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Characterization of antibodies reacting with HIV *gag* proteins occasionally found in the serum of non-infected subjects

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SUMMARY

The use of serological tests for the diagnosis of HIV infection has revealed that some non-infected persons have antibodies that react with HIV-1 *gag* proteins. Here, the sera of three non-infected subjects reacting with p17 and 11 non-infected subjects reacting with p24 were investigated, using an enzyme immunoassay (EIA) with six recombinant *gag* antigens and Western blot analysis of proteolytic peptides of two of these *gag* antigens. The results indicate that whereas all p17-reactive sera could react with an unique epitope, individual p24-reactive sera recognize different epitopes. Investigations by EIA also demonstrated the role of sequences located far from the epitopes in making these epitopes accessible to the antibodies or in providing them with an antigenic conformation. In addition to the 14 subjects mentioned above, another subject was shown to have antibodies reacting with the p9 (NC) *gag* protein. Several proteins are known as having homology with HIV-1 *gag* proteins. Their possible role in eliciting cross-reactive antibodies is discussed.

Keywords gag core cross-reaction false-positive

INTRODUCTION

The diagnosis of infection with HIV is based on the detection of antibodies to viral proteins coded by the *gag*, *pol* and *env* open reading frames. With first-generation tests, in which whole virus lysates are used as the antigen, several false-positive results have been observed. Most of them are due to antibodies against lymphocytic proteins which contaminate the viral preparations (Wittwer *et al.*, 1987). In second-generation tests, highly purified recombinant proteins replace viral lysates, leading to a substantial decrease of the rate of false-positive results (Lelie, Reesink & Huisman, 1989). However, it has become clear that some non-infected persons, most of them healthy, have antibodies that react with one or more viral proteins, most often the *gag*-encoded p17 (membrane associated (MA)) or p24 (core associated (CA)) or their precursor p55 as seen in Western blot (Dock *et al.*, 1988; Tribe *et al.*, 1988; Josephson *et al.*, 1989). Whereas the specificity of these reactions is established, their mechanism remains unclear (Dock *et al.*, 1988; Tribe *et al.*, 1988; Josephson *et al.*, 1989). In order to characterize these reactions better, we selected sera from several subjects reacting with HIV *gag* proteins without evidence of HIV infection. One important question addressed here is whether all sera reacting, e.g. with p24, recognize the same epitope. Such a finding could be indicative of a molecular mimicry mechanism. Thus, cross-reactive antibodies would result from exposure to an immunogenic protein displaying a short sequence homology with p24, but otherwise unrelated to p24 (Guldner *et al.*, 1990).

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SUBJECTS AND METHODS

Subjects

Seventeen sera from 15 non-infected subjects were selected for this study. None of the subjects belonged to a high risk group for HIV infection. Thirteen were healthy and two (subjects 11 and 12) had systemic lupus erythematosus (SLE). The sera of these two SLE patients were collected in 1976, before the spread of HIV infection in Switzerland. All sera were reactive with p17 and/or p24 and/or p55 by Western blot analysis for HIV-1 antibodies (Bio-Rad Laboratories, Richmond, CA). None of the subjects had antibodies to *pol* or *env* proteins in HIV-1 or HIV-2 (Diagnostics Pasteur, Marnes-la-Coquette, France) Western blot. All were negative for antibodies to env and core proteins using a competitive recombinant enzyme immunoassay (EIA) (Envacor; Abbott Laboratories, North Chicago, IL). None had circulating HIV-1 p24 antigen (HIVAG-1 EIA; Abbott Laboratories). HIV serological tests were repeated after 17 days (subject 1) or after 3 months or more (12 subjects) and no qualitative change was observed. Although the World Health Organization (1990) recommends a 6-month follow up in the case of indeterminate Western blots with p24 only, we consider from our experience that an unchanged serology after a

Table 1. Reactivity of the sera with proteolytic peptides by Western blot analysis

Subject no.	HIV-1 Western blot	HIV-2 Western blot	LIA p17	LIA p24	Antigen II *S. aureus* protease*	Antigen VI *S. aureus* protease†	Antigen VI chymotrypsin‡
1	p17/p55w	Negative	−	±	−/−/−	−/−	−
2	p17/p24w/p55w	Negative	−	±	+/+/+	±/?	+
3	p17	Negative	±	−	NT	−/−	±
4	p24	Negative	−	±	+/−/−	−/?	+
5	p24/p55w	Negative	−	±	+/+/+	−/−	+
6	p24/p55	Negative	−	+	+/+/+	+/+	+
7	p24/p39w/p41w/p55	Negative	−	+	+/+/+	+/+	NT
8	p24	p26	−	±	+/+/+	+/+	+
9	p24	p26	−	±	+/+/+	+/+	+
10	p24/p55	Negative	−	±	+/+/+	−/−	+
11/serum 3	p24/p39w/p41w/p55	NT	±	±	+/+/+	+/+	+
12	p24	NT	+	±	+/+/+	+/±	+
13	p24/p39w/p41w/p55	Negative	−	+	+/+/+	−/−	+
14	p24/p55	Negative	−	±	±/±/±	−/−	−
16	p55	Negative	−	±	−/−/−	−/?	−
Negative control	neg	NT	−	−	−/−/−	−/−	−
HIV-1 seropositive	All major *gag*, *pol* and *env* bands		+	+	+/+/+	+/+	+

*Reactivity with the uncleaved antigen II and two proteolytic peptides, respectively (Fig. 2).

†Reactivity with 2 proteolytic peptides (Fig. 2).

‡Reactivity with any of the proteolytic peptides.

p39, p41, processing intermediates of p55 *gag*; w, weak; ±, very weak; NT, not tested; LIA, line immunoassay (LiaTek HIV 1+2).

3-month interval strongly argues against HIV infection. Two subjects (nos. 4 and 8) were not available for follow up, but were considered as not being at risk by the attending physician. In the case of subject no. 14, peripheral blood mononuclear cells could be obtained and were sent to Dr L. Perrin (Laboratoire Central de Sérologie Virale, Hôpital Cantonal Universitaire de Genève, Switzerland) for HIV DNA amplification (Perrin *et al.*, 1990). The result was negative. Non-reactive normal sera and sera from HIV-1-infected subjects were used as controls.

Commercially available immunoassays

In addition to those mentioned above, a Western blot for HTLV-I antibodies (Biotech Research Labs, Rockville, MD) and a line immunoassay for HIV antibodies (LiaTek HIV 1+2; Organon Teknika, Boxtel, The Netherlands) were used. The latter test provides five recombinant or chemically synthesized peptides containing sequences of gp41 *env*, p32 *pol*, p24 *gag*, p17 *gag* and gp36 *env* (HIV-2).

Competition experiments with thymosin α-1

These experiments were performed using a Western blot kit (New Lav-Blot I) from Diagnostics Pasteur, France. Twenty microlitres of serum were pre-incubated in 500 μl diluent for 1 h at 37 °C, in the presence or absence of 35 μg (11·3 nmol) thymosin α-1 (Serva, Heidelberg, Germany) (Schüpbach, Sarngadharan & Gallo, 1984). These 0·5 ml were then added to 1·5 ml of diluent in wells containing nitrocellulose strips coated with HIV-1 antigens and incubation was performed for 2 h at room temperature. All the following steps were done according to the manufacturer.

Recombinant antigens

Six recombinant proteins, numbered I–VI with sequences encompassing various portions of the p55 *gag* precursor of HIV-1 were synthesized in *Escherichia coli* and purified by metal ion affinity chromatography (Hochuli *et al.*, 1988). To make the purification possible, the proteins were endowed with six consecutive histidine residues at their C terminus (Hochuli *et al.*, 1988). They were kindly provided by Hoffmann-La Roche, Basle, Switzerland.

Enzyme immunoassay

Polystyrene beads coated with one of each of the six recombinant proteins were supplied by Hoffmann-La Roche. The coating efficiency did not differ significantly from one protein to another, as judged by a modification of the BCA™ protein assay of Pierce (Oud-Beijerland, The Netherlands). The beads were incubated at 37°C with the patient's serum for 20 min (1/10 dilution) or 30 min (1/25) with shaking. After washing, the beads were incubated with a horseradish peroxidase anti-human IgG conjugate for 20 min at 37°C. Following another washing step, OPD and H_2O_2 were added. The enzymatic reaction was allowed to proceed for 20 min at room temperature, stopped with sulphuric acid, and the absorbances read at 492 nm. All measurements were performed in duplicate. Specimen diluent, conjugate and buffer for the enzymatic reaction were identical to

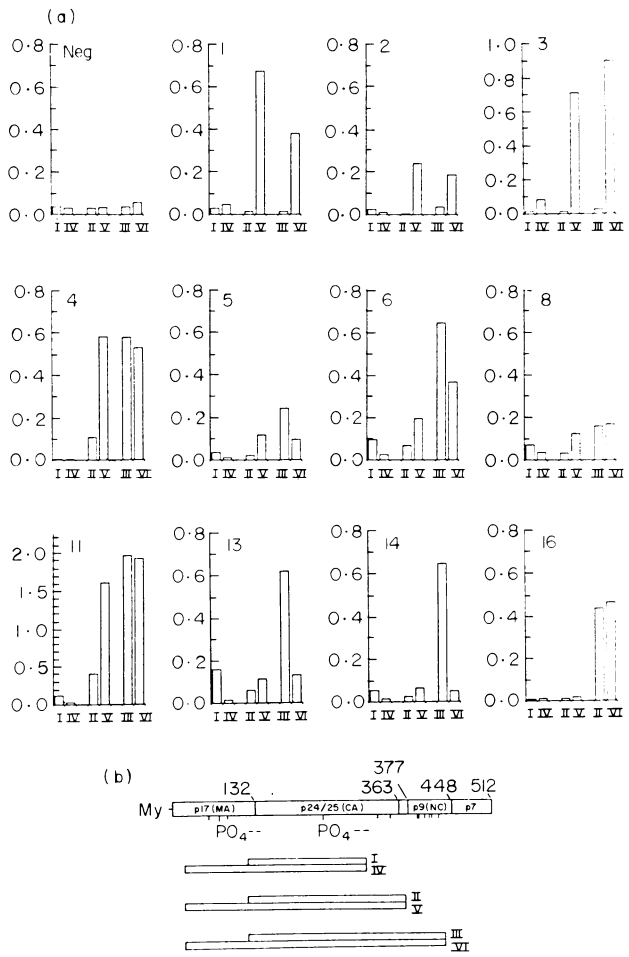

Fig. 1. Results by EIA using six recombinant antigens. (a) Plots of the absorbances measured with each of the six antigens. Subjects are numbered as in Table 1. Neg., non-reactive control subject. Subjects 10 and 12 both gave a pattern similar to subject 5. Subjects 9 and 7 gave results similar to subjects 6 and 8, respectively; (b) schematic view of the p55 *gag* precursor of HIV-1 (Mervis *et al.*, 1988) and delineation of the six antigens. Numbers above p55 indicate the C terminal amino acids of the mature proteins, and vertical dashes indicate the position of cysteine residues. Phosphoester groups present in the native *gag* proteins are depicted. However, recombinant antigens are not phosphorylated.

those supplied with the anti-HIV-1/HIV-2 EIA kit of Hoffmann-La Roche.

Western blotting after limited proteolysis

Recombinant proteins II and VI were digested for 2 h at 37 °C with *Staphylococcus aureus* Glu-C endoproteinase (Boehringer, Mannheim, Germany; enzyme/substrate 6:10, w/w) in the presence of 0·4% (w/v) SDS or with bovine chymotrypsin A4 (Boehringer Mannheim; enzyme/substrate 3:10, w/w) in the presence of 0·1% SDS. Other conditions were as described (Cleveland, 1983). The resulting peptides were separated in 15% polyacrylamide gels in the presence of SDS (Laemmli, 1970) and either silver stained (Merril, Goldman & van Keuren, 1984) or transferred onto zeta-probe membranes (Bio-Rad Laboratories) (Karey & Sirbasku, 1989), to which they were attached covalently using glutaraldehyde (Karey & Sibasku, 1989). Blocking was performed with 5% non-fat milk with or without 10% normal goat serum. Sera were incubated overnight at a 1/50 or 1/12·5 dilution. Antibodies were detected using an alkaline phosphatase goat anti-human IgG conjugate (Bio-Rad Laboratories), and 5-bromo-4-chloro-3-indolyl phosphate (*p*-toluidine salt) and nitroblue tetrazolium as the substrate and the chromogen, respectively (Ey & Ashman, 1986).

RESULTS

Table 1 summarizes the reactivity of the sera in HIV Western blots. In addition to p17 and/or p24 and/or p55 of HIV-1, some sera also reacted with two sharp minor bands in the range of 39–41 kD, which presumably represents processing intermediates of p55 *gag* (Mervis *et al.*, 1988; Gowda, Stein & Engleman, 1989; Erickson-Viitanen *et al.*, 1989). These bands are unambiguously distinct from the broad *env*-encoded gp41 on Western blot strips from Bio-Rad. In competition experiments with thymosin α-1 and sera from subjects 1–3, no decrease of the staining intensity of the p17 band was observed in the presence of thymosin α-1. Even when serum 1 was diluted 1/3, which resulted in a faint staining of p17, no competition was observed.

Results with the line immunoassay (LIA) were qualitatively concordant with those obtained using HIV-1 WB, except for subjects 1, 2, 11, 12, and 16 (Table 1). Even after overnight incubation, none of the 15 sera reacted with the *env* and *pol* antigens in the LIA.

No serum reacted with HTLV-I proteins in Western blot except that of subject 14, which reacted weakly with p19 *gag* (MA).

Results obtained by EIA with six recombinant proteins

The results are shown in Fig. 1. Subjects are numbered as in Table 1. Sera from subjects reacting mainly or exclusively with p17 in HIV-1 Western blot (nos 1–3) did not react with antigens I, II and III. They produced a strong reaction with antigens V and VI. However, they did not react significantly with antigen IV, which nevertheless contains the same sequence of p17 as antigens V and VI (Fig. 1). This suggests that the C terminal part of p24, present in antigens V and VI, is necessary to induce the antigenic conformation of the p17 epitope(s) or to expose it at the surface.

Three features were common to all sera (nos 4–14) that react with p24 in HIV-1 Western blot: the reaction with the two antigens that lack the C terminal part of p24 (I, IV) was weak or absent; the reaction was stronger when the antigen included most of p17 (V *versus* II); and the reaction was also stronger when the antigen extended into p9 (III *versus* II). Again, non-antigenic sequences seem necessary either to expose an otherwise buried determinant or to bring about the correct folding of the polypeptide to form the epitope. The establishment of disulphide bridges (see position of cysteine residues in Fig. 1) may play a role in stabilizing an antigenic conformation. While the three trends described above were general, their magnitude differed substantially from one serum to another, as did the reactivity with antigen VI. Subject 11, who had SLE, was followed over several years. Antibodies reacting with p24 appeared in his serum within 5 weeks and remained detectable during the following 7 months. The three samples collected during that period displayed a similar pattern of reaction with the six antigens.

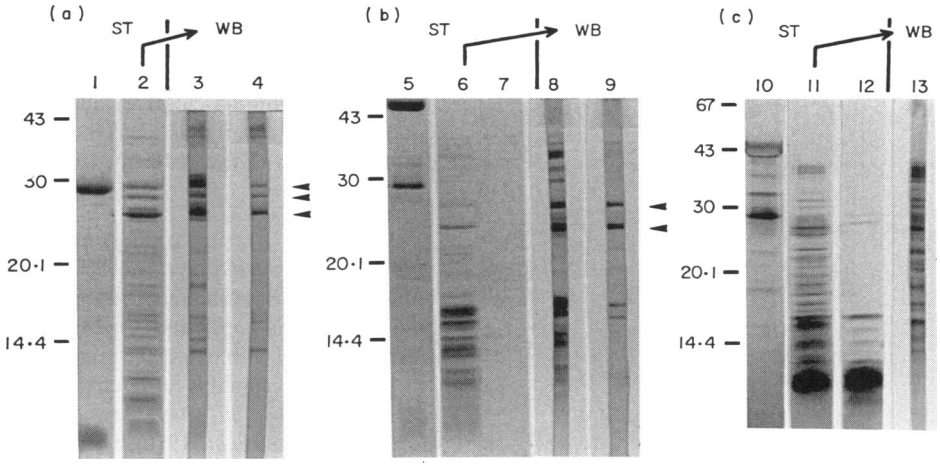

Fig. 2. Results by Western blot (WB) analysis using proteolytic peptides of antigens II and VI. (a) Antigen II, *S. aureus* protease; (b) antigen VI, *S. aureus* protease; (c) antigen VI, chymotrypsin. Silver stained (ST) gels demonstrate the pattern of bands generated by proteolytic cleavage of the antigens (lanes 2, 6, 11) and used for WB; antigens incubated in the absence of protease (lanes 1, 5, 10) as well as protease alone (lanes 7, 12) are shown for comparison. Lanes 3, 8, 13, WB with the serum of an HIV-1-infected individual. Lanes 4, 9, WB with the serum of subjects 8 and 6 (Table 1), respectively. Arrowheads point at bands discussed in the text.

The reaction pattern of sample 16 in HIV-1 WB and with the recombinant antigens strongly suggests that it recognizes an epitope present at the p25/p9 boundary or within p9.

Results by Western blot analysis of proteolytic peptides

The peptides produced by limited proteolysis of antigens II and VI are shown in Fig. 2. With *S. aureus* protease, antigen II was only partially cleaved. Its apparent molecular mass (28·7 kD) is slightly larger than that expected from Fig. 1. This is due at least in part to the presence of additional histidine residues at the C terminus of the recombinant proteins used here. Several HIV-1$^+$ sera reacted strongly with the uncleaved proteins and two peptides with apparent molecular masses of 27·5 and 25·1 kD (Fig. 2a). All the p24-reactive sera also showed these three bands, except serum from subject 4 which did not react with the 27·5- and 25·1-kD peptides (Table 1). The three bands appeared very faint when serum 14 was tested (Table 1). However, this serum reacted strongly with p24 in HIV-1 Western blot and with antigen III by EIA. Thus, the nature of the solid phase or the covalent attachment of the peptides may have altered the particular epitope recognized by this serum. *S. aureus* protease cleavage of antigen VI produced, among others, two peptides with apparent molecular masses of 26·4 and 23·8 kD which were strongly reactive with sera from HIV-1-infected persons (Fig. 2b). Only some of the p24-reactive sera recognized these two bands (Table 1). Other peptides observed upon *S. aureus* protease treatment were not considered, because some of them reacted also with control sera, suggesting that they represent staphylococcal peptides originating from the protease preparation. This problem was not encountered when chymotrypsin was used. Therefore, all immunoreactive bands (12–40 kD) generated from antigen VI by chymotrypsin digestion could be taken into account as *gag*-specific (Fig. 2c). All of the 11 p24-reactive sera tested but one (no. 14) reacted with at least one chymotryptic peptide. Among these 10 reactive sera, seven generated a distinctive pattern of bands with chymotryptic peptides. At least one p17-reactive sample and the p9-reactive sample did not react with any of the proteolytic peptides, possibly because the epitopes involved were cleaved by the proteases or altered following covalent attachment to zeta-probe membranes.

DISCUSSION

We show here that the various sera that contain antibodies reacting with p24 recognize several different epitopes. This conclusion is based on the different reaction patterns observed by EIA using six recombinant antigens and on the different sets of peptides recognized by Western blot analysis after limited proteolysis of two of these antigens. Additional features, such as reaction with p26, the HIV-2 homologue of HIV-1 p24, and reactivity with antigens after covalent binding to cationic membranes, add to the diversity observed. Therefore, our working hypothesis that p24 shares a unique continuous epitope with an unrelated immunogenic protein, as the result of a short sequence homology, is not supported. More elaborate hypotheses must be put forward to account for the diversity of patterns observed among non-infected subjects. It is possible that all subjects were exposed to the same immunogenic protein. The latter must then exhibit a stretch of homology with p24 long enough to provide several cross-reactive epitopes. Alternatively, the various subjects may have been exposed to different immunogens, each possessing a distinct short region of homology with p24. The following proteins have been reported as having some homology with HIV-1 p24 and could therefore have elicited the cross-reacting antibodies: the B lysozyme from *Bacillus subtilis* (Kamei *et al.*, 1988), the low affinity IL-2 receptor (Weinberg *et al.*, 1988), the VP2 coat protein of picornaviruses (Argos, 1989), and the *gag* proteins of exogenous lentiviruses other than the HIVs. *Gag* proteins encoded by endogenous retroviruses and expressed under some circumstances should also be considered as possible immunogens (Perl *et al.*, 1989). Exposure to a retrovirus closely related to HIV-1 is likely in the case of subject 2, whose serum reacted with both p17 and p24. In the case of the two SLE patients, cross-reactivity between p24 and proteins constitutive of the Sm autoantigen may be involved, as suggested recently by Talal *et al.* (1990). Since the sera of these SLE patients also reacted with p17 in the

LIA, expression of a defective endogenous retrovirus, as described by Garry *et al.* (1990), is a possibility.

Our results are consistent with the possibility that antibodies that react with p17 recognize only one continuous protease-sensitive epitope. Thymosin α-1 is known as having an 18-amino-acid-long homology with p17 (Sarin *et al.*, 1986). However, competition experiments presented here do not favour autoantibodies to thymosin α-1 as the cause of reactivity with p17. Finally, the possibility that antibodies reacting with HIV-1 *gag* proteins recognize discontinuous epitopes rather than continuous epitopes as discussed above should also be considered.

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