Anti-gp160 IgG and IgA Antibodies Associated with a Large Increase in Total IgG in Cervicovaginal Secretions from Human Immunodeficiency Virus Type 1–Infected Women

Xu-Sheng Lu, Laurent Bélec, and Jacques Pillot *Unité d'Immunologie Microbienne, Institut Pasteur, Paris, France*

To study the specific local immune response in vaginal fluids, 19 women infected with human immunodeficiency virus (HIV) type 1 and 23 seronegative controls were selected. Vaginal fluids were tested by ELISA for total IgG, IgA, and IgM levels and for specific IgG, IgA, and IgM antibodies to gp160. Total IgG, IgA, and IgM concentrations were 6.8-, 5.0-, and 2.5-fold higher, respectively, in HIV-1–infected women than in controls, with a positive correlation between IgG and IgA levels. IgG or IgA antibodies or both to gp160 were detected in 12 subjects (63%), whereas no IgM antibodies to gp160 were found. Anti-gp160 IgG strongly predominated. Serum samples were available for 11 women whose total IgG vaginal levels strongly correlated with total IgG in sera. These results suggest that transudation of serumborne antibodies is the main source of gp160-specific antibodies in the vaginal fluid of HIV-1–infected women.

Human immunodeficiency virus (HIV) transmission through heterosexual contact appears to be relatively infrequent in the absence of sexually transmitted diseases or other cofactors [1–3]. It has been suggested that specific mucosal immunity against HIV could have a significant inhibitory effect on heterosexual transmission [4]. Indeed, specific IgG and IgA antibodies to HIV have been demonstrated in vaginal fluids of HIV-infected individuals and may be involved in a putative neutralization process [4–6].

Previous studies on genital immunity to HIV have been done with techniques originally developed for serum, such as the radioimmunoprecipitation assay [7] and Western blot [4–6], which allow only qualitative detection of antibodies to HIV of the IgG and IgA isotypes. Moreover, in a study of African women, cervicovaginal fluids of some HIV-1–seronegative controls contained antibodies that bound to *pol*- and *gag*-encoded HIV proteins on Western blot strips [4], suggesting that the risk of false-positive reactions in the characterization of specific antibodies to HIV proteins is possible with this assay.

We evaluated specific IgG, IgA, and IgM antibodies to gp160 in vaginal secretions from 19 heterosexual HIV-1–infected African women.

Materials and Methods

Patients and cervicovaginal fluids. Fluids were collected from 19 heterosexual HIV-1–infected African women living in the Central African Republic. According to the Centers for Disease Control 1986 classification, they were stages II ($n = 11$), III ($n = 6$), and IV-C-2 ($n = 2$). A group of 23 healthy HIV-1–seronegative African women served as controls. Both HIV-infected and control women did not have evidence of sexually transmitted diseases at genital examination. They lacked absence of vaginal discharge, genital ulcerations, vulvar or vaginal condilomata, and other inflammatory lesions. Genital fluids were obtained by vaginal washing with 3 mL of sterile saline, centrifuged, and stored at −25°C until use. Samples were not collected during menstruation. The washing procedure was evaluated as corresponding to a 1:10 dilution of normal genital secretion [4]. Serum samples were collected from 11 HIV-infected women and controls and stored at −25°C until use.

Immunologic reagents. IgG and monomeric and polymeric IgA were purified as previously described [8, 9]. Secretory IgA (sIgA) was purified from human colostrum [8].

Anti-γ– (rabbit), -α– (sheep), and -μ (goat)–specific antibodies were purified by passage of the immune sera on the corresponding immunosorbent. Complete anti-μ specificity was achieved by additional immunosorption on insoluble IgA. For mouse monoclonal antibodies, ascitic fluids were produced and fractionated on Sepharose–protein A [10]. Polyclonal or monoclonal IgG were peroxidase-labeled by the periodate method [11].

Estimation of total immunoglobulin content by ELISA. A sandwich ELISA was used. The wells of microtiter plates (Nunc, Kampstrup, Denmark) were coated overnight at 4°C with 100 μL of polyclonal (4–8 μg/mL) or monoclonal (2 μg/mL) anti-γ, -α, or -μ IgG antibodies, in PBS. The wells were washed with PBS with 0.1% Tween 20 and overcoated with 350 μL of skim milk powder at 4% concentration in PBS as blocking solution for 3 h at 37°C. Wells were then washed and incubated for 1 h at 37°C with 100 μL of duplicate twofold serial dilutions of genital fluids or of standard purified immunoglobulin isotypes. After additional washings, 100 μL of peroxidase-conjugated anti-isotype–specific antibodies were added in the blocking solution. Immunodetection was done with orthophenylenediamine as substrate.

Received 17 June 1992; revised 3 December 1992.
Grant support: Agence Nationale de Recherche sur le Sida (ANRS 91-141).
Reprints or correspondence: Dr. Jacques Pillot, Unité d'Immunologie Microbienne, Institut Pasteur, 75724 Paris Cedex 15, France.

The Journal of Infectious Diseases 1993;167:1189–92
© 1993 by The University of Chicago. All rights reserved.
0022-1899/93/6705-0030$01.00

Downloaded from http://jid.oxfordjournals.org/ at University of Iowa Libraries/Serials Acquisitions on March 18, 2015

Petitioner Merck, Ex. 1185, p. 1189
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00033

Dimeric sIgA is the predominant molecular form of IgA in human mucosal secretions. However, the presence of monomeric forms of IgA without secretory component, as established in saliva, or of high polymeric forms associated with the component, cannot be excluded in cervicovaginal fluid. Preliminary experiments were done with various sandwich ELISAs using our polyclonal and monoclonal anti-α reagents to determine the combination recognizing both monomeric and polymeric forms of IgA with or without secretory component. The best ELISA used a monoclonal anti-α antibody (F10D9) on the solid-phase and polyclonal rabbit antibodies labeled with peroxidase for immunodetection.

For detection of IgG and IgM, polyclonal anti-γ or anti-μ antibodies were used in solid and liquid phases, respectively. Immunoglobulin concentrations were assigned as the mean of two measurements after determining the dilution factor for samples and standards that gave equal optical densities (OD) in the linear region of the standard curve.

ELISA for antibodies to HIV. Nunc plates were coated with an optimal concentration of gp160 (2.5 μg; Diagnostics Pasteur, Marnes-la-Coquette, France) and saturated with 4% skim milk powder. Cervicovaginal fluids were tested at two dilutions, 1:4 and 1:16 for the IgA and IgM isotypes and 1:40 and 1:160 for the IgG isotype, in 4% skim milk. Serum samples were tested at 1:100 for the IgA and IgM isotypes and 1:1000 for the IgG isotype in 4% skim milk. For immunodetection, labeled polyclonal anti-α, anti-γ and anti-μ antibodies were used under the same conditions.

For each pair of diluted cervicovaginal secretions, paired wells without antigen, that is, coated only with the blocking solution, served as negative control. Lack of gp160 antigen on the solid phase appeared to be an indispensable control since cervicovaginal IgA could nonspecifically bind the antigen-free solid phase. Comparing wells with and without gp160 antigen, the cervicovaginal fluids collected from 19 HIV-infected and 23 healthy HIV-free women showed a significant difference in OD ($P < .001$). ODs above the maximum (IgG, maximum = 0.099, mean = 0.050; IgA, maximum = 0.11, mean = 0.056) observed with the antigen-free control well for 42 tested secretions from HIV-positive and -negative women were considered as the threshold of positivity. For each sample, results are OD, which expresses the difference between the antigen-coated well and the antigen-free control well.

Statistical analysis. Statistical analyses were done using Student's t test and the rank order correlation test.

Results

Cervicovaginal secretions were evaluated for total IgG, IgA, and IgM levels. Total IgG was significantly higher in the 19 HIV-1–infected women (mean, 1489 μg/mL) than in controls (mean, 217 μg/mL; $P < .001$); similarly, IgA was higher (mean, 473 vs. 96 μg/mL; $P < .001$), as was IgM (195 vs. 74 μg/mL; $P = .028$). In vaginal fluids of infected individuals, the total IgG-to-IgA ratio was always >1, reaching 100 in some patients, and total IgG and IgA levels were positively correlated ($P = .042$). No correlation was found between IgG and IgM levels, or between IgA and IgM levels.

HIV-1 gp160-specific IgG or IgA antibodies were detected in the cervicovaginal secretions of 12 (63%) of the 19 HIV-1–infected women (table 1). No IgM antibodies to gp160 were found. Eleven (61%) of the 18 cervicovaginal fluid samples containing anti-gp160 IgG antibodies also had anti-gp160 IgA antibodies. In 1 patient (no. 17), only anti-gp160 IgA antibodies were detected. In 6 patients, IgG and IgA antibodies to gp160 were absent (no. 11, 14–16, 18, and 19). Quantitatively, anti-gp160 IgG antibodies largely predominated. The ODs were much higher for IgG antibodies at a dilution of 1:400 than for IgA at a dilution of 1:40 in 8 (66%) of the 12 cervicovaginal fluid samples with anti-gp160 antibodies. All uninfected women lacked IgG, IgA, and IgM antibodies to gp160.

The relationships between total levels of IgG and IgA and the presence of anti–HIV-1 antibodies of each isotype were evaluated. All 8 tested infected women with IgG levels above the median had anti-gp160 IgG; in contrast only 2 of 9 infected women had IgG levels below the median ($P < .005$). There was a positive correlation between the level of anti-gp160 IgG antibodies evaluated by the value of the OD at a

Table 1. Anti-gp160 IgG and IgA antibodies and total IgG and IgA levels in cervicovaginal secretions of 19 African women infected with HIV-1.

Patient	IgG dilution* 1:400	IgG dilution* 1:1600	Total IgG† (μg/mL)	IgA dilution* 1:40	IgA dilution* 1:160	Total IgA† (μg/mL)
1	1.500	1.144	3300	0.340	0.199	900
2	0.242	0.210	3200	0.311	0.193	176
3	0.801	0.411	3125	0.039	0	124
4	0.012	0	3100	0.130	0.060	842
5	1.901	1.901	2600	0.202	0.078	1800
6	0.028	0	1600	0.022	0	1800
7	1.901	1.775	1600	0.274	0.100	[284]
8	1.491	0.985	1590	0.349	0.199	268
9	ND	ND	1523	0	0	734
10	1.901	1.901	[1283]	0.399	0.199	45
11	0	0	1212	0	0	186
12	0.912	0.553	982	0.250	0.181	487
13	0.036	0	803	0.050	0.050	312
14	0	0	702	0	0	33
15	0	0	582	0	0	383
16	0	0	582	0	0	5
17	0	0	262	0.130	0	498
18	0	0	190	0	0	111
19	0	0	12.8	0	0	0
Controls (n = 23)						
Mean			216			96
Median			121			38.4

NOTE. ND, not done. 0 indicates optical density (OD) ≤ threshold OD.

* Results are OD, expressing differences between antigen-coated well and antigen-free control well.

† IgG is shown by decreasing values. Median distribution is in brackets. OD for anti-gp160 with total IgG or IgA levels in vaginal secretions below median values of distribution are underlined.

Downloaded from http://jid.oxfordjournals.org/ at University of Iowa Libraries/Serials Acquisitions on March 18, 2015

Petitioner Merck, Ex. 1185, p. 1190
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00033

dilution of 1:400 and the level of total IgG ($P < .002$). Anti-gp160 IgA antibodies were detected in 7 of 9 patients with IgA levels above the median and in 4 of 9 patients with levels below the median (no significant difference). Hence, no relationship could be established between total IgA levels and the level of anti-gp160 IgA (OD at 1:40). Anti-gp160 IgG (OD at 1:400) and IgA levels (OD at 1:40) appeared to be closely correlated ($P < .001$).

Serum samples were available for 11 HIV-1–infected women (table 2). Total IgG serum levels (mean, 21,779 μg/mL; range, 6000–40,200) were higher than in controls (data not shown; mean, 14,467 μg/mL; range, 12,900–19,500) ($P < .05$), but total IgA serum levels (mean, 2211 μg/mL; range, 390–5200) did not differ significantly from the controls (data not shown; mean, 2652 μg/mL; range, 1600–3520). Total IgG serum levels were correlated with total IgG in vaginal secretions (mean, 1963 μg/mL; $P < .001$). In contrast, total IgA serum levels did not correlate with IgA in vaginal secretions (mean, 476 μg/mL). Similarly, total IgM serum levels (mean, 2302 μg/mL; range, 560–6020) did not correlate with IgM in vaginal secretions (data not shown). Serum anti-gp160 IgG and IgA were 0.3–3.0 and 0.6–2.0 OD units, respectively. No correlation was found between the levels of anti-gp160 IgG antibodies in serum (OD at 1:1000) and in vaginal secretions (OD at 1:40). There was also no correlation between the total IgA serum levels and the levels of anti-gp160 IgA antibodies in vaginal secretions (OD at 1:40) and between the levels of anti-gp160 IgA antibodies in serum (OD at 1:100) and in vaginal secretions (OD at 1:40).

Discussion

Total IgG, IgA, and IgM concentrations in vaginal secretions were respectively 6.8-, 5.0-, and 2.5-fold higher in HIV-1–infected women than in controls. HIV-infected women and controls did not have sexually transmitted diseases that could have induced mucosal inflammation. Increased immunoglobulin levels in vaginal fluids from HIV-infected women demonstrate that local immunity is markedly enhanced at the non-AIDS stage of the disease. In 11 HIV-infected women, the IgG levels in vaginal fluids correlated positively with the total IgG serum levels. Higher IgG and IgA levels in infected women than in controls could be due to passive transudation of IgG and monomeric IgA from the plasma with a diffuse hyperimmunoglobulinemia secondary to the systemic polyclonal B activation characteristic of the HIV infection [12]. Increased IgM levels in vaginal secretions are probably locally produced, because passive transudation of the mainly pentameric serum immunoglobulins is unlikely. Therefore, it is possible that the polyclonal B cell activation induced by HIV [12] could also affect the secretory immune system of the female genital tract and contribute to increased release of locally produced IgM, IgG, and IgA.

Table 2. Anti-gp160 IgG and IgA antibodies and total IgG and IgA levels in serum of 11 African women infected by HIV-1.

Case	Total IgG (μg/mL)	IgG antibody to gp160* (1:1000)	Total IgA (μg/mL)	IgA antibody to gp160* (1:100)
1	32,000	2.87	1360	1.7
2	40,200	3	1450	1.9
3	39,700	>3	3300	2
4	22,900	2.8	1380	2
5	24,800	2.7	4240	1.6
7	22,600	2.7	2360	1.7
8	14,300	1.6	2240	0.8
10	19,000	0.3	5200	0.15
12	6000	2.1	420	1
13	8700	2.7	390	1.6
19	9370	1.7	1980	0.6

* Serum anti-gp160 values are in optical densities, expressing difference between antigen-coated well and antigen-free control well.

The presence of antibodies against the virus envelope precursor gp160 was found in the cervicovaginal secretions of about two-thirds of the HIV-1–infected women studied. Specific antibodies against HIV have been found by qualitative tests in the cervicovaginal secretions of women at risk for HIV infection through infected male partners [4–7]. Our assay had a sensitivity similar to that of the Western blot used previously to detect antibodies to gp160 in vaginal secretions from HIV-infected women living in Central Africa [4]. However, our ELISA had higher specificity than the Western blot. Use of a control without antigen ruled out nonspecific binding of immunoglobulins from mucosal secretions to the solid phase. Furthermore, nonimmunologic binding to proteins adsorbed on the solid phase was largely overcome by the use of skim milk powder as diluent instead of the classical Tween–bovine serum albumin mixture.

Our ELISA was particularly useful for quantifying the IgA isotype, since the anti-α reagent used recognized the different molecular forms of IgA in contrast to the usual anti-α reagents formerly used in Western blots. Our data clearly showed that anti-gp160 IgG antibodies were strongly predominant; in most cases levels were 10- to 100-fold those of IgA antibodies to gp160. The faint binding of IgA antibodies previously observed on Western blot nitrocellulose strips [4] could be explained by the excess of specific IgG antibodies to HIV, which block the binding of the lower titer IgA in vaginal secretions.

IgG and anti-gp160 IgG antibody levels were strongly correlated in most vaginal secretions. Evaluation of the respective quantities of total IgG and IgA showed an IgG concentration ranging from 0.5- to 100-fold that of IgA. Such a predominance of specific and nonspecific IgG in vaginal fluids of HIV-1–infected women was unexpected, since the vaginal mucosa is generally thought to belong to the secretory immune system, which is mainly characterized by a rela-

Downloaded from http://jid.oxfordjournals.org/ at University of Iowa Libraries/Serials Acquisitions on March 18, 2015

Petitioner Merck, Ex. 1185, p. 1191
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00033

tive predominance of sIgA [13]. It remains uncertain whether immunoglobulins from plasma can contribute to local immunity in cervicovaginal secretions [13].

Moreover, correlations between IgA and IgG levels in vaginal fluids and between IgA and IgG antibodies to gp160 strongly indicate that high amounts of both nonspecific and specific monomeric IgA could have transudated from sera to the vaginal mucosa. Taken together, these data show that, at least in HIV-infected women, IgA-producing cells do not appear in the cervicovaginal tract as the predominant source of specific antibodies to HIV, which more likely originate from sera. However, the lack of correlation between total IgA and anti-gp160 IgA antibodies in vaginal secretions and between total IgA serum levels and anti-gp160 IgA antibodies in vaginal secretions seems to indicate that the origin of local IgA antibodies to HIV may depend on both systemic transudation and secretory local production. Finally, although increased IgM levels were found in vaginal fluids, no antibodies of this isotype to HIV were detectable, suggesting that the local immune system produces only nonspecific IgM in HIV-infected women.

Local antibodies to HIV may be functionally important in antiviral immunity. In HIV-infected individuals, local antibodies to gp160, which contains the major epitopes involved in virus neutralization, could decrease infectivity of genital secretions leading to alteration of transmission of HIV in heterosexuals. If the transudation of immunoglobulins from serum to vaginal secretions observed in HIV-infected women is a physiologic process, antibodies induced after systemic vaccination against HIV could also passively transudate to the surface of the vaginal mucosa. Their neutralizing power, if present in serum, might also be apparent in genital secretions and act to block viral attachment, entry into susceptible cells of the mucosa, or both. Furthermore, since some local immunity to HIV may have a secretory origin, with specific IgA antibodies, secretory antibodies induced by a local vaccine against HIV could be also effective for immune exclusion. Thus, design of an efficient vaccine against HIV may require both parenteral and local immunizations.

Acknowledgments

We thank S. Iscaki and J. P. Bouvet for helpful contributions, H. Hamdi for manuscript preparation, and the Pasteur Institute of Bangui for collection of biologic samples.

References

1. Al-Nozha M, Ramia S, Al-Frayh A, Arif M. Female to male: an inefficient mode of transmission of human immunodeficiency virus. J Acquir Immune Defic Syndr **1990**;3:193–4.
2. Padian NS, Shiboski SC, Jewel NP. Female-to-male transmission of human immunodeficiency virus. JAMA **1991**;226:1664–7.
3. European Study Group on Heterosexual Transmission of HIV. Comparison of female to male and male to female transmission of HIV in 563 stable couples. BMJ **1992**;304:809–13.
4. Belec L, Georges AJ, Steenman G, Martin PMV. Antibodies to human immunodeficiency virus in vaginal secretions of heterosexual women. J Infect Dis **1989**;160:385–91.
5. Belec L, Peghini M, Georges AJ, Barabe P, Martin PMV. Antibodies to HIV-2 in genital secretions. Res Virol **1989**;140:15–21.
6. O'shea S, Cordery M, Barrett WY, Richman DD, Bradbeer C, Batnavala JE. HIV excretion patterns and specific antibody responses in body fluids. J Med Virol **1990**;31:291–6.
7. Archibald DW, Witt DJ, Craven DE, Vogt MW, Hirsch MS, Essex M. Antibodies to human immunodeficiency virus in cervical secretions from women at risk for AIDS [letter]. J Infect Dis **1987**;156:240–1.
8. Bouige P, Iscaki S, Pillot J. Immune complexes as immunizing agents to increase the number of monoclonal antibody producing hybrids and to deviate the response to poorly immunogenic epitopes. Hybridoma **1990**;9:519–26.
9. Bouvet JP, Pirès R, Pillot J. A modified gel filtration technique producing an unusual exclusion volume of IgM: a simple way for preparing monoclonal IgM. J Immunol Methods **1984**;66:299–305.
10. Ey PL, Proxse SJ, Jenkin DR. Isolation of pure IgG1, IgG2a and IgG2b immunoglobulins from mouse serum using protein A–Sepharose. Immunochemistry **1978**;15:429–36.
11. Nakane PK, Kawaoi A. Peroxidase labelled antibody, a new method of conjugation. J Histochem Cytochem **1974**;22:1084–91.
12. Lane HC, Masur H, Edgar LC, Whalen G, Rook AH, Fauci AS. Abnormalities of B-cell activation and immunoregulation with the acquired immunodeficiency syndrome. N Engl J Med **1983**;309:453–8.
13. Mestecky J, Kutteh WH, Ladjeva I, Peterman JH. The common mucosal immune system in the human genital tract and mammary gland. In: Metter L, Billington WD, eds. Reproductive immunology. Amsterdam: Elsevier, **1989**;251–8.

Downloaded from http://jid.oxfordjournals.org/ at University of Iowa Libraries/Serials Acquisitions on March 18, 2015