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Detection of very early antibody to native HIV antigens by HIV neutralization and live-cell immunofluorescence assays

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Abstract

Very early detection of HIV infection could help decrease the spread of HIV, improve safety of the blood supply, and permit earlier treatment. Early detection was reported when native gp41 antigen was used to detect antibodies that occurred 2–6 weeks earlier than detection of antibodies to denatured antigens by the current EIA or WB tests or detection by the HIV RNA test. We hypothesized that early antibodies to native gp41/160 could be detected, not only by the reported live-cell immunofluorescence (IFA) but also by a neutralization test, since virions as well as HIV-infected cells contain native gp41/160. To test this hypothesis, we did an initial test of concept study to compare the neutralization test with other tests, using sera from 12 high-risk patients. The neutralization test reproducibly detected early antibodies (characterized) in the sera of 10 of 12 (83%) high-risk subjects. Importantly, the EIA and WB tests that use denatured antigens missed the early diagnosis in 12 of the 13 subjects (92%). The findings support the concept that native HIV antigens can detect polyclonal HIV neutralizing antibodies and live-cell IFA antibodies earlier than currently available tests that use denatured antigens.

Keywords: HIV; Early diagnosis; Antibody; Native antigens; Neutralization test

1. Introduction

To reduce transmission of human immunodeficiency virus (HIV), early detection of HIV infection is critical for early diagnosis and for insuring the safety of blood products and donated organs (Chen et al., 2002; Ling et al., 2000; Sharma et al., 2001). Enzyme-linked immunosorbent assays (EIA), and further confirmation by Western blot (WB) are currently the most common serological methods utilized for diagnosis of HIV infection (Nielsen and Bryson, 2000). However, at the very early stages of HIV infection, patients may still remain undiagnosed because of negative EIA and WB tests (Chen et al., 2002; Race et al., 1991). This delayed antibody conversion was shown in contaminated needle stick studies in health care personnel, where the average estimated time for seroconversion, using the current denatured antigens in the EIA and WB tests, was 2–3 months.

To improve early diagnosis, more sensitive and specific tests that use polymerase chain reaction (PCR) amplification of plasma HIV RNA (Le Corfec et al., 1999; Pilcher et al., 2005), or detection of both HIV p24 antigen and antibodies against HIV antigens, have been reported (Ascher et al., 1992; Busch and Hecht, 2005; Ledergerber et al., 2000; Shima et al., 2001). However, in several comparative studies, they detected infection only 1–2 weeks earlier than EIA and WB (Fiebig et al., 2003; Guay et al., 2000; Saville et al., 2001), perhaps because HIV viremia occurs subsequent to the initial infection at the portals of HIV entry. Consequently, the need for alternative screening methods was identified (Pilcher et al., 2004). As an alternative test, very early detection of HIV antibody was reported when native gp41/160, rather than the current denatured HIV

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antigens, was used. This early antibody to the conformational epitope of gp41 occurs 2–6 weeks earlier than antibodies reacting to denatured antigens that are detected by the current EIA or WB tests or by the HIV RNA test (Chen et al., 2002; Race et al., 1991). Thus, these studies have shown that the first antibodies induced following HIV infection are usually antibodies directed at conformational epitopes of gp41, including antigens in virion envelopes, and these antibodies are not detected with denatured antigens.

We hypothesized that early antibodies to native gp41 would be detected not only by the reported live-cell immunofluorescence (IFA) but also by a neutralization test, since both HIV-infected cells and virions contain native gp41/160. Native gp41 is known to be a good target for neutralization. Importantly, detection of early neutralizing antibody would help confirm the previous reports that early HIV antibody can be detected by tests that use native antigens. To test this hypothesis, we did an initial test of concept study to compare the neutralization test with the live-cell IFA, EIA and WB tests using sera from 12 high-risk patients who were previously shown to develop antibodies to native gp41/160 (see Section 2 below).

We found that the neutralization test detected very early antibody in the sera of 11 of the 12 high-risk subjects. The findings support the concept that native HIV antigens can detect HIV antibody 2–6 weeks earlier than current tests that use denatured antigens.

2. Methods

We conducted our initial study to compare the neutralization test with other tests using selected sera from 12 high-risk patients, 11 of whom were previously shown to develop antibodies to native gp41 (Chen et al., 2002; Race et al., 1991). The IFA quantification and a photo of the fluorescence have been reported (Cloyd and Bigner, 1977). The seronegative high-risk subjects had been bled every 1–2 months and their sera were stored frozen. Subject 11 had been bled more frequently. In our retrospective study, the stored samples were tested for antibodies using the live-cell IFA, neutralization, EIA, and WB tests. The first serum samples that were antibody-positive are reported, as well as any adjacent samples that are relevant (subjects 3, 10, and 11). As reported from several laboratories (Chen et al., 2002; Lopalco et al., 2000; Race et al., 1991; Tenenbaum et al., 2005), some of the high-risk patients later developed antibodies to denatured antigens and others stopped producing antibody to native antigen.

Serum samples were obtained under NIH and IRB guidelines. Serum was stored at −20 °C. HIV strains 213, RF, JRCSF, and HIVC were propagated in the human T-cell lines H9 or VB and stored at −70 °C until used. To determine the neutralizing antibody titer of the serum or plasma, 25 μl of threefold serial dilutions (starting at 1:2 or 1:4) were mixed with 25 μl of virus containing 75 Fluorescent Forming Unit (FFU) of infectious HIV, and incubated for 1 h at 37 °C to allow antibody to inactivate (neutralize) infectivity of HIV. Twenty-five microliters of the mixture were then added to two replicates of Ghost CCR-5 cells obtained from the NIH-NCI reagents division. The Ghost CCR-5 cells produce green fluorescent protein after infection. The cultures were incubated for 2 days and counted under a fluorescent microscope for the number of infected fluorescent cells (typically 75 FFC). The 50% HIV neutralizing endpoint is calculated as the reciprocal of the highest dilution of sample that resulted in a 50% reduction of the number of fluorescent Ghost CCR-5 cells in comparison with a negative serum control. Each assay was confirmed two to three times and the titers were determined to be statistically highly valid.

To remove antibody from samples, sequential absorptions with protein G and anti-IgG agarose were performed. Agarose beads first were washed and equilibrated with phosphate buffered saline. A 100 μl sample was then mixed and incubated with 25 μl of protein G beads (Sigma) for 4 h, and then centrifuged to remove IgG. The procedure was repeated twice on each sample. The supernatant from the final step was assayed for residual neutralizing activity and also assayed for neutralizing antibody.

3. Results

3.1. The neutralization assay

The neutralization assay detects antibody that inactivates the infectivity of HIV (Dianzani et al., 2002). This assay is a well-established method in which serial dilutions of serum are mixed with infectious HIV and then assayed for residual HIV-infectivity. Fig. 1 illustrates the neutralization assay for two specimens—a low titer serum early after infection and a high titer serum late after progression to chronic infection. The lowest dilutions of both sera inactivate the ability of HIV to infect the cells. As shown, at higher dilutions, the neutralizing activity of the sera diminishes sharply and becomes undetectable. The neutralizing titer is calculated as the reciprocal of the dilution of serum which inactivates 50% of HIV infectivity. In Fig. 1, the neutralizing antibody titer for an early neutralizing serum example is 8 and the titer for a late neutralizing serum example is 2000.

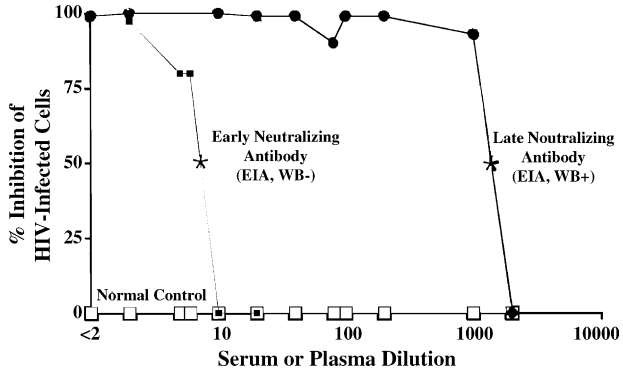

Fig. 1. Examples of two neutralization assays for antibodies against HIV. The neutralizing titer is calculated as the reciprocal of the dilution of serum which inactivates 50% of HIV infectivity as assayed on Ghost CCR5 cells. (* = 50% endpoints) The titer of the early neutralizing antibody sample is 8 units/ml, and the titer of the late neutralizing antibody sample is 2000 units/ml.

Table 1
Comparative times of detection of anti-HIV antibodies by EIA, WB, neutralization, and live-cell IFA assays

Subject	Serum number	Day of sequential serum	Antibody detection tests			
			EIA	WB	Neutralization[a]	Live-cell IFA[a]
Earliest detection by neutralization and/or IFA tests						
1	114-7	0	−	−	20	10
2	HR 179	0	−	−	≥12	70
3	HR 22	0	−	−	≥12	40
	HR 53	70	+	+	ND	640
4	R312	0	−	−	≥12	80
5	R299	0	−	−	8	100
6	152-3	0	−	−	20	10
7	HR43	0	−	−	12	−
8	HR137	0	−	−	8	−
9	HR95	0	−	−	6	±
10	RAW-1	0	−	−	5	ND
	RAW-2	34	+	+	10	ND
11	PRB 935-1	0	−	−	<4	10
	PRB 935-2	10	−	−	<4	5
	PRB 935-3	16	−	−	<4	10
	PRB 935-4	21	−	−	<4	20
	PRB 935-5	24	−	−	<4	40
	PRB 935-6	28	−	−	<4	60
	PRB 935-7	43	+	+	<4	80
Earliest detection simultaneously by EIA, WB, and IFA tests						
12	PRB 925-1	0	+	+	<4	30
Normal controls	(12)[b]	−	−	−	−	−

ND = not done; + = positive; ± = equivocal; − = negative.
[a] Neutralization or IFA titers are presented as the reciprocal of the highest dilution of sample giving 50% reduction of HIV infectivity or IFA in comparison with a negative serum control. EIA and WB were assayed with the commercial tests as reported (Chen et al., 2002; Race et al., 1991).
[b] Difference in positive neutralization findings between subjects and normal controls is significant at $p < 0.001$ by the Fisher exact probability test.

3.2. Detection of early antibody in the sera of 12 high-risk subjects

We compared the neutralization assay to the live-cell IFA, EIA, and Western blot assays using the sera from 12 high-risk subjects. The results are shown in Table 1 and summarized in Fig. 2. Among the total of 12 sera, the number of sera that contained neutralizing antibody prior to detection by the EIA and WB tests was 10 (83%). In an additional serum (subject 11) early antibodies were detected first by the live-cell IFA test. In subject 12, earliest detection occurred simultaneously with tests using native or denatured antigens. The total number of sera that detected early antibody by the neutralization test or the live-cell IFA test (native antigens) prior to detection by the EIA and WB was 11 (92%). The intervals between detection by tests using native antigens and the tests using denatured antigens in the three subjects where serial bleedings were available (subjects 3, 10, and 11), were 70, 34, and 43 days, respectively. These findings support the hypothesis that neutralization test, like the live-cell IFA test, can detect HIV antibody 2–6 weeks earlier than the current tests that use denatured antigens. As noted in the discussion, larger studies are planned.

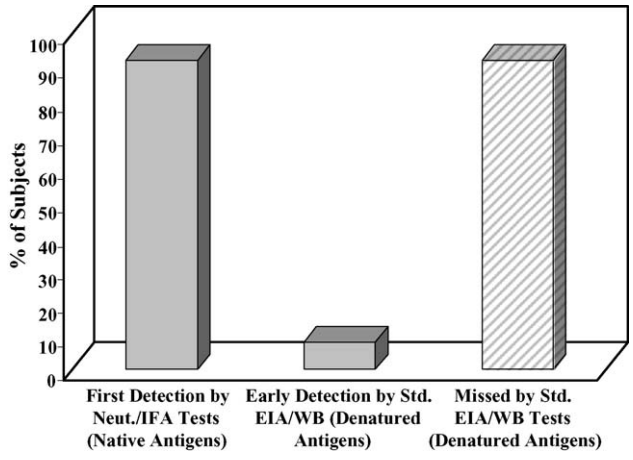

Fig. 2. Summary of comparative time of detection of anti-HIV antibodies by EIA, WB, neutralization, and live-cell IFA assays. The differences between the neutralization test using native antigen and the EIA/WB tests using denatured antigen are significant at $p = 0.001$ by the Fisher exact probability test.

3.3. Characterization of the neutralizing activity in sera: occurrence in the antibody-immunoglobulin G (IgG) component of sera

To determine whether HIV-neutralizing activity of sera occurred in the IgG fraction of serum, the sera were serially absorbed using protein G and anti-human IgG agarose beads. HIV neutralizing activity for HIV was reduced by an average of 60% in the samples when IgG was depleted (Table 2).

As additional evidence for the effectiveness of absorption of IgG, the reduction of another antibody, poliovirus-neutralizing

Table 2
HIV inhibition in patient sera is reduced by sequential absorptions with protein G and anti-human IgG agarose beads

Patient/diagnosis	Anti-HIV titer			Anti-poliovirus antibody percentage reduction[b]
	Pre-absorption	Post-absorption[a]	Percent reduction	
ALT-1/chronic	8	<3	92	70
RAW-1/high-risk	7	<3	87	90
HR 124/high-risk	7	<3	81	50
ALT-3/chronic	5	<3	68	70
DDD/chronic	8	2	68	90
ALT-2/chronic	5	<3	64	70
RAW-3/high-risk	140	51	64	90
HR 22/high-risk	<3	<3	6	0
Average reduction			60	60

[a] The samples were serially absorbed with protein G and anti-human IgG agarose to remove antibody.
[b] The percentage reduction of poliovirus antibody in the same sample.

activity, in the sera was determined using a poliovirus plaque-reduction assay. Poliovirus IgG antibodies in these samples also were reduced an average of 60% by the absorptions (Table 2), indicating the effectiveness of the absorption of IgG in reducing another antibody. Taken together, these findings indicate that the HIV neutralizing activity in the high-risk sera occurs mainly in the IgG-antibody fraction as reported for live-cell IFA antibodies (Chen et al., 2002; Race et al., 1991).

3.4. Antigenic specificity

Among the viral-inhibitory substances in sera, antibodies are distinguished by their high antigenic specificity. As a control for antigenic specificity of the HIV-neutralization activity of the sera, and to rule out non-specific inhibitors (Singh et al., 1992), the sera were tested for neutralization of a virus that does not occur in our geographic area, and for which specific antibodies would not be expected. For this test, we used the antigenically HIV-unrelated Banzi virus (a flavivirus). The samples' inhibitory titers against Banzi virus were determined with the standard virus plaque neutralizing assay in Vero cells (Green monkey kidney cells) (Singh et al., 1989). The titers of the 12 high-risk sera were <2 neutralizing antibody units/ml, indicating that the neutralizing activity in the sera of our subjects is specific for HIV.

3.5. Avidity for virus

Another specific characteristic of virus-neutralizing antibodies is their high avidity for virus. Due to the high avidity, simple dilution of the virus–antibody mixture does not reverse the inactivation of virus as occurs with non-antibody inhibitors in serum (Singh et al., 1992). To test this property of non-reversibility, we diluted neutralized virus-serum mixtures 10- and 100-fold and assayed for reactivation of virus infectivity. As shown in Fig. 3, dilution did not reverse neutralization by reactivating virus infectivity, thereby indicating that the specimens inactivated HIV irreversibly as do true antibodies, but not non-specific inhibitors in serum (Singh et al., 1992). Thus, the neutralizing activity in the sera occurs in the IgG fraction and has the biological specifications of antibody.

3.6. Sensitivity of the neutralization test is not increased by use of other strains of HIV

It is possible that the sensitivity of the neutralization test might be increased if HIV strains, in addition to strain 213 were used in the assay. To test this possibility, additional strains of HIV were used. The results showed that the neutralization titers of positive and negative sera were not increased when other strains of HIV (RF, JRCSF, and HIVC) were compared to strain 213 (data not shown).

3.7. Comparative titers of neutralizing antibodies in early high-risk (EIA−, WB−) and chronically infected (EIA+, WB+) subjects

The neutralizing early antibody titers in these high-risk subjects were compared to late antibody titers in chronically infected subjects that we studied previously (Dianzani et al., 2002). To minimize variation between assays, all neutralization tests included a standard serum to control for inter-assay variability. As shown in Table 3, the titers of HIV early neutralizing antibodies ranged from <4 to ≥20 U/ml (average 10 U/ml) and the titers

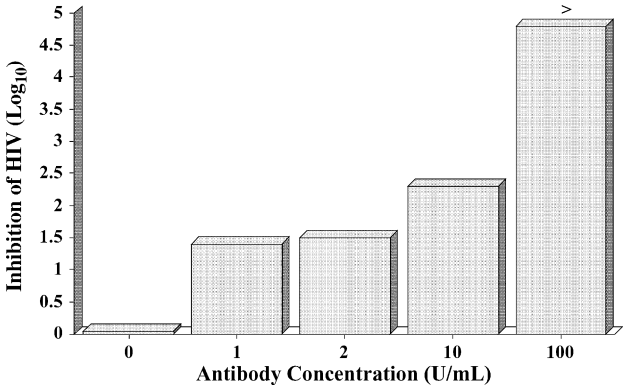

Fig. 3. Irreversible inactivation of HIV by human antisera. Representative undiluted sera were mixed with an equal volume of HIV containing 10^5 infective units, incubated for 1 h at 37 °C, and then 10-fold dilutions were used to determine residual infectivity on Ghost CCR5 cells. The figure shows the residual $\log_{10}$ inhibition of HIV by different concentrations of neutralizing antibody after serial dilution of the mixture of serum and virus.

Table 3
Comparative neutralizing antibody titers in high-risk (EIA−, WB−) and chronically infected (EIA+, WB+) subjects

EIA−, WB− high-risk subjects		EIA+, WB+ chronically infected subjects	
Serum number (subject)	Neutralization titer (units/ml)[a,b]	Subject	Neutralization titer (units/ml)[a,b]
114-7 (1)	20	A	>1024
152-3 (6)	20	B	1024
HR179 (2)	≥12	C	600
HR 22 (3)	≥12	D	>128
R312 (4)	≥12	E	96
HR43 (7)	12	F	96
R299 (5)	8	G	60
HR137 (8)	8	H	>20
HR95 (9)	6	I	>20
RAW-1 (10)	5	J	16
PRB925 (12)	<4	K	12
PRB935 (11)	<4	L	10
		M	10
		N	6
		O	6
		P	6
Average	10		50
Range	<4–≥20		6–>1024

[a] HIV challenge dose, 75 infectious units.
[b] The titer is the reciprocal of highest dilution which inactivated 50% of the challenge dose of HIV.

in chronically infected subjects ranged from 6 to >1024 U/ml (average 50 U/ml). (A few sera with titers of >4 or >1024 could not be re-titered due to exhaustion of the sample. These sera were assigned titers of 50 or 2049, respectively, for statistical averaging.) Overall, the range and average early neutralizing antibody titers in high-risk subjects tended to be lower than those of late neutralizing antibody titers in chronically infected subjects but the difference did not reach significance.

4. Discussion

4.1. Detection of early neutralizing antibody in the sera of 12 high-risk subjects

An urgent problem is the lack of early diagnostic tests to prevent newly infected persons from transmitting HIV by sexual contact or by blood or organ donation during the early stages of infection when they are EIA and WB negative (Cohen et al., 1989; Ensoli et al., 1991), and when infectious HIV titers are highest (Wawer et al., 2005). As an example, for blood safety, the NAT RNA test of blood donors is now used widely in addition to the EIA and WB tests to reduce the political and legal repercussions of even the infrequent transfusion–transmissions (Busch and Hecht, 2005). Also, such early diagnosis might permit very early treatment when the infection could be aborted. Previously, very early detection of HIV infection was reported when native gp41 rather than the current denatured HIV antigens were used to detect antibody. This early antibody to the conformational epitope of gp41/160 occurs 2–6 weeks earlier than antibodies reacting to denatured antigens that are detected by the current EIA or WB tests or even by the HIV RNA test (Chen et al., 2002). We hypothesized that some of the early antibodies to native gp41/160 would be detected not only by live-cell immunofluorescence (IFA) but also by a neutralization test, since both HIV-infected cells and virions contain native gp41/160 (Sahu et al., 2005). Native gp41/160 is known to be a good target for neutralization. We compared a neutralization assay with live-cell IFA, EIA, and WB assays. The findings show that most patients produced detectable neutralizing antibodies before seroconversion by EIA and WB antibody assays that use denatured HIV antigens. Specifically, the use of native antigens in the neutralization and/or IFA assays detected antibodies to native antigens in the sera of 11 of 12 subjects at early times, whereas denatured antigens that are used in the current EIA and/or WB assays did not detect these antibodies (Table 1 or Fig 2). In comparison, using denatured antigens (EIA and WB), initial detection of antibodies occurred in only one subject, and simultaneously with the tests using native antigen. Importantly, early diagnosis was missed by EIA and WB in 92% of the subjects. To maximally detect early antibodies, simultaneous use of more than one type of test may be necessary due to the known polyclonal nature and timing of the different types of antibody responses to HIV and other viruses, e.g., poxviruses (Baxby, 1996; Henderson and Moss, 1999) and to the different sensitivity of the different tests.

Thus, in this initial test of concept study of 12 high-risk sera, the ability of native antigens to detect very early antibodies is supported. To maximally detect early antibodies and reduce false negatives, simultaneous use of more than one type of test may be necessary based on the known polyclonal nature of the antibody response to HIV and other viruses. Further study of the potential advantage of the neutralization and other tests that use native antigens for detection of early antibody is planned using seroconversion panels and larger numbers of subjects to confirm the potential clinical value.

4.2. Characterization of the neutralizing activity in sera

Neutralizing titers below 1:10 must be characterized as true antibody since non-specific inhibitors in serum may occur (Singh et al., 1999). The neutralization activity of the samples was characterized as antibody in that (a) the anti-HIV activity was in an antibody fraction of serum since the activity was reduced by absorption of IgG antibody; (b) the anti-HIV activity had the specificity of antibody in not cross-neutralizing the antigenically unrelated Banzi virus; (c) the anti-HIV activity had high avidity since the activity was not reversed by dilution of the serum–virus mixture; and (d) normal subjects' sera were uniformly negative in the neutralization assay using a starting dilution of 1:2.

4.3. Time of production of antibodies in relation to viremia

The finding that early antibodies are produced before viremia is consistent with the immunopathogenesis of HIV infection. HIV, like many viruses, provides the first antigenic stimulation by multiplying at the portal of entry before later spreading to

the bloodstream (Baron et al., 1996; Boldogh et al., 1996). The portals of entry for HIV are subcutaneous for needle-stick infection and submucosal for sexual transmission. HIV multiplication can be detected at these portals by infectivity, by viral antigen and RNA assays (Acheampong et al., 2005; Ambrose et al., 2001; Blauvelt, 1997; Miller et al., 2005; Veazey and Lackner, 2005). This local multiplication is sufficient to provide the initial stimulation for production of early local or serum antibodies that generally occur about 7 days after many viral infections (Dianzani and Baron, 1996). Subsequent to the local multiplication and antigenic stimulation, HIV spreads to the bloodstream where it is detected by infectivity or RNA tests (Busch et al., 1991), even in the presence of partially neutralizing antibody (Dianzani et al., 2002) (also, see Section 4.4 below). Thus, after HIV infection, early antibody may be produced during the time interval between its initial multiplication at the portal of entry and the later viremia.

4.4. Role of neutralizing antibodies in host defense

An important question is whether early neutralizing antibody participates in the defense against HIV infection (Haase, 2001). Early neutralizing antibody functions inhibit HIV infectivity and correlates with the partial control of viremia during the early acute stage of infection (Dianzani et al., 2002). Also, this early control of virus may, in part, account for reported transient infections (Sahu et al., 2001; Tenenbaum et al., 2005) in some subjects.

Although neutralizing antibody may reduce infectious HIV in serum up to 100-fold (Dianzani et al., 2002), the neutralization is not complete and infectious circulating immune complexes containing HIV RNA persist due to viral evasive mechanisms. This persistence of circulating HIV in the presence of partially neutralizing antibodies probably explains the detection of infectious HIV and HIV RNA in serum (Busch and Hecht, 2005) after production of early and late antibodies. Another mechanism for HIV persistence in the presence of antibodies is that HIV mutates periodically to further escape neutralization until new antibody is produced (Dianzani et al., 2002). Thus, despite the production of neutralizing antibodies as well as cell-mediated immunity, infection progresses in most patients and the levels of late neutralizing antibodies do not correlate with progression of disease (Cloyd, 1996; Margalith et al., 2001).

4.5. Study limitations

We believe that our study has some limitations that will be addressed in future studies. The limited number of samples that were available to carry out this test of concept study should be increased. Also, the limited number of serial samples on the same subject should be increased. We plan to use serial conversion panels in future confirmation studies. Use of masked samples would also be desirable and is planned. Also important would be the development of commercially applicable binding assays (e.g., ELISA) using purified recombinant antigens produced by recombinant technology. This is under active development.

5. Conclusions

We hypothesized that early antibodies to native gp41 would be detected not only by the live-cell immunofluorescence test, but also by a neutralization test, since both HIV-infected cells and HIV virions contain the more sensitive native gp41/160. We found that the neutralization test detected very early antibody in the sera of 11 of the 12 high-risk subjects. Importantly, the results demonstrate that early diagnosis was missed in 92% of the subjects by EIA and WB tests that use denatured antigens. The findings support the concept that native HIV antigens can be used to detect HIV antibody 2–6 weeks earlier than current tests that use denatured antigens.

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References

Acheampong, E.A., Parveen, Z., Muthoga, L.W., Wasmuth-Peroud, V., Kalayeh, M., Bashir, A., Diecidue, R., Mukhtar, M., Pomerantz, R.J., 2005. Molecular interactions of human immunodeficiency virus type 1 with primary human oral keratinocytes. J. Virol. 79, 8440–8453.

Ambrose, Z., Larsen, K., Thompson, J., Stevens, Y., Finn, E., Hu, S.L., Bosch, M.L., 2001. Evidence for early local viral replication and local production of antiviral immunity upon mucosal simian-human immunodeficiency virus SHIV (89.l6) infection in *Macaca nemestrina*. J. Virol. 75, 8589–8596.

Ascher, D.P., Roberts, C., Fowler, A., 1992. Acidification modified p24 antigen capture assay in HIV seropositives. J. Acquir. Immune Defic. Syndr. Hum. Retrovirol. 5, 1080–1083.

Baron, S., Fons, M., Albrecht, T., 1996. Viral pathogenesis. In: Baron, S. (Ed.), Medical Microbiology. The University of Texas Medical Branch, Galveston, TX, pp. 575–584.

Baxby, D., 1996. Poxviruses. In: Baron, S. (Ed.), Medical Microbiology. University of Texas Medical Branch at Galveston, Galveston, TX, pp. 839–848.

Blauvelt, A., 1997. The role of skin dendritic cells in the initiation of human immunodeficiency virus infection. Am. J. Med. 102, 16–20.

Boldogh, I., Albrecht, T., Porter, D.D., 1996. Persistent viral infections. In: Baron, S. (Ed.), Medical Microbiology. The University of Texas Medical Branch, Galveston, TX, pp. 585–596.

Busch, M.P., Eble, B.E., Khayam-Bashi, H., Heilbron, D., Murphy, E.L., Kwok, S., Sninsky, J., Perkins, H.A., Vyas, G.N., 1991. Evaluation of screened blood donations for human immunodeficiency virus type 1 infection by culture and DNA amplification of pooled cells. N. Engl. J. Med. 325, 1–5.

Busch, M.P., Hecht, F.M., 2005. Nucleic acid amplification testing for diagnosis of acute HIV infection: has the time come? AIDS 19, 1317–1319.

Chen, J., Wang, L., Chen, J.J.-Y., Sahu, G.K., Tyring, S., Ramsey, K., Indrikovs, A.J., Petersen, J.R., Paar, D., Cloyd, M.W., 2002. Detection of antibodies to Human Immunodeficiency Virus (HIV) that recognize conformational epitopes of Glycoproteins 160 and 41 often allows for early diagnosis of HIV infection. J. Infect. Dis. 186, 321–331.

Cloyd, M.W., 1996. Human retroviruses. In: Baron, S. (Ed.), Medical Microbiology. The University of Texas Medical Branch, Galveston, TX, pp. 761–775.

Cloyd, M.W., Bigner, D.D., 1977. Contained indirect viable-cell membrane immunofluorescence microassay for surface antigen analysis of cells infected with hazardous viruses. J. Clin. Microbiol. 5, 86–90.

Cohen, N.D., Munoz, A., Reitz, B.A., et al., 1989. Transmission of retroviruses by transfusion of screened blood in patients undergoing cardiac surgery. N. Engl. J. Med. 320, 1172–1176.

Dianzani, F., Antonelli, G., Riva, E., Turriziani, O., Antonelli, L., Tyring, S., Carrasco, D.A., Lee, H., Nguyen, D., Pan, J., Poast, J., Cloyd, M., Baron, S., 2002. Is human immunodeficiency virus RNA load composed of neutralized immune complexes? J. Infect. Dis. 185, 1051–1054.

Dianzani, F., Baron, S., 1996. Nonspecific defenses. In: Baron, S. (Ed.), Medical Microbiology. The University of Texas Medical Branch at Galveston, Galveston, pp. 631–640.

Ensoli, F., Fiorelli, V., Mezzaroma, I., D'Agati, V., D'Offizi, G., Rainaldi, L., Luzi, G., 1991. Plasma viraemia in seronegative HIV-1-infected individuals. AIDS 5, 1195–1199.

Fiebig, E.W., Wright, D.J., Rawal, B.D., Garrett, P.E., Schumacher, R.T., Peddada, L., Heldebrant, C., Smith, R., Conrad, A., Kleinman, S.H., Busch, M.P., 2003. Dynamics of HIV viremia and antibody seroconversion in plasma donors: implications for diagnosis and staging of primary HIV infection. AIDS 17, 1871–1879.

Guay, L.A., Hom, D.L., Kabengera, S.R., Piwowar-Manning, E.M., Katasha, P., Ndugwa, C., Marum, L.H., Kalyesubula, I., Jackson, J.B., 2000. HIV-1 ICD p24 antigen detection in Ugandan infants: use in early diagnosis of infection and as a marker of disease progression. J. Med. Virol. 62, 426–434.

Haase, A.T., 2001. The pathogenesis of sexual mucosal transmission and early stages of infection: obstacles and a narrow window of opportunity for prevention. AIDS 15, S10–S11.

Henderson, D.A., Moss, B., 1999. Smallpox and vaccinia. Vaccines, 74–97.

Le Corfec, E., Le Pont, F., Tuckwell, H.C., Rouzioux, C., Costagliola, D., 1999. Direct HIV testing in blood donations: variation of the yield with detection threshold and pool size. Transfusion 39, 1141–1144.

Ledergerber, B., Flepp, M., Boni, J., Tomasik, Z., Cone, R.W., Luthy, R., Shupbach, J., 2000. Human immunodeficiency virus type 1 p24 concentration measured by boosted ELISA of heat-denatured plasma correlates with decline in CD4 cells, progression to AIDS, and survival: comparison with viral RNA measurement. J. Infect. Dis. 181, 1280–1288.

Ling, A.E., Robbins, K.E., Brown, T.M., Dunmire, V., Thoe, S.Y., Wong, S.Y., Leo, Y.S., Teo, D., Gallarda, J., Phelps, B., Chamberland, M.F., Busch, M.P., Folks, T.M., Kalish, M.L., 2000. Failure of routine HIV-1 tests in a case involving transmission with preseroconversion blood components during the infectious window period. J. Am. Med. Assoc. 284, 238–240.

Lopalco, L., Pastori, C., Cosma, A., Burastero, S.E., Capiluppi, B., Boeri, E., Beretta, A., Lazzarin, A., Siccardi, A.G., 2000. Anti-cell antibodies in exposed seronegative individuals with HIV type 1-neutralizing activity. AIDS Res. Hum. Retroviruses 16, 109–115.

Margalith, M., Levy, E., Rinaldo, C.R., Detels, R., Phair, J., Kaslow, R., Saah, A.J., Sarov, B., 2001. HIV-1 IgA specific serum antibodies and disease progression during HIV-1 infection. J. Hum. Virol. 4, 269–277.

Miller, C.J., Li, Q., Abel, K., Kim, E.Y., Ma, Z.M., Wietgrefe, S., La Franco-Scheuch, L., Compton, L., Duan, L., Shore, M.D., Zupancic, M., Busch, M.P., Carlis, J., Wolinksy, S., Haase, A.T., 2005. Propagation and dissemination of infection after vaginal transmission of simian immunodeficiency virus. J. Virol. 79, 9217–9227.

Nielsen, K., Bryson, V.J., 2000. Diagnosis of HIV infection in children. Pediatr. Clin. North Am. 47, 39–63.

Pilcher, C.D., Fiscus, S.A., Nguyen, T.Q., Foust, E., Wolf, L., Williams, D., Ashby, R., O'Dowd, J.O., McPherson, J.T., Stalzer, B., Hightow, L., Miller, W.C., Eron Jr., J.J., Cohen, M.S., Leone, P.A., 2005. Detection of acute infections during HIV testing in North Carolina. N. Engl. J. Med. 352, 1873–1883.

Pilcher, C.D., Price, M.A., Hoffman, I.F., Galvin, S., Martinson, F.E., Kazembe, P.N., Eron Jr., J.J., Miller, W.C., Fiscus, S.A., Cohen, M.S., 2004. Frequent detection of acute primary HIV infection in men in Malawi. AIDS 18, 517–524.

Race, E.M., Ramsey, K.M., Lucia, H.L., Cloyd, M.W., 1991. Human immunodeficiency virus infection elicits early antibody not detected by standard tests: implications for diagnostics and viral immunology. Virology 184, 716–722.

Sahu, G.K., Chen, J.J., Huang, J.C., Ramsey, K.M., Cloyd, M.W., 2001. Transient or occult HIV-1 infection in high-risk adults. AIDS 15, 1175–1177.

Sahu, G.K., McNearney, T., Evans, A., Weaver, S., Huang, J.C., Baron, S., Cloyd, M.W., 2005. Transient or occult HIV infections may occur more frequently than progressive infections: changing the paradigm about HIV persistence. Arch. Virol. Suppl. 19, 131–145.

Saville, R.D., Constantine, N.T., Cleghorn, F.R., Jack, N., Bartholomew, C., Edwards, J., Gomez, P., Blattner, W.A., 2001. Fourth-generation enzyme-linked immunosorbent assay for the simultaneous detection of human immunodeficiency virus antigen and antibody. J. Clin. Microbiol. 39, 2518–2524.

Sharma, U.K., Schreiber, G.B., Glynn, S.A., Nass, C.C., Higgins, M.J., Tu, Y., Bethel, J., Williams, A.E., 2001. Knowledge of HIV/AIDS transmission and screening in United States Blood Donors. Transfusion 41, 1341–1350.

Shima, T., Kondo, M., Saito, T., Kawata, K., Ito, A., Sakamoto, M., Sagara, H., Imai, M., 2001. Evaluation of an HIV screening assay kit for the combined detection of anti-HIV-1/2 antibodies and HIV p24 antigen. Kansenshogaku Zasshi 75, 1014–1024.

Singh, I.P., Chopra, A.K., Coppenhaver, D.H., Ananatharamaiah, G.M., Baron, S., 1999. Lipoproteins account for part of the broad nonspecific antiviral activity of human serum. Antiviral Res. 42, 211–218.

Singh, I.P., Coppenhaver, D.H., Chopra, A.K., Baron, S., 1992. Further characterization of a broad-spectrum antiviral substance in human serum. Viral. Immunol. 5, 293–303.

Singh, I.P., Coppenhaver, D.H., Sarzotti, M., Sriyuktasuth, P., Poast, J., Levy, H.B., Baron, S., 1989. Postinfection therapy of arbovirus infections in mice. Antimicrob. Agents Chemother. 33, 2126–2131.

Tenenbaum, S.A., Morris, C.A., Alexander, S.S., McFerrin, H.E., Garry, R.F., Leissinger, C.A., 2005. Evidence of HIV exposure and transient seroreactivity in archived HIV-negative severe hemophiliac sera. Virol. J. 2, 65.

Veazey, R.S., Lackner, A.A., 2005. HIV swiftly guts the immune system. Nat. Med. 11, 469–470.

Wawer, M.J., Gray, R.H., Sewankambo, N.K., Serwadda, D., Li, X., Laeyendecker, O., Kiwanuka, N., Kigozi, G., Kiddugavu, M., Lutalo, T., Nalugoda, F., Wabwire-Mangen, F., Meehan, M.P., Quinn, T.C., 2005. Rates of HIV-1 transmission per coital act, by stage of HIV-1 infection, in Rakai, Uganda. J. Infect. Dis. 191, 1403–1409.