kaul R, Rutherford J, Rowland-Jones SL, Kimani J, Onyango JI, Fowke K, *et al*. **HIV-1 Env-specific cytotoxic T-lymphocyte responses in exposed, uninfected Kenyan sex workers: a prospective analysis.** *AIDS* 2004; **18**:2087–2089.
23. Kaul R, Kimani J, Nagelkerke NJ, Fonck K, Ngugi EN, Keli F, *et al*. **Monthly antibiotic chemoprophylaxis and incidence of sexually transmitted infections and HIV-1 infection in Kenyan sex workers: a randomized controlled trial.** *JAMA* 2004; **291**:2555–2562.
24. Devito C, Hinkula J, Kaul R, Lopalco L, Bwayo JJ, Plummer F, *et al*. **Mucosal and plasma IgA from HIV-exposed seronegative individuals neutralize a primary HIV-1 isolate.** *AIDS* 2000; **14**:1917–1920.
25. Moulard M, Phogat SK, Shu Y, Labrijn AF, Xiao X, Binley JM, *et al*. **Broadly cross-reactive HIV-1-neutralizing human monoclonal Fab selected for binding to gp120-CD4-CCR5 complexes.** *Proc Natl Acad Sci U S A* 2002; **99**:6913–6918.
26. Zhang MY, Shu Y, Rudolph D, Prabakaran P, Labrijn AF, Zwick MB, *et al*. **Improved breadth and potency of an HIV-1-neutralizing human single-chain antibody by random mutagenesis and sequential antigen panning.** *J Mol Biol* 2004; **335**:209–219.
27. Binley JM, Wrin T, Korber B, Zwick MB, Wang M, Chappey C, *et al*. **Comprehensive cross-clade neutralization analysis of a panel of antihuman immunodeficiency virus type 1 monoclonal antibodies.** *J Virol* 2004; **78**:13232–13252.
28. Neilson JR, John GC, Carr JK, Lewis P, Kreiss JK, Jackson S, *et al*. **Subtypes of human immunodeficiency virus type 1 and disease stage among women in Nairobi, Kenya.** *J Virol* 1999; **73**:4393–4403.
29. Kaul R, Dong T, Plummer FA, Kimani J, Rostron T, Kiama P, *et al*. **CD8(+) lymphocytes respond to different HIV epitopes in seronegative and infected subjects.** *J Clin Invest* 2001; **107**:1303–1310.
30. Holford TR, White C, Kelsey JL. **Multivariate analysis for matched case–control studies.** *Am J Epidemiol* 1978; **107**:245–256.
31. Allison PD, Christakis N. **Logit models for sets of ranked items.** *Sociological methodology* 1994; **24**:199–228.
32. Kaul R, Kimani J, Nagelkerke NJ, Fonck K, Keli F, MacDonald KS, *et al*. **Reduced HIV risk-taking and low HIV incidence after enrollment and risk-reduction counseling in a sexually transmitted disease prevention trial in Nairobi, Kenya.** *J Acquir Immune Defic Syndr* 2002; **30**:69–72.
33. Skurnick JH, Palumbo P, DeVico A, Shacklett BL, Valentine FT, Merges M, *et al*. **Correlates of nontransmission in US women at high risk of human immunodeficiency virus type 1 infection through sexual exposure.** *J Infect Dis* 2002; **185**:428–438.
34. Kaul R, Trabattoni D, Bwayo JJ, Arienti D, Zagliani A, Mwangi FM, *et al*. **HIV-1-specific mucosal IgA in a cohort of HIV-1-resistant Kenyan sex workers.** *AIDS* 1999; **13**:23–29.
35. Hu DJ, Baggs J, Downing RG, Pieniazek D, Dorn J, Fridlund C, *et al*. **Predominance of HIV-1 subtype A and D infections in Uganda.** *Emerg Infect Dis* 2000; **6**:609–615.

36. Morison L, Buvé A, Zekeng L, Heyndrickx L, Anagonou S, Musonda R, *et al*. **HIV-1 subtypes and the HIV epidemics in four cities in sub-Saharan Africa.** *AIDS* 2001; **15 (Suppl 4)**:S109–S116.
37. Mesteckey J. **Humoral immune responses to the human immunodeficiency virus type-1 (HIV-1) in the genital tract compared to other mucosal sites.** *J Reprod Immunol* 2007; **73**:86–97.
38. Kaul R, Rowland-Jones SL, Kimani J, Dong T, Yang HB, Kiama P, *et al*. **Late seroconversion in HIV-resistant Nairobi prostitutes despite preexisting HIV-specific CD8+ responses.** *J Clin Invest* 2001; **107**:341–349.
39. Lo Caputo S, Trabattoni D, Vichi F, Piconi S, Lopalco L, Villa ML, *et al*. **Mucosal and systemic HIV-1-specific immunity in HIV-1-exposed but uninfected heterosexual men.** *AIDS* 2003; **17**:531–539.
40. Mazzoli S, Lopalco L, Salvi A, Trabattoni D, Lo Caputo S, Semplici F, *et al*. **Human immunodeficiency virus (HIV)-specific IgA and HIV neutralizing activity in the serum of exposed seronegative partners of HIV-seropositive persons.** *J Infect Dis* 1999; **180**:871–875.
41. Kaslow RA, Dorak T, Tang JJ. **Influence of host genetic variation on susceptibility to HIV type 1 infection.** *J Infect Dis* 2005; **191 (Suppl 1)**:S68–S77.