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Intraepithelial Cell Neutralization of HIV-1 Replication by IgA[1]

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HIV is transmitted sexually through mucosal surfaces where IgA Abs are the first line of immune defense. In this study, we used paired IgA and IgG mAbs against HIV gp160 to study intraepithelial cell neutralization and inhibition of HIV replication. African green monkey kidney cells, Vero C1008, polarizable epithelial cells transfected to express the polymeric Ig receptor (pIgR), were transfected with HIV proviral DNA, and intracellular neutralization mediated by the mAbs was assessed. D47A and D19A IgA, which neutralized HIV in a conventional assay, potently inhibited intracellular HIV replication as assessed by infecting HeLa-CD4-long terminal repeat/β-galactosidase cells (human cervical carcinoma cell line) and CEMx174 cells (human T cell line) with apical supernatant, basolateral medium, and cell lysate from transfected cells. D47A also inhibited the production of virus as assessed by direct assay of p24. In contrast, D47 and D19 IgG, sharing the same V regions, but which were not transcytosed by the pIgR, did not inhibit intracellular HIV replication, nor did D47A and D19A IgA in pIgR$^-$ cells, incapable of transcytosing IgA. Confocal immunofluorescence microscopy showed prominent colocalization of HIV protein and D47A, in agreement with the intracellular neutralization data. D10A, which did not neutralize HIV in the conventional assay, and irrelevant IgA did not show intracellular neutralization or colocalization. Control studies with two kinds of conditioned medium confirmed that HIV neutralization had indeed occurred inside the cells. Thus, during its transcytosis through epithelial cells, HIV-specific IgA can neutralize HIV replication. ***The Journal of Immunology,*** **2005, 174: 4828–4835.**

Human immunodeficiency virus, either free or inside cells, predominantly infects as a result of sexual contact through the genital and rectal mucosal surfaces. Several routes of entry have been proposed, including direct transcytosis through epithelial cells, infection of target cells beneath the epithelium via breaks in the lining, transepithelial transport by Langerhans cells, dendritic cells, or M cells, and transcytosis through epithelial tight junctions (1–5). In addition, epithelial cells derived from colon, uterus, oral cavity, and salivary gland have been reported to support HIV replication (6–15). All these routes could allow spread of virus to lymphoid target cells. Nevertheless, in sexually acquired HIV infection in humans, the primary locus of transmission and the initial site of replication of virus remain uncertain.

Because mucosal transmission is such an important route of infection, a strong and effective mucosal immune response is a desirable goal for an HIV vaccine. The major mucosal Ab is IgA, which provides immune defense by blocking adherence of virus to epithelial cells, neutralizing virus within epithelial cells, and binding virus in the lamina propria or inside epithelial cells and transporting it in an immune complex to the luminal side (16–22). By these means, IgA Abs can limit the spread of virus. Moreover, IgA against HIV has been detected in a number of mucosal secretions, including parotid saliva and fluids from the genital and intestinal tracts, and IgA Abs collected from these sites have been shown to neutralize HIV in vitro (23–27). Furthermore, the presence of HIV-specific mucosal IgA correlates with HIV resistance in sex workers and in uninfected sexual partners of infected individuals (25, 26, 28–32).

With respect to HIV, IgA (and IgM) Abs placed at either the apical or basolateral surface have been reported to block transcytosis of virus from the apical to the basolateral side in a tight epithelial cell monolayer (16, 24, 33–36). These findings suggest that in vivo HIV-specific IgA can both exclude HIV from mucosal epithelial cells and prevent transcytosing HIV from spreading to the lamina propria, where abundant T cells and macrophages are potential targets of infection. During transport of IgA through mucosal epithelial cells, after polymeric Ig receptor (pIgR)[4]-mediated endocytosis, IgA Abs may also be able to interact intracellularly with viral proteins to inhibit viral replication. Evidence for such intraepithelial cell neutralization has been demonstrated in vitro with IgA mAbs against Sendai and measles viruses (paramyxoviruses), influenza virus (an orthomyxovirus), and rotavirus (a reovirus) (17–22, 37), but not yet with Abs to HIV (a retrovirus). In addition, neutralization (presumably intracellular) was shown in mice by an IgA mAb against a rotavirus internal protein, which was able both to prevent infection and cure persistent infection (17).

In the current study, pairs of IgA and IgG mAbs bearing the same V regions (Ig class switch variants) against envelope protein of HIV were used to investigate inhibition of virus replication inside epithelial cells.

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[4] Abbreviations used in this paper: pIgR, polymeric Ig receptor; p.i., postinfection; LTR, long terminal repeat; β-Gal, β-galactosidase; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; CM, conditioned medium.

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Materials and Methods

Cell culture and viruses

We evaluated a number of human and primate epithelial cell lines for use in our experiments. For various technical reasons African green monkey kidney cells, Vero C1008 (CRL 1587; American Type Culture Collection), were the most suitable. These were obtained from the American Type Culture Collection. They were transfected and stably expressed human pIgR (38). The cells were grown on tissue culture-treated, 0.4-μm pore size Transwell polyester membranes (Costar). Polarization of cell monolayers was tested by monitoring electrical resistance between the apical and basal chambers (38). HeLa (CD4-long terminal repeat (LTR)/β-galactosidase (β-Gal)) cells expressing human CD4 and the HIV LTR fused to a *LacZ* reporter gene (39) were obtained from the National Institutes of Health AIDS Research and Reagent Program and grown in DMEM containing 10% FBS, 0.2 mg/ml geneticin, and 0.1 mg/ml Hygromycin B (Invitrogen Life Technologies). Human T cell line CEMx174 was cultured in RPMI 1640 medium with antibiotics and 10% FBS. HIV subtype B (pKS242 molecular clone supplied by the National Institutes of Health AIDS Research and Reagent Program) proviral DNA (40) was used to transfect Vero C1008 cells, and virus was propagated in CEMx174 cells.

The mAbs

Hybridomas secreting anti-HIV gp160 IgG Abs were generously provided by Dr. P. Earl (Laboratory of Viral Diseases, National Institutes of Health, Bethesda, MD) (41–43): D19, D25, D47 (anti-gp120), and D10 (anti-gp-41). IgA mAbs were obtained by repetitive cycles of limiting dilution and spontaneous isotype switching of the IgG hybridomas (44). Production and purification of mAbs were performed as described (22). Four pairs, each sharing the same V regions, of IgA and IgG mAbs were used in this study.

Ab transcytosis through polarized cell monolayers

To assess the transcytosis of mAbs across a polarized epithelial cell monolayer, 12 μg of purified IgA or IgG in 120 μl (100 μg/ml) was added to the basolateral chamber below pIgR$^+$ or pIgR$^-$ Vero C1008 cells, and the cells were incubated at 37°C. Apical supernatants were collected at intervals and analyzed by ELISA for Ig content.

Traditional HIV neutralization

Neutralization of HIV subtype B (pKS242) was analyzed with an assay based on the infection of reporter CD4-LTR/β-Gal HeLa cells as described (45). Briefly, ~400 blue cell-forming units of virus were mixed with different concentrations of mAbs and incubated at room temperature for 2 h before inoculation onto the reporter cells. Cells were stained 48 h postinfection (p.i.) by addition of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Diagnostics Hybrids) to the monolayer. The blue infected cells were then enumerated.

Intracellular HIV neutralization

Unpolarized Vero C1008 cells expressing CD4 were able to be infected efficiently by SIV (38), although not by HIV, probably due to the lack of a suitable co-receptor or other factor. Therefore, transfection of polarized Vero C1008 cells with HIV proviral DNA was used in this study. About 6% of the cells were productively infected as measured by staining the tranfected cells with HIV mAbs. Polarized pIgR$^+$ and pIgR$^-$ Vero C1008 cell monolayers on polyester membranes were transfected from the apical side with 2 μg of HIV proviral DNA (pKS242) in 12 μl of TransIT-LT1 transfection reagent (Mirus). After 4 h of incubation at 37°C, each monolayer was washed three times followed by the addition of fresh medium to the apical chamber and 120 μl of mAb or medium to the basolateral chamber. After incubation at 37°C for 18 h, both the apical and basolateral chambers were washed three times and replaced with fresh medium followed by incubation at 37°C for another 12 h before specimen collection for virus assay. The apical supernatant and basolateral medium were collected. The cell monolayer was then washed three times before collection of the cells by scraping into medium. Cell lysates were prepared by freeze-thawing three times, and centrifuging for 10 min at 1900 $\times$ *g* to remove cellular debris. Virus was quantified in the apical supernatant, basolateral medium, and cell lysate by infecting both CD4-LTR/β-Gal HeLa cells and CEMx174 T cells. The CD4-LTR/β-Gal cell assay was done as described (39). Briefly, samples were incubated with CD4-LTR/β-Gal cells for 2 h, medium was added, and then at 48 h p.i., the cells were fixed and stained with X-gal, and blue cells were enumerated. Supernatants from infected CEMx174 cells were collected at 48 h p.i. and p24 Ag was assayed according to the manufacturer's instructions (Beckman Coulter). In some experiments, p24 was assayed directly in apical or basolateral fluids or lysates of transfected Vero C1008 cells.

Preparation of conditioned media

To confirm that intracellular neutralization was mediated by IgA Ab acting intracellularly during transcytosis, two kinds of conditioned medium (CM) were made. CM 1 was prepared by aspirating apical supernatants from uninfected pIgR$^+$ Vero C1008 cell monolayers that had transcytosed D47A or D19A mAb after basolateral exposure for 18 h, apical and basolateral surface washes, and incubation for another 12 h after addition of fresh medium. CM 1 was the apical medium from the final 12-h period. CM 2 was prepared by three cycles of freeze-thawing of uninfected pIgR$^+$ Vero C1008 cell monolayers that had been exposed to mAb D47A or D19A for 18 h, washed, and incubated for another 12 h with fresh medium. CM 2 was the supernatant of the cell lysates after centrifugation for 10 min at 1900 $\times$ *g*.

Intracellular colocalization of IgA Ab and HIV protein

Polarized pIgR$^+$ Vero C1008 cell monolayers were transfected and incubated with Ab as above. After 18–24 h, the mAbs were washed off and the polarized cells were fixed and permeabilized as described (22). Two-color immunofluorescence (Case Western Reserve University/Ireland Comprehensive Cancer Center confocal microscopy facility) was used to detect HIV protein and IgA simultaneously. IgA was identified with FITC-conjugated goat anti-mouse IgA (Southern Biotechnology). HIV protein was identified with D19 IgG (anti-gp120) followed by rhodamine-conjugated goat anti-mouse IgG (Pierce). A Zeiss 510 laser scanning confocal microscope with a 40$\times$ (numerical aperture, 1.2) Apochromat objective lens (Zeiss) was used. Confocal images of fluorescence were collected with a 488-nm excitation light from an argon laser, a 488-nm dichroic mirror, and a 500- to 550-nm band-pass barrier filter. Imagines of rhodamine fluorescence were collected with 543-nm excitation from a He/Ne laser, a 543-nm dichroic mirror, and a 560-nm long pass filter.

Data analysis

All parameters from each of three independent experiments (all in duplicate) were subjected to two-way ANOVA using Statview 11 software (Abacus Concepts). In all cases, experiment-wise variation was not significant; therefore, the means $\pm$ SEM of the pooled data from all three experiments are reported. All post hoc comparisons are based on Dunnett's *t* statistics.

Results

The mAbs

By limiting dilution and spontaneous isotype switching of IgG hybridomas, four pairs, i.e., sharing V regions, of IgA- and IgG-secreting hybridomas were obtained (Table I). All mAbs were >90% pure as determined by densitometry after SDS-PAGE.

Transcytosis of IgA across a polarized epithelial cell monolayer

To assess the transcytosis of mAbs across a polarized cell monolayer, 12 μg of purified IgA or IgG in 120 μl (100 μg/ml) was added to the basolateral chamber of pIgR$^+$ or pIgR$^-$ Vero C1008

Table I. *HIV mAbs*

Hybridoma	Ag Specifity	Percentage of Oligomeric IgA[a]
D10A	gp-41	70
D10G	gp-41	
D19A	gp-120	43
D19G	gp-120	
D25A	gp-120	65
D25G	gp-120	
D47A	gp-120	64
D47G	gp-120	

[a] The original IgG hybridomas were established and characterized by Earl et al. (41– 43). The hybridomas were isotype switched to IgA and their specificities confirmed by Western blotting. The percentage of the total IgA that was oligomeric was assessed by size exclusion liquid chromatography.

Table II. *HIV neutralization after different times of IgA Ab exposure*[a]

Exposure to mAb (100 μg/ml)	Apical Supernatant				Cell Lysate			
	Blue cell	% Virus reduction[b]	p24 Level (pg/ml)	% Virus reduction[b]	Blue cell	% Virus reduction[b]	p24 Level (pg/ml)	% Virus reduction[b]
6 h								
Control	130 ± 7	0	44 ± 4	0	2181 ± 62	0	533 ± 30	0
D47A	27 ± 5[c]	79	19 ± 2[c]	57	932 ± 150[c]	57	226 ± 41[c]	58
12 h								
Control	135 ± 8	0	51 ± 2	0	1968 ± 155	0	532 ± 25	0
D47A	29 ± 9[c]	79	17 ± 1[c]	67	734 ± 67[c]	63	320 ± 71[c]	43
18 h								
Control	128 ± 8	0	45 ± 3	0	1733 ± 74	0	521 ± 28	0
D47A	25 ± 4[c]	80	16 ± 2[c]	64	370 ± 58[c]	79	226 ± 9[c]	57

[a] mAbs were added for 6, 12, or 18 h to the basolateral surface of polarized monolayers of pIgR$^+$ Vero C1008 cells that had been transfected with HIV proviral DNA for 4 h. HIV levels in apical supernatants and cell lysates 30 h post-transfection were determined by infecting target HeLa cells ("blue cell" assay) and CEMx174 T cells (p24 assay). Data are means ± SEM pooled from three experiments, each with duplicate determinations.

[b] Compared with no Ab control.

[c] Mean virus titer significantly less ($p < 0.01$, $t > 2.8$) than control.

cells followed by incubation at 37°C. Apical supernatants were sampled at intervals and analyzed by ELISA for Ig content. Amounts of IgA transported across pIgR$^+$ cells to the apical chamber at 4, 8, and 12 h were 88 ± 5, 211 ± 6, and 381 ± 19 ng, respectively, for D10; 69 ± 3, 203 ± 13, and 360 ± 20 ng, respectively, for D19; 101 ± 5, 223 ± 11, and 435 ± 25 ng, respectively, for D25; and 94 ± 4, 223 ± 10, and 396 ± 21 ng, respectively, for D47. In contrast, the most IgG reaching the apical chamber at 12 h was 20 ± 3 ng. Transport of IgA was mediated by the pIgR since pIgR$^-$ cells did not transport IgA (≤22 ± 4 ng in the apical chamber at 12 h).

Traditional HIV neutralization

Neutralization of HIV subtype B (pKS242 molecular clone) was analyzed by an assay based on the infection of HeLa cells as described in *Materials and Methods*. Cells were stained 48 h p.i. by addition of X-gal. The reduction of blue cells compared with a virus control (no mAb) indicates the virus neutralized by the mAb. D19 and D47 IgA at 0.075 μg/ml neutralized HIV 70 and 85%, respectively, whereas the same concentration of D19 and D47 IgG neutralized HIV 20 and 60%, respectively. D10 and D25, both IgA and IgG, showed no significant neutralization activity even at 50 μg/ml (data not shown).

Intracellular HIV neutralization

Polarized pIgR$^+$ Vero C1008 cell monolayers on polyester membranes were transfected with HIV proviral DNA (pKS242). After 4 h, IgA mAb was added to the basolateral chamber for 6, 12, or 18 h; cells were washed and incubated for 24, 18, and 12 h, respectively, before collection of apical supernatant, basolateral medium, and cell lysate for virus assay. Virus was quantified in the apical supernatant, basolateral medium, and cell lysate by infecting HeLa-CD4-LTR/β-Gal cells and CEMx174 cells. Infected HeLa cells were stained, and blue cells were enumerated 48 h p.i. Supernatants from infected CEMx174 cells were collected 48 h p.i., and the quantity of p24 Ag released was analyzed by HIV-1 p24 Ag assay. Virus titers were reduced by 79, 79, and 80% in the apical supernatants and by 57, 63, and 79% in the cell lysates after 6, 12, and 18 h of IgA mAb (D47A) incubation as measured by infected HeLa cells (Table II). Virus titers were reduced by 57, 67, and 64% in the apical supernatants and by 58, 43, and 57% in the cell lysates as measured by p24 assay of infected CEMx174 cell

Table III. *Intracellular neutralization of HIV by transcytosing IgA*[a]

mAb (100 μg/ml)	Apical Supernatant				Cell Lysate			
	Blue cell	% Virus reduction[b]	p24 Level (pg/ml)	% Virus reduction[b]	Blue cell	% Virus reduction[b]	p24 Level (pg/ml)	% Virus reduction[b]
pIgR$^+$ cells								
Control	154 ± 5	0	45 ± 3	0	1796 ± 107	0	511 ± 26	0
D47A	15 ± 1[c]	90	27 ± 3[c]	40	354 ± 25[c]	80	224 ± 13[c]	56
D47G	142 ± 9	8	39 ± 4	13	1683 ± 84	6	482 ± 30	6
D19A	19 ± 1[c]	88	18 ± 3[c]	60	341 ± 40[c]	81	233 ± 18[c]	54
D19G	144 ± 11	6	46 ± 3	0	1835 ± 88	0	568 ± 23	0
Control	153 ± 9	0	35 ± 2	0	1765 ± 80		454 ± 39	0
D47A	22 ± 4[c]	86	11 ± 2[c]	69	368 ± 22[c]	79	183 ± 7[c]	60
D10A	137 ± 10	10	33 ± 1	6	1716 ± 104	3	463 ± 31	0
D25A	138 ± 5	10	31 ± 1	6	1746 ± 110	3	443 ± 6	2
Irrelevant IgA	146 ± 12	5	31 ± 2	11	1856 ± 98	0	453 ± 16	0
pIgR$^-$ cells								
Control	157 ± 3	0	42 ± 2	0	2085 ± 117	0	575 ± 18	0
D47A	142 ± 9	10	49 ± 5	0	1968 ± 115	6	518 ± 11	10
D19A	151 ± 16	4	42 ± 3	0	1906 ± 145	9	561 ± 44	2

[a] Experiments as in Table II except that pIgR$^-$ Vero cells were also included. Cells were exposed to mAbs basolaterally for 18 h. Data are means ± SEM pooled from three experiments, each with duplicate determinations.

[b] Compared with no Ab control.

[c] Mean virus titer significantly less ($p \leq 0.0005$, $t > 4.1$) than control.

Table IV. *IgA Ab concentration dependence of intracellular virus neutralization*[a]

mAb (μg/ml)	Apical Supernatant				Cell Lysate			
	Blue cell	% Virus reduction[b]	p24 Level (pg/ml)	% Virus reduction[b]	Blue cell	% Virus reduction[b]	p24 Level (pg/ml)	% Virus reduction[b]
Control	151 ± 6	0	37 ± 3	0	1682 ± 52	0	547 ± 11	0
D47A (300)	18 ± 3[c]	88	13 ± 1[c]	65	163 ± 16[c]	90	161 ± 5[c]	71
D47A (100)	23 ± 3[c]	85	15 ± 1[c]	59	233 ± 18[c]	86	186 ± 13[c]	66
D47A (25)	33 ± 2[c]	78	24 ± 2[c]	35	422 ± 25[c]	75	311 ± 18[c]	43
D47A (6.25)	96 ± 8[c]	36	26 ± 2[c]	30	1224 ± 74[c]	27	356 ± 11[c]	35

[a] Experiments as in Table II except that different amounts of mAbs were added to the basolateral surface (exposure for 18 h). Data are means ± SEM pooled from three experiments, each with duplicate determinations.
[b] Compared with no Ab control.
[c] Mean virus titer significantly less ($p < 0.0005$, $t > 4.1$) than control.

supernatants (Table II). These are all statistically significant reductions vs no Ab controls (all p values <0.01; all t values >2.8). The amount of virus collected from the basolateral chamber was too low to be quantified (data not shown). In most cases exposure to IgA mAb in the basolateral chamber for an initial period of 18 h (compared with 12 and 6 h) yielded better inhibition of HIV infection. Therefore, subsequent experiments were performed after 18 h of initial exposure to Ab in the basolateral chamber.

The mAbs D47A, D47G, D19A, and D19G all neutralized HIV in the conventional neutralization assay. These Abs were assessed for intraepithelial cell neutralization as described above. Virus was quantified in the apical supernatants, and cell lysates by infection of HeLa-CD4-LTR/β-Gal cells and CEMx174 cells. The virus titer in the apical supernatants was reduced 90 and 88% by D47A and D19A IgA mAbs, respectively, as measured by HeLa cell infectivity (Table III). By the same assay, the virus titers were reduced 80 and 81% in the cell lysates. The virus titer reduction as measured by infection of CEMx174 cells was 40 and 60% in the apical supernatants and 56 and 54% in the cell lysates by mAbs D47A and D19A, respectively (all p values <0.0005; all t values >4.1 vs no Ab control). The IgG mAbs D47G and D19G, which are not transcytosed by the pIgR, yielded virus titers comparable with the controls with no Ab. Native Vero cells not expressing pIgR showed no significant reductions in virus titers after exposure to the D47A or D19A IgA mAbs.

IgA mAbs D10A (anti-gp41) and D25A (anti-gp120) both lacked conventional neutralization activity against HIV pKS242 virus. They were also tested for intracellular virus neutralization as described above. Both Abs failed to produce significant virus reduction in apical supernatants as well as in cell lysates as measured by infection of HeLa cells and CEMx174 cells (Table III). An irrelevant IgA mAb (anti-measles virus hemagglutinin) also yielded no significant reduction in virus titers (Table III).

Concentration dependence of intracellular neutralization by IgA Ab

To show that intracellular virus reduction by IgA Ab is concentration dependent, different concentrations of D47A Ab were placed in the basolateral chamber for 18 h after proviral DNA transfection for 4 h. After another 12 h of incubation with fresh medium, the apical supernatants and cell lysates were analyzed. As measured by HeLa cell infection, the virus titer reductions were 88, 85, 78, and 36% in the apical supernatants and 90, 86, 75, and 27% in the cell lysates compared with the no Ab controls (all p values <0.0001; all t values >7.4) (Table IV). Similar comparisons measured by infection of CEMx174 cells were 65, 59, 35, and 30% reductions in the apical supernatants and 71, 66, 43, and 35% reductions in the cell lysates (all p values <0.0005; all t values >4.1). These results indicate that the extent of intracellular neutralization was dependent on the concentration of specific IgA Ab in the basolateral chamber and plateaued at $\sim$100 μg/ml.

Confirmation that virus neutralization by IgA Ab is mediated intracellularly

To confirm that neutralization was mediated by IgA Ab acting intracellularly during transcytosis rather than by Ab interacting with HIV outside cells or after endocytosis from the apical compartment, two kinds of CM were tested. First, we tested whether neutralization was caused by Ab that had been transported into the

Table V. *Intracellular virus neutralization mediated by intracellular IgA vs CM*[a]

mAb (100 μg/ml)	Apical Supernatant				Cell Lysate			
	Blue cell	% Virus reduction[b]	p24 Level (pg/ml)	% Virus reduction[b]	Blue cell	% Virus reduction[b]	p24 Level (pg/ml)	% Virus reduction[b]
Control	133 ± 10	0	40 ± 4	0	1677 ± 91	0	485 ± 30	0
D47A	23 ± 4[c]	83	15 ± 1[c]	63	263 ± 28[c]	84	224 ± 20[c]	54
D19A	32 ± 4[c]	76	18 ± 3[c]	55	292 ± 17[c]	83	197 ± 13[c]	59
C.M.1 - D47A[d]	44 ± 5[c]	67	20 ± 2[c]	50	1597 ± 118	5	488 ± 32	0
C.M.1 - D19A[d]	45 ± 5[c]	66	16 ± 1[c]	60	1684 ± 76	0	465 ± 38	4
C.M.2 - D47A[e]					1589 ± 88	0	475 ± 24	1
C.M.2 - D19A[e]					1743 ± 73	5	479 ± 28	2

[a] Experiments as in Table II; mAbs were added basolaterally for 18 h. In some cases CM was added to the apical chamber or cell lysate without mAb having been added to the basolateral surface. Data are means ± SEM pooled from three experiments, each with duplicate determinations.
[b] Compared with no Ab control.
[c] Mean virus titer significantly less ($p < 0.0001$, $t > 5.6$) than control.
[d] CM 1, apical supernatant containing transcytosed IgA (see text).
[e] CM 2, lysate from IgA-transcytosing cells (see text).

Table VI. *Intracellular virus neutralization as measured directly from Vero cells by p24 assay*[a]

mAb (100 μg/ml)	Apical Supernatant		Basal Medium		Cell Lysate	
	p24 Level (ng/ml)	% Virus reduction[b]	p24 Level (ng/ml)	% Virus reduction[b]	p24 Level (ng/ml)	% Virus reduction[b]
Control	7.1 ± 0.2	0	9.1 ± 0.3	0	35.6 ± 1.1	0
D47A	5.1 ± 0.5[c]	28	6.5 ± 0.4[c]	29	25.3 ± 1.2[c]	29
D10A	6.1 ± 0.7	14	8.2 ± 0.4	10	32.3 ± 3.0	9
Irrelevant IgA	6.1 ± 0.3	14	9.0 ± 0.3	1	35.9 ± 0.9	0

[a] mAbs were added for 18 h to the basolateral surface of polarized monolayers of pIgR$^+$ Vero C1008 cells that had been transfected with HIV proviral DNA for 4 h. HIV levels in apical supernatants, basolateral media, and cell lysates 30 h post-transfection were determined directly by p24 assay. Data are means ± SEM pooled from three experiments each with duplicate determinations.

[b] Compared with no Ab control.

[c] Mean virus titer significantly less ($p < 0.005$, $t > 3.0$) than control.

apical medium. CM 1, transcytosed IgA (see *Materials and Methods*), was added to the apical chamber of HIV proviral DNA-transfected pIgR$^+$ cells not exposed to basolateral Ab, and the cells were incubated for another 12 h. The apical supernatants and cell lysates were prepared for virus assay. The decrease in virus titer in the apical supernatants mediated by CM 1 was not significantly different statistically from the decrease by transcytosing mAb D47A or D19A added to the basolateral chamber as measured by HeLa cell infection (blue cells) (Table V). However, importantly, CM 1 did not reduce the infectious virus in cell lysates (only 5 vs 84% for transcytosing D47A and 0 vs 83% for transcytosing D19A). The virus titers measured by p24 assay after infection of CEMx174 cells showed analogous results. Thus, CM 1 (containing already transcytosed IgA Ab) added to the apical cell surface was able to neutralize virus released into the apical medium, but had no effect on the virus content of cell lysates. In contrast, IgA Ab added to the basolateral medium profoundly reduced virus content of cell lysates. Therefore, to mediate virus reduction intracellularly, IgA Abs must be in the normal path of basolateral to apical transcytosis.

It was also important to confirm that the neutralization was not due to free Ab released from cells during the freeze-thaw cycle binding to HIV released at the same time. When CM 2 (lysate of uninfected cells transcytosing IgA) was added to monolayers of HIV-transfected cells just before collection and preparation of lysates, it did not reduce the virus titer in the lysate (0 vs 84% for D47A during transcytosis and 5 vs 83% for D19A during trancytosis) as measured by infection of HeLa cells (Table V). CM 2 also

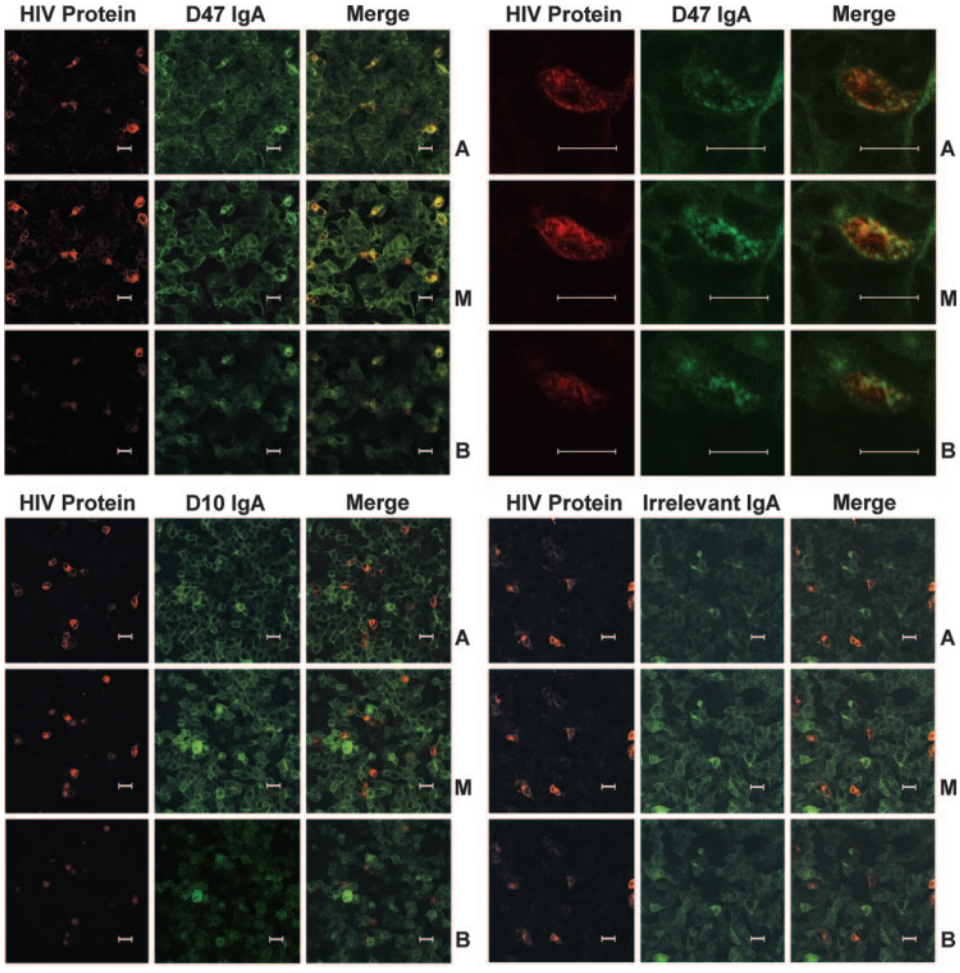

FIGURE 1. Polarized pIgR$^+$ Vero C1008 cell monolayers were transfected with HIV proviral DNA for 4 h and incubated with mAbs at the basolateral surface for 18–24 h before being fixed, permeabilized, and stained. Intracellular colocalization of IgA and HIV gp160 was observed by confocal immunofluorescence microscopy. Apical (A), middle (M), and basal (B) horizontal sections through the cell monolayer were imaged. Each section is shown as red channel (HIV gp160), green channel (IgA Ab), and merged red and green channels. Where red and green signals colocalize, the color is orange to yellow depending on relative intensities. Neutralizing D47A (anti-gp120) mAb showed colocalization at all three cell levels whereas non-neutralizing D10A (anti-gp41) mAb showed little to no colocalization, as did irrelevant IgA mAb (anti-measles hemagglutinin). The images in the *upper right quadrant* are from a replicate experiment of the *upper left quadrant* at greater magnification. (Bar = 20 μm).

produced no significant decrease in virus titer as measured by p24 assay after infection of CEMx174 cells (Table V). These results with CM 2 confirm that transcytosing IgA Ab in its natural path from the basolateral to the apical surface neutralized HIV by an intracellular action.

Intracellular IgA Ab inhibits virus replication

The above experiments showed that IgA mAb can inhibit HIV intracellularly as assessed by quantifying infectious virus, as did previous studies of intracellular neutralization with other viruses (19–22). Since IgA Ab is capable of intercepting HIV that is transcytosing epithelial cells in the apical to basolateral direction and redirecting virus to the apical surface (16), it was important to determine whether this phenomenon could account for the virus reduction by IgA that was observed in the experiments described above. Accordingly, D47A, D10A, or irrelevant IgA was placed in the basolateral chamber of polarized Vero cell monolayers as described above. The apical supernatants, basolateral media, and cell lysates were analyzed directly for viral p24. D47A significantly reduced the amount of p24 in all three compartments, by 28% in the apical supernatants and by 29% in the basolateral media and cell lysates compared with the no Ab controls (all p values <0.005; all t values >3.0) (Table VI). D10A also reduced the amount of p24 in all three compartments but not statistically significant. Irrelevant IgA produced no significant reductions in p24 levels. The fact that D47A reduced the quantity of p24 in all three compartments (apical, basolateral, and cell lysate) indicates that the Ab inhibited replication of virus.

Interestingly, by direct assay the basolateral medium contained more p24 than the apical supernatant (~9 vs 6 ng/ml). However, by infection assay of HeLa-CD4-LTR/β-Gal cells and CEMx174 cells, no infectious virus was detectable in the basolateral medium (data not shown), contrary to the apical supernatant, which contained moderate amounts of infectious virus as measured by infection of HeLa and CEMx174 cells (Tables II–V). These results suggest that the p24 detected in the basolateral medium represented p24 Ag but not whole virus particles. Another possibility would be noninfectious particles.

Intracellular colocalization of IgA Ab and viral Ag

To demonstrate that transcytosing IgA can interact with newly synthesized viral protein (gp160) inside infected epithelial cells, two-color immunofluorescence with a confocal laser scanning microscope was used (Fig. 1). Polarized $pIgR^+$ Vero C1008 cell monolayers grown on polyester membranes were transfected with 2 μg of pKS242 HIV DNA for 4 h. After washing, IgA mAbs (D47A, D10A, or irrelevant IgA) were added to the basal surface (12 μg/120 μl) for 18 h, the monolayer was washed, fixed in 4% formaldehyde in PBS, pH 7.4, and stained and observed as previously described (22).

HIV protein, visualized in red by rhodamine staining, was primarily located in the apical and middle portions of the cells. Neutralizing Ab D47A (anti-gp120), stained green with FITC, was distributed through all three horizontal sections (apical and middle more than basal) with the most intensity in the middle section. As shown in the merged images, HIV gp160 and D47A IgA were prominently colocalized in the apical and especially the middle sections (orange to yellow), suggesting the principle site, perhaps in the apical recycling endosome (16, 46), of interaction between IgA Ab and HIV protein. Both non-neutralizing D10A (anti-gp41) and irrelevant IgA (anti-measles hemagglutinin) showed no colocalization with HIV protein, suggesting little or no interaction with HIV protein even though both IgAs were transported efficiently to the apical side.

Discussion

Mucosal surfaces are the major site through which HIV is transmitted. IgA, the major mucosal Ab class, is present in various mucosal and exocrine fluids, including parotid saliva, cervicovaginal and intestinal secretions, milk, and bronchial lavage fluid, and can contain Abs to HIV (23–25, 47–49). IgA from parotid saliva, cervicovaginal fluid, and plasma of naturally infected individuals has been shown to be capable of neutralizing T cell line-adapted as well as primary HIV isolates (23, 27, 28, 50, 51), as has rectal IgA from mucosally immunized mice (52–54).

In addition to the conventional neutralization activity mentioned, human IgA Ab has also been shown to be able to act intracellularly to block HIV transcytosis from the apical to the basolateral side of epithelial cell monolayers, suggesting the potential to inhibit spread of HIV from mucosal epithelium to the lamina propria (16, 24, 33, 35, 36). The ability of IgA Ab to act in this manner has been referred to as "intracellular neutralization" (16). In these experiments, after complexing with IgA Ab intracellularly, HIV was delivered to the apical side of the cell and released as an immune complex. In this regard, we have previously described the ability of IgA Abs to bind Ags beneath epithelial cells and transport them as immune complexes to the apical surface (luminal surface in vivo), a process we termed "immune excretion" (22, 55, 56). In our view, the important phenomenon described by Bomsel et al. (16) can be regarded as a variant of immune excretion, in this case virus excretion. We have instead used intracellular neutralization to refer to the ability of transcytosing antiviral IgA Abs to inhibit the production of virus by epithelial cells (19–22), which has not heretofore been demonstrated with HIV.

In the present study, we used pairs of IgA and IgG mAbs and HIV proviral DNA transfection of polarized epithelial cells to demonstrate the capacity of HIV-specific IgA acting intracellularly to disrupt the synthesis of HIV. Varying the duration of exposure to IgA Ab in the basolateral chamber showed that longer exposure tended to increase virus neutralization (Table II). In vivo, mucosal epithelium is continuously exposed to IgA Ab produced by plasma cells in the underlining lamina propria. With different concentrations of IgA placed in the basolateral chamber, we found that virus neutralization is a function of Ab concentration (Table IV). Interestingly, neutralization plateaued at ~100 μg/ml, which is similar to the IgA content of human external secretions (57) and dog mesenteric lymph (58), suggesting that after appropriate immunization, physiological concentrations of IgA should be able to produce significant intraepithelial virus neutralization in HIV as well as other viral infections (22).

The intraepithelial cell neutralization demonstrated in this study was mediated by IgA that had been internalized by the pIgR since paired IgG Abs containing the same V regions, but not capable of binding and transcytosis by the pIgR, showed no intracellular neutralization (Table III) even though both the IgA and IgG Abs mediated significant conventional neutralization. Furthermore, the same IgA mAbs placed in the basolateral chamber below $pIgR^-$ Vero C1008 cells, unable to bind and endocytose IgA, showed no intraepithelial cell neutralization. These results confirmed that the intracellular neutralization of HIV observed was mediated by IgA Ab that had been endocytosed and transcytosed via the pIgR.

We isolated four IgA mAbs, of which two, D19 and D47, neutralized HIV conventionally as well as intracellularly. In contrast, two IgA mAbs, D10 and D25, did not neutralize HIV conventionally or intracellularly. Therefore, it was critical to demonstrate that the intracellular neutralization mediated by D19 and D47 was not caused by Ab that had already transcytosed to the apical chamber during the 12-h incubation period. Accordingly, CM 1 containing

IgA that had trancytosed uninfected cells was placed on the apical side of HIV-transfected cells that had not been exposed to Ab. CM 1 neutralized virus in the apical supernatant significantly, as did the Ab placed in the basolateral chamber, but it did not decrease the virus titer in the cell lysate (Table V). These results suggest that although Ab transcytosed to the apical side can neutralize virus in the apical supernatant, virus neutralization can also be mediated by IgA inside epithelial cells. In the current study, CM 1 reduced apical virus to a greater extent than in previous reports with other viruses (19, 20, 22). This is probably due to the smaller amount of HIV reaching the apical surface (6% of cells infected) compared with the previous reports, where most of the cells were infected. Additional experiments with CM 2, containing lysates of uninfected cells that had been transcytosing IgA (Table V), confirmed that robust HIV neutralization had indeed occurred inside the cells.

Finally, direct measurement of p24 levels in apical supernatants, basolateral media, and lysates of transfected epithelial cells exposed to D47A anti-gp120 Ab showed reduction of p24 in all three compartments (Table VI). This result further confirms that replication of HIV is being inhibited by an intracellular action of IgA Ab and that the reduced virus titers in cell lysates cannot be attributed to transcytosis (excretion) of virus-IgA immune complexes from inside the cell to the apical surface as was described (16).

The ability of IgA Ab against viral envelope protein to block replication of virus intracellularly is presumed to be due to, at least in part, the intersection of vesicles transcytosing IgA with post-Golgi vesicles in the secretory path carrying newly synthesized envelope protein, perhaps meeting at the level of the apical recycling endosome (16, 46). In contrast, the mechanism whereby the same Ab disrupts the synthesis of a viral core protein, like p24 that is made in the cytosol, is unknown.

In summary, with HIV proviral DNA transfection of polarized $pIgR^+$ epithelial cells and paired IgA and IgG mAbs, we demonstrated a new function for HIV-specific IgA, namely that it can neutralize virus replication inside epithelial cells. The current and previous works have thus demonstrated multiple ways in which IgA Abs can inhibit HIV. These studies collectively provide a rationale for including the mucosal immune system in regimens for immunizing against HIV.

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Disclosures

The authors have no financial conflict of interest.

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