Relevance of Anti-*nef* Antibody Detection as an Early Serologic Marker of Human Immunodeficiency Virus Infection

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The relevance, previously suggested by some authors, of anti-*nef* antibody detection as an early marker of human immunodeficiency virus (HIV) infection was examined with a sensitive liquid phase radioimmunoassay by investigating: (1) the kinetics of appearance of anti-*nef* antibodies in a set of 77 longitudinal sera collected from 12 HIV-infected donors at the time of seroconversion; and (2) *nef* serology in a population of 32 HIV seropositive and *three* seronegative hemophiliacs and their seronegative or seropositive sexual partners. The results obtained showed that anti-*nef* antibodies could not be detected in the sera tested independently of the appearance of antibodies specific to HIV structural proteins. Thus, the detection of anti-*nef* antibodies appears to be of little diagnostic value.

THE DIAGNOSIS of human immunodeficiency virus (HIV) infection is usually assessed by serologic detection of antibodies directed against HIV structural proteins *(gag, pol, env)*. However, some recent studies based on the detection of HIV DNA in peripheral blood mononuclear cells using gene amplification by the polymerase chain reaction (PCR), or virus isolation by culture techniques, have reported that some virus-infected individuals do not present any detectable amount of such anti-HIV antibodies.[1-3] This immunologically silent period could extend for several months,[4] from 5 to 21 months for Horsbough et al,[5] or even for greater than 35 months.[2] However, due to technical limitations, routine application of either PCR technique or virus culture for HIV infection diagnosis is yet unrealizable.

The *nef* gene product is a nonstructural protein of 25 to 27 Kd that is N-terminus myristylated and can be phosphorylated, at least in some viral isolates.[6] As a negative factor of HIV replication, *nef* appears to be a viral protein expressed early by HIV-infected cells and thus could be one of the first targets recognized by the host immune system. However, the role of *nef* as a negative factor of HIV replication has been recently questioned.[7,8] Some reports have suggested that antibodies to *nef* could be detected among seronegative individuals at high risk for HIV infection long before the appearance of antibodies to viral structural proteins.[9,10]

To investigate this point, we report the results of a kinetic study of the appearance of anti-*nef* antibodies in a set of 77 longitudinal sera collected from 12 HIV-infected donors at the time of seroconversion, and a study of *nef* serology in HIV-seropositive and seronegative hemophiliacs and their seropositive or seronegative sexual partners. In addition, epitope mapping of antibody recognition was performed by using a panel of synthetic peptides spanning the entire *nef* sequence from HIV-1 (LAV_{Bru} strain).

MATERIALS AND METHODS

Production of HIV-1 nef (LAV_{Bru} strain). The *nef* protein was obtained from Transgène S.A. (Strasbourg, France). It was produced in *Escherichia coli* bacteria as a nonmyristylated molecule. The protein from the lysed bacteria was extracted with urea in the presence of benzamidine and, after dialysis and centrifugation, chromatographed on cibracon blue. The *nef*-rich fraction was finally purified by ultrafiltration. Homogeneity of *nef* was about 75% to 90% as determined by high pressure liquid chromatography (HPLC).

Iodination of HIV recombinant proteins nef and gp160. Ten micrograms of *nef* in 50 μL of phosphate-buffered saline (PBS), pH 7.4, and 1 mCi of ^{125}INa (13 to 17 mCi/μg) were added to a tube precoated with 75 nmol of iodogen and incubated for 10 minutes at room temperature. The reaction was stopped by adding 10 μL of tyrosine (9 mg/mL). The iodinated protein was desalted from free ^{125}INa by filtration through a sephadex G25 column PD 10 equilibrated with PBS, 0.5% (wt/vol) bovine serum albumin (BSA). Specific radioactivity of ^{125}I *nef* was about 50 μCi/μg. The same protocol was used for labeling soluble recombinant gp160 (obtained from Transgène) with a specific radioactivity of about 30 μCi/μg.

Radioimmunoassay (RIA). Fifty microliters of ^{125}I *nef* (6×10^4 to 10^5 cpm) were incubated with 50 μL of various serum dilutions and incubated for 2 hours at 37°C. The preformed complexes between ^{125}I *nef* and anti-*nef* antibodies were immunoprecipitated with 100 μL of a suspension of protein A-Sepharose (1 vol/3 vol) in PBS buffer pH 7.4, 0.5% (wt/vol) BSA for 1 hour at 37°C. After two washes with PBS buffer pH 7.4, 0.5% (wt/vol) BSA, 0.05% (wt/vol) NaN_3, 0.01% (vol/vol) tween 20, bound radioactivity was counted in a gamma counter. Inhibition of the binding of anti-*nef* antibodies to ^{125}I *nef* by unlabeled protein was examined with the same procedure, except that unlabeled protein was added in a competition assay. RIA with ^{125}Igp160 (60,000 to 80,000 cpm) was performed under the same conditions as above.

*Enzyme-linked immunosorbent assay (ELISA) for the detection of anti-*nef *antibodies.* Microtiter plates, 96-well (Nunc, Rotskild, Denmark), were coated for 2 hours at 37°C with 50 ng of *nef* or with 500 ng of synthetic peptides per well, in 50 μL of PBS (pH 7.4). After saturation with 400 μL of PBS (pH 7.4), 5% (wt/vol) casein for 1 hour at 37°C and washing with PBS (pH 7.4), 0.01% (vol/vol) tween 20, 50 μL of sera at different dilutions was added for 2 hours at 37°C. After washing, 50 μL of mouse anti-human IgG (H + L)-peroxydase 1:1,000 were incubated for 1 hour at 37°C. After further washing, 100 μL of O-phenylenediamine (OPD) was added for 30 minutes at room temperature in the dark. The enzyme reaction was stopped by adding 50 μL of 4N sulphuric acid, and the plates were

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read for their optical density ratio of 492 nm:620 nm. Wells coated with casein were used as negative controls.

Synthetic peptides. *Nef* synthetic overlapping peptides spanning the entire sequence of the molecule were derived from the amino acid sequence of LAV_{Bru} in which the N-terminus amino acid is a glycine. The primary structure of these peptides is shown in Table 1. They were synthesized using an Applied Biosystems peptide synthesizer (model 430A; Foster City, CA) according to the solid phase method developed by Merrifield,[11] as previously described by Sabatier et al.[12,13]

Serum samples. Two groups of sera were tested blindly under code. (1) Group one consisted of sera from 35 hemophilic males, 32 of whom were seropositive for HIV-1 antibodies, and their female sexual partners (Tables 2 and 3). Samples were collected between 1986 and 1989 and kept frozen at −20°C or below until tested. For most of these couples, the presence of HIV DNA sequences was investigated by PCR and the results reported elsewhere.[14] (2) Sequential samples from 12 individuals who seroconverted to HIV-1 structural proteins were collected at 3- to 45-day intervals. HIV antibody, antigen, and viral isolation results from the first seven subjects have been previously reported.[15]

RESULTS

*Anti-*nef *antibodies and heterosexual HIV transmission.* To investigate the relevance of anti-*nef* antibodies as early markers of HIV infection before the development of other serologic markers, we examined serum samples from 35 couples composed of seropositive or seronegative hemophiliacs and their sexual partners. As shown in Tables 2 and 3, 16 of the 32 HIV-seropositive men were anti-*nef* positive (50%), as were all but one of the seropositive women (5 of 6 or 83%). In contrast, none of the 29 seronegative women had detectable anti-*nef* antibodies. In addition, three seronegative hemophiliacs who had been in contact with nonheat-treated plasma derivatives between 1981 and 1985 were anti-*nef* negative.

An excess of HIV-seropositive females (5 of 16) was noted when males were negative for anti-*nef* antibodies, as compared with sexual partners of anti-*nef* antibody-positive males (1 of 16), but this did not reach statistical significance ($P = .154$ by the Fisher exact test).

*Analysis of anti-*nef *antibodies in a set of longitudinal sera from individuals who seroconverted.* To investigate whether anti-*nef* antibodies may appear before antibodies to structural antigens (envelope gp160, gp120, gp41, core p25, p18, or polymerase p68, p51, p34) in sera of HIV-infected subjects, we tested by RIA the immunoreactivity against *nef* of a set of 77 sera collected at frequent intervals from 12 plasma donors at the time of seroconversion. In addition, these sera were tested for the presence of antibodies to structural HIV antigens by Western blot and for antibodies to gp160 by a specific RIA (Table 4). The first available sample from all donors, except that of donor 1 which was slightly over the cutoff,[15] was negative in the ELISA screening assay. When tested by Western blot, however, the initial samples from donors 1 and 6 were positive (according to the criteria of the French Laboratoire National de la Santé); those from donors 3, 4, and 8 were borderline; and that from donor 5 was indeterminate. Initial samples from the six other donors were negative. Subsequently, all donors became frankly positive. Anti-*nef* antibodies were noted in all 12 cases. They were present in 5 of the 6 initial sera that were

Table 1. Amino Acid Sequences of the Synthetic Peptides of *nef* From HIV-1 (LAV_{Bru} isolate)

33-mer:including sequence 1-31:
PF11 = GGKWSKSSVVGWPTVRERMRRAEPAADGVGAYC(acm)
66-mer:sequence 1-66:
PF12 = GGKWSKSSVVGWPTVRERMRRAEPAADGVGAASRDLEKHGAITSSNTAATNAAC(acm)AWLEAQEEEEVG
33-mer:sequence 32-64:
PF13 = ASRDLEKHGAITSSNTAATNAAC(acm)AWLEAQEEEE
46-mer including sequence 65-109:
PF14 = C(acm)VGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHSQRRQDIL
51-mer including sequence 118-167:
PF15 = CGYFPDWQNYTPGPGVRYPLTFGWC(acm)YKLVPVEPDKVEEANKGENTSLLHPV
35-mer:sequence 171-205:
PF16 = GMDDPEREVLEWRFDSRLAFHHVARELHPEYFKNC(acm)
65-mer:sequence 141-205:
PF17 = C(acm)YKLVPVEPDKVEEANKGENTSLLHPVSLHGMDDPEREVLEWRFDSRLAFHHVARELHPEYFKNC(acm)
31-mer including sequence 93-122:
PF18 = C(acm)KGGLEGLIHSQRRQDILDLWIYHTQGYFPD
26-mer:sequence 147-172:
PF62 = VEPDKVEEANKGENTSLLHPVSLHGM
21-mer:sequence 185-205:
PF63 = DSRLAFHHVARELHPEYFKNC(acm)
19-mer including sequence 1-17:
PF3 = GGKWSKSSVVGWPTVREYC(acm)
22-mer including sequence 17-37:
PF5 = YERMRRAEPAADGVGAASRDLE
18-mer:sequence 35-52:
PF6 = DLEKHGAITSSNTAATNA
19-mer:sequence 88-106:
PF8 = HFLKEKGGLEGLIHSQRRQ

The acetamidomethyl (acm) corresponds to the side chain protecting group of cysteinyl residue.

Table 2. Antibody Characterization of the Sera from 35 Couples of HIV-Infected Hemophilic Men and Their Seronegative or Seropositive Sexual Partners

Couple	Sex	HIV Ab	*nef* Ab
1	M	+	−
	F	+	+
2	M	+	+
	F	−	−
3	M	+	−
	F	−	−
4	M	+	+
	F	−	−
5	M	+	+
	F	−	−
6	M	+	+
	F	−	−
7	M	+	+
	F	+	+
8	M	+	+
	F	−	−
9	M	+	−
	F	−	−
10	M	+	−
	F	+	−
11	M	+	+
	F	−	−
12	M	+	−
	F	−	−
13	M	+	+
	F	−	−
14	M	+	−
	F	+	+
15	M	+	−
	F	−	−
16	M	+	−
	F	−	−
17	M	+	−
	F	−	−
18	M	+	−
	F	+	+
19	M	+	+
	F	−	−
20	M	+	+
	F	−	−
21	M	+	−
	F	−	−
22	M	+	+
	F	−	−
23	M	+	+
	F	−	−
24	M	−	−
	F	−	−

(Continued in next column)

Table 2. (Cont'd)

Couple	Sex	HIV Ab	*nef* Ab
25	M	+	−
	F	−	−
26	M	+	−
	F	−	−
27	M	+	−
	F	+	+
28	M	−	−
	F	−	−
29	M	−	−
	F	−	−
30	M	+	+
	F	−	−
31	M	+	−
	F	−	−
32	M	+	−
	F	−	−
33	M	+	+
	F	−	−
34	M	+	+
	F	−	−
35	M	+	+
	F	−	−

Sera were tested for their immunoreactivity to HIV structural proteins (HIV Ab) and *nef* (*nef* Ab). Positive and negative reactivities are indicated by + and −, respectively.

already positive, borderline, or indeterminate in Western blot (donors 1, 3, 5, 6, and 8), and they occurred at the fourth bleed, on day 12, for donor 4. In the cases in which initial sera were negative, anti-*nef* occurred simultaneously with antibodies to *gag* p25 and soluble *env* gp 160 (as detected in RIA) in donors 2, 9, 10, and 12; antibodies to gp160 and p25 were detected before anti-*nef*, by one bleed in donor 7, and in donor 11 anti-*gag* and anti-gp160 preceded anti-*nef* by two bleeds. Altogether, detection of anti-gp160 was more sensitive by RIA than by Western blot.

To study the specificity and the potential variations in the avidity of anti-*nef* during the early stages of IgG immune response, we evaluated the inhibition of ^{125}I *nef* binding to anti-*nef* antibodies by competition with increasing concentrations of unlabeled *nef*. Serum samples from donors 1 and 9 were selected. In each case, five samples positive for anti-*nef*

Table 3. Summary of the Results Described in Table 2

Hemophiliacs			Female Partners		
HIV Ab	*nef* Ab	Number	HIV Ab	*nef* Ab	Number
+	+	16	+	+	1
			−	−	15
+	−	16	+	+	4
			+	−	1
			−	−	11
−	−	3	−	−	3

Table 4. Serologic Analysis by Western Blot and Liquid-Phase RIAs of a Set of 77 Longitudinal Sera Collected at Various Dates From 12 HIV-Infected Donors at the Time of Seroconversion

		Western Blot Tests									RIAs	
		env			*pol*			*gag*				
Donor	Days of Donation	160	120	41	68	51	34	55	25	18	gp160	*nef*
1	1	+	−	−	−	−	−	−	+	−	+	+
	6	+	−	−	−	−	−	−	+	−	+	+
	10	+	−	−	−	−	−	−	+	−	+	+
	16	+	+	−	−	−	−	−	+	−	+	+
	20	+	+	−	−	−	−	−	+	−	+	+
3	1	±	−	−	−	−	−	−	+	−	+	+
	5	±	−	−	−	−	−	−	+	−	+	+
	8	+	−	−	−	−	−	−	+	−	+	+
4	1	±	−	−	−	−	−	−	+	−	+	−
	5	±	−	−	−	−	−	−	+	−	+	−
	9	±	−	−	−	−	−	−	+	−	+	−
	12	±	−	−	−	−	−	−	+	−	+	+
	16	+	−	−	−	−	−	+	+	−	+	+
	19	+	−	−	−	−	−	−	+	+	+	+
	23	+	−	−	−	+	−	−	+	+	+	+
	26	+	−	−	−	+	−	−	+	−	+	+
5	1	−	−	−	−	−	−	−	+	−	−	+
	8	−	−	−	+	−	−	−	+	−	+	+
	10	−	−	−	+	−	−	−	−	−	+	+
	17	+	−	−	+	+	−	−	−	−	+	+
	22	+	−	−	+	+	−	−	−	−	+	+
6	5	+	+	−	+	+	−	−	+	−	+	+
	9	+	+	−	+	+	−	+	+	−	+	+
8	1	±	−	−	−	−	−	−	+	−	+	+
	3	±	−	−	−	−	−	−	+	−	+	+
	10	±	−	−	−	−	−	−	+	−	+	+
	16	+	−	−	−	−	−	−	+	+	+	+
	22	+	−	−	−	−	−	−	+	+	+	+
	24	+	−	−	−	−	−	−	+	+	+	+
2	1	−	−	−	−	−	−	−	−	−	−	−
	6	−	−	−	−	−	−	−	−	−	−	−
	8	−	−	−	−	−	−	−	−	−	−	−
	14	−	−	−	−	−	−	−	+	−	+	±
	17	±	−	−	−	−	−	−	+	−	+	+
	21	±	−	−	−	−	−	−	+	−	+	+
	24	±	−	−	−	−	−	−	+	−	+	+
7	1	−	−	−	−	−	−	−	−	−	−	−
	3	−	−	−	−	−	−	−	−	−	−	−
	8	−	−	−	−	−	−	−	+	−	±	−
	10	−	−	−	−	−	−	−	+	−	+	+
	17	+	−	−	+	−	−	−	+	−	+	+
	20	+	−	−	+	−	−	−	+	−	+	+
9	1	−	−	−	−	−	−	−	−	−	−	−
	4	−	−	−	−	−	−	−	−	−	−	−
	11	±	−	−	−	−	−	+	+	−	+	+
	16	±	−	−	−	−	−	+	+	−	+	+
	18	±	−	−	−	−	−	+	+	−	+	+
	23	+	−	−	−	+	−	+	+	−	+	+
	25	+	−	−	−	+	−	+	+	−	+	+

(*Continued on following page*)

were collected over a period of 20 and 16 days, respectively (Figs 1A and 1B). For these sera, the concentration of recombinant *nef* able to inhibit 50% of ^{125}I *nef* binding (ie, $K_{0.5}$) ranged between 10^{-7} to 10^{-8} mol/L.

To further confirm the anti-*nef* specificity of the antibodies, and as a way to approach B-cell epitope mapping at the time of early antibody response, we tested seven longitudinal sera from donor 9 in an ELISA using synthetic fragments of

Table 4. Serologic Analysis by Western Blot and Liquid-Phase RIAs of a Set of 77 Longitudinal Sera Collected at Various Dates From 12 HIV-Infected Donors at the Time of Seroconversion (Cont'd)

		Western Blot Tests									RIAs	
		env			*pol*			*gag*				
Donor	Days of Donation	160	120	41	68	51	34	55	25	18	gp160	*nef*
10	1	−	−	−	−	−	−	−	−	−	−	−
	3	−	−	−	−	−	−	−	−	−	−	−
	14	−	−	−	−	−	−	−	−	−	−	−
	21	−	−	−	−	−	−	−	−	−	−	−
	51	±	−	+	−	−	−	+	+	+	+	±
	55	±	−	+	−	−	−	+	+	+	+	−
	59	±	−	+	−	−	−	+	+	+	+	±
11	1	−	−	−	−	−	−	−	−	−	−	−
	3	−	−	−	−	−	−	−	−	−	−	−
	7	−	−	−	−	−	−	−	−	−	−	−
	10	−	−	−	−	−	−	−	−	−	−	−
	21	+	±	−	−	−	−	−	+	−	+	−
	24	+	±	±	±	−	−	±	+	−	+	−
	28	+	±	+	+	−	±	+	+	−	+	+
	34	+	±	+	+	±	+	+	+	−	+	+
	41	+	±	+	+	+	+	+	+	±	+	+
	48	+	±	+	+	+	+	+	+	+	+	+
12	1	−	−	−	−	−	−	−	−	−	−	−
	28	−	−	−	−	−	−	−	−	−	−	−
	77	−	−	−	−	−	−	−	−	−	−	−
	104	−	−	−	−	−	−	−	−	−	−	−
	142	−	−	−	−	−	−	−	−	−	−	−
	191	−	−	−	−	−	−	−	−	−	−	−
	241	+	−	+	−	−	−	+	+	−	+	±
	273	+	+	+	+	+	+	+	+	+	+	±
	301	+	+	+	+	+	+	+	+	+	+	+
	352	+	+	+	+	+	+	+	+	+	+	+
	440	+	+	+	+	+	+	+	+	+	+	+

Positive, intermediate, and negative reactivities are indicated by +, ±, and −, respectively.

the molecule (Table 1). As expected from the RIA results, the two preseroconversion samples did not recognize the full-length protein nor the peptides in ELISA; the next five anti-*nef* positive samples collected during the first 2 weeks of seroconversion (numbers 45, 46, 47, 48, and 49) reacted with peptide PF12 (ie, sequence 1–66), and the last sera collected at days 23 and 25 (numbers 48 and 49) gave a significant reaction with peptide PF13 (Fig 2), thus allowing us to localize the major epitope in the region spanning from amino acid 32 to 64. No other short peptides showed any significant immunoreactivity.

DISCUSSION

nef is the 27-Kd product of one of the HIV regulatory genes and has been named after its apparent function as a negative regulator of viral replication in infected cells. Using radioimmunoprecipitation, Western blot, or ELISA, several groups of investigators have shown that *nef* was antigenic and that 30% to 70% of HIV-infected seropositive subjects at various clinical stages presented with anti-*nef* IgG.[12,16-18] More recently, the presence of anti-*nef* was detected in the sera of individuals exposed to HIV through homosexual or heterosexual activity who were negative for antibody to HIV structural proteins (envelope, core, or polymerase). This situation was observed during the months or weeks preceding seroconversion, defined as a positive screening ELISA, confirmed by Western blot showing positive IgG bands against viral envelope and at least another structural gene product.[10] These findings were not reproduced by other investigators using similar antigen sources but different assay formats.[18,19] In addition, the group of investigators who found anti-*nef* before seroconversion noticed that the presence of these antibodies correlated with the presence of proviral sequences detected by PCR in peripheral blood lymphocytes.[9]

In view of these conflicting results, we decided to reexamine the issues of the respective temporal sequence of occurrence of antibodies to *nef* and HIV structural proteins and the value of anti-*nef* as a marker of HIV infection in exposed, but yet persistently seronegative, individuals. For this purpose, we used an RIA with recombinant ^{125}I *nef*. With this assay, we determined that 70% of randomly selected HIV-positive sera were anti-*nef* positive,[12] a percentage of reactivity similar to that reported by Reiss et al,[18] and higher than prevalences previously reported by others (30% to 50%).[6,16,17] This difference might be indicative of a higher sensitivity of the RIA. No anti-*nef* reaction was noted in negative control sera nor in the sera of 550 asymptomatic seronegative people at risk for HIV infection,[19] indicating high specificity of our assay.

No anti-*nef* antibodies were found in 26 seronegative women who were sexual partners of seropositive hemophiliacs;

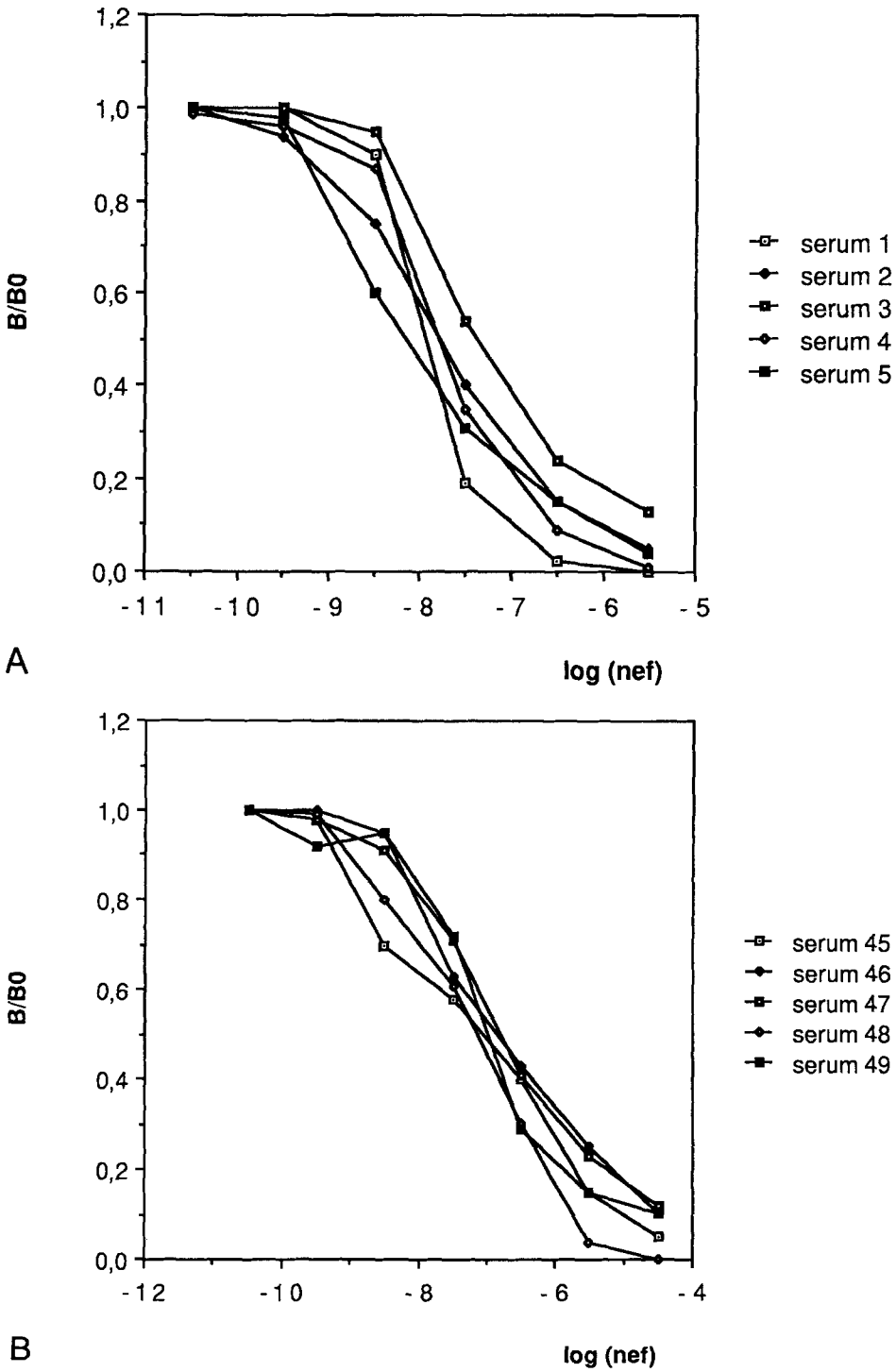

Fig 1. Liquid phase RIA of ^{125}I *nef* with five sequential anti-*nef* positive serum samples (1: 200 dilution) collected from both donors 1 (A) and 9 (B) at the time of seroconversion. Serum samples 1 to 5 were collected from donor 1 on days 1, 6, 10, 16, and 20, respectively, while sera 45 to 49 were collected from donor 9 on days 11, 16, 18, 23, and 25, respectively. The specificity of ^{125}I *nef* binding is demonstrated by the capacity of nonradiolabeled *nef* to inhibit binding of anti-*nef* antibodies to ^{125}I *nef*. The concentration of *nef* able to inhibit 50% of ^{125}I *nef* binding (ie, $K_{0.5}$) ranged between 10^{-7} to 10^{-8} mol/L for these sera.

in contrast, 5 of the 6 seropositive women were anti-*nef* positive. Anti-*nef* does not appear to be a marker of contact with HIV-1 unless full seropositivity characterized by antibodies to structural proteins is also present (Table 4). In addition, 22 of the 26 exposed seronegative women were independently tested for the presence of HIV genome by PCR using serum (n = 22) and cells (n = 7) as a source of viral DNA[14]: half of them (11 subjects) were positive for HIV DNA, but none of those was found to be anti-*nef* positive; thus, anti-*nef* does not correlate with silent HIV infection evidenced by PCR positivity. Interestingly, when the HIV status of female sexual partners of seropositive hemophiliacs was examined according to the anti-*nef* status of the male partner, an excess of seropositive women (5 of 6), as well as of PCR positive women (11 of 16),[14] was noted when males were anti-*nef* negative. Longitudinal follow-up of these women is currently under way.

The issue of anti-*nef* as an early marker of HIV-1

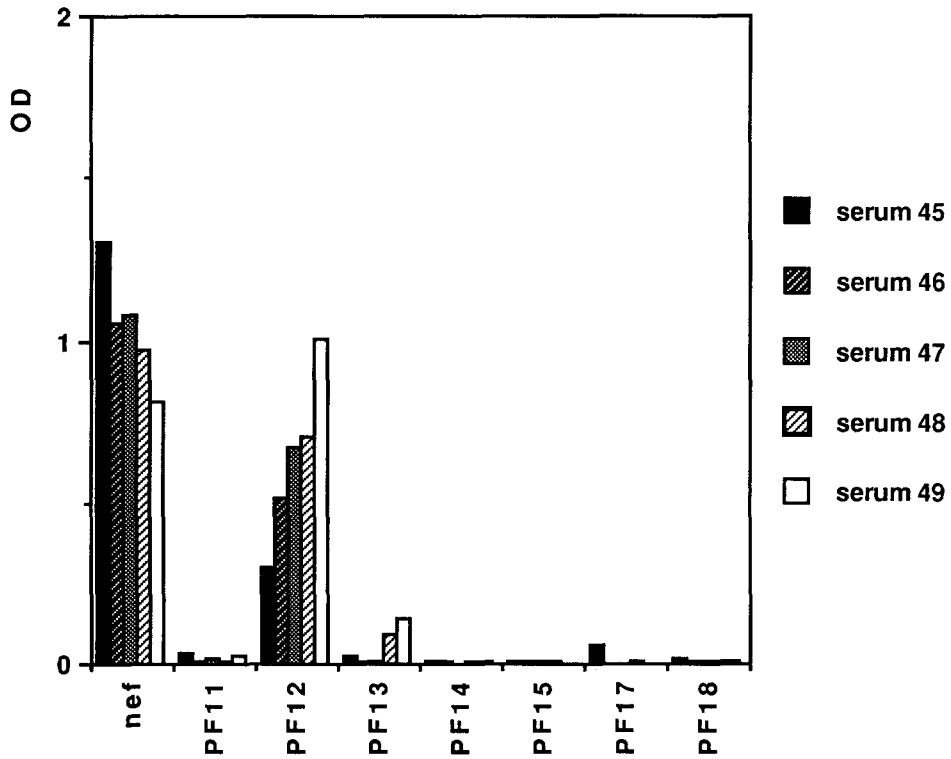

Fig 2. ELISA using recombinant *nef* or precoated synthetic peptides (PF11 to PF18) of five longitudinal anti-*nef* positive serum samples (1:200) collected from donor 9 at the time of seroconversion. Serum samples 45 to 49 were collected from donor 9 on days 11, 16, 18, 23, and 25, respectively. Absorbance:optical density ratio (492 nm:620 nm).

infection, as suggested by several investigators, was revisited in samples collected at close interval from 12 plasma donors who fully seroconverted, six of whom presented with HIV-negative initial serum samples. Although each donor seroconverted to *nef*, these specific antibodies were detected either simultaneously with, or later than, anti-*gag* detected in the Western blot or anti-*env* antibodies tested with a highly sensitive method. The specificity of anti-*nef* antibodies was determined in two ways: (1) the ability of unlabeled *nef* to inhibit the formation of ^{125}I *nef*–anti-*nef* antibody complexes in a dose-dependent manner; and (2) the capacity to react with synthetic peptides of the molecule, some of which were known to be recognized by anti-*nef* sera from HIV positive individuals.[12] The latter allowed to map one epitope, recognized by sequential sera of one of the donors, in the region of amino acid residues 32 to 64 which is included in one of the four epitopic regions previously individualized.[12]

Altogether, our data clearly indicate that IgG antibodies to *nef* develop with high frequency after HIV infection, but not earlier than antibodies to structural proteins. These results contrast those published previously[1,9,10] even though the assay used here appears more sensitive and specific than the one used by Ameisen et al[9,10] and Khalife et al.[17] However, they are similar to the data presented by Reiss et al[18] as well as to our recent results showing the lack of anti-*nef* antibody detection in a transversal study of 550 HIV-seronegative high-risk people.[19]

The frequency of anti-*nef* detected at the time of seroconversion (12 of 12) contrasts with the lower prevalence of these antibodies found in seropositive hemophiliacs (16 of 32 or 50%) and in the seropositive women (5 of 6 or 83%) we examined. Similar prevalences (30% to 70%) were found in larger groups of seropositive subjects,[9,10,12,19] suggesting that, over time post-seroconversion, a substantial number of individuals lose anti-*nef* or that titers of these antibodies decrease below the threshold of detection of the assays used. Nonetheless, if no obvious variation of the avidity of antibodies to *nef* was found over a period of 2 to 3 weeks, it is of note that the $K_{0.5}$ values of serum samples collected further away from conversion were in the 10^{-9} mol/L range,[12] one logarithm of magnitude lower than those obtained for the sequential sera from donors 1 and 9. This may suggest that over a longer period of time significant variations of anti-*nef* avidity may be observed.

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