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Fine specificity of IgG subclass response to group antigens in HIV-1-infected patients

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SUMMARY

There is an association between the clinical stage of HIV-1 infection and the presence of antibodies against viral gag proteins (p17 and p24). The IgG subclass (G1 and G3) pattern against these antigens was analysed in stable patients and HIV patients progressing to AIDS. Antibodies were analysed with whole viral or peptide ELISA (using sequentially overlapping peptides) and Western Blots. IgG1 was found to be the dominant anti-HIV-1 IgG subclass and IgG1 antibodies declined in progressing patients against all HIV antigens evaluated in Western blot, including p17, p24, p31, gp41, p64, gp120 and gp160. In contrast IgG3 antibodies, which were found to be predominantly directed against gag proteins, and which could be detected in almost all patients, remained in the circulation during disease progression. By peptide assays distinct immunogenic regions were found in p17 in contrast to more evenly distributed epitopes in p24. A decreased divergence of antibody reactivity to both p17 and p24 peptides in the group of patients who developed AIDS was seen. No reaction to any single gag epitope related to disease progression. The difference between IgG1 and IgG3 anti-gag antibodies in relation to clearance during disease progression may depend on different properties of immune complexes formed by these two IgG subclasses.

Keywords IgG subclass HIV AIDS

INTRODUCTION

Human immunodeficiency virus (HIV) is the etiological agent of the acquired immunodeficiency syndrome (AIDS) and a spectrum of related disorders (Barré-Sinoussi *et al.*, 1983; Gallo *et al.*, 1984). The virus infects humans cells expressing the CD4 receptor, mainly helper T cells and macrophages. Progressive numerical and functional depletion of CD4 cells leads to impaired immune responsiveness. In about 20% of seropositive individuals AIDS develops within 5 years. The HIV infection is associated with a variety of clinical manifestations: acute infection (Centers for Disease Control, 1986) (CDC group I), asymptomatic carrier state (CDC group II), persistent generalized lymphadenopathy, PGL (CDC group III), and neurological manifestations, opportunistic infections (OI) and secondary cancers (CDC group B, C and D).

The humoral response to HIV has been analysed in detail. Within 4–12 weeks after exposure to HIV, sensitized individuals typically develop antibodies to the viral core proteins and to envelope glycoproteins (Gaines *et al.*, 1987). The major anti-HIV antibodies are directed to specific viral proteins (Schüpbach *et al.*, 1984): gp160, gp120 and gp41 encoded by the env gene; p55, p24 and p17 by the gag gene; p64, p53 and p31 by the pol gene, and also to several small regulatory proteins.

The four subclasses of human IgG are induced in response to various types of antigen (Linde *et al.*, 1983; Sundqvist *et al.*, 1984; 1986). In viral diseases IgG1 and 3 appear to predominate, while IgG2 may be the predominant response to bacterial polysaccharides. IgG4 has been detected sporadically against most viruses and typically following repeated antigenic stimulation. IgG1 has been shown to be the dominant anti-viral subclass in healthy seropositive individuals and in patients with ongoing viral infections. IgG3 was detected mainly in individuals with current infection, and in lower titres than IgG1. Anti-viral IgG3 is shown to be of special interest due to its high relative neutralizing capacity to other viruses, i.e. Herpes simplex (Mathiesen *et al.*, 1988) and CMV (Gilljam & Wahren, 1989), but so far no neutralizing capacity has been shown by IgG3 against HIV. In HIV infection a progression of clinical symptoms has been correlated with a loss of the minor virus-specific subclasses (Khalife *et al.*, 1988; Sundqvist *et al.*, 1986). This study was undertaken to analyse differences in the HIV-specific IgG1 and IgG3 in stable and progressive HIV infection.

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MATERIALS AND METHODS

Patients

Serum samples from a group of 20 homosexual men with HIV infection and PGL taking part in a prospective longitudinal clinical study were analysed retrospectively. Clinical examination and serum collection were carried out on 4–10 occasions during 8–29 (mean 21) months. All patients suffered from persistent generalized lymphadenopathy at entry. Nine patients remained fairly stable both clinically and immunologically during the observation period (Table 1). The median time of observation was 21·7 (range 16–30) months in this group which we call 'stable' PGL patients. Eleven of the patients progressed to opportunistic infection during the observation period. Eight developed major opportunistic infections, AIDS/OI, and three developed minor OI, in this case oral candidiasis (Table 1). The median time of observation was 21·5 (range 9–28) months in this group of patients progressing from PGL to OI. This group of patients is called OI or progressors. No further monitoring with regard to IgG subclasses took place after the diagnosis of AIDS/OI or after the start of anti-viral treatment. Serum samples from healthy laboratory personnel were used as negative controls.

ELISA for IgG subclasses

HIV-specific IgG and IgG subclasses (Sundqvist *et al.*, 1986) were investigated by using HIV antigen coated plates of a commercially available kit (Organon Teknika, Netherlands). Serum samples were diluted in 10-fold steps using the dilution buffer provided with 20% fetal goat serum. One-hundred microlitres of sample dilutions were added to wells of microplates, and incubated for 120 min at 37° C. After washing, 100 μl of monoclonal antibodies (MoAb) directed against the different IgG subclasses were added for 105 min at 37° C. The following dilutions were used: IgG1 clone NL16 1:2000 (Oxoid Ltd, Basingstoke, Hampshire, UK), IgG2 clone HP 6002 1:50 000 (Center for Disease Control, Atlanta, GA), IgG3 clone ZG4 (Oxoid) 1:1500 and IgG4 clone RJ4 1:800 (Oxoid). The plates were washed and 100 μl of peroxidase-conjugated rabbit anti-mouse IgG 1:3000 (Dakopatts, Copenhagen, Denmark) were added for 105 min at 37° C. The substrate orthophenylenediamine, activated by H_2O_2 was added, and the reaction was terminated after 30 min at room temperature by adding 100 μl 2·5 M H_2SO_4. Absorbance was measured at 492 nm. The background level was set at mean optical density (OD) + 3 standard deviations for HIV seronegative controls.

HIV antigen ELISA

Antigen in serum was measured in our HIV p24-antigen ELISA assay (Sundqvist *et al.* (manuscript), 1988). Briefly, micro-ELISA plates were coated with selected ammonium sulphate precipitated immunoglobulins of HIV-1 seropositive humans. Samples were added and the plates were incubated overnight in room temperature. HRPO-conjugated anti-p24 mouse monoclonal antibodies (MoAbs) were added for 2 h at 37°C. The substrate orthophenylenediamine was added for 30 min at room temperature, and the reaction was terminated with 2·5 M H_2SO_4. All volumes were 100 μl and the plates were carefully washed six times between each step. Positive and negative standard sera were used as controls. The absorbance was measured at 492 nm.

Table 1. Clinical outcome and the number of $CD4^+$ cells oif 20 patients with an initial diagnosis of persistent generalized lymphadenopathy

Patients		CDC stage				$CD4^+$ cells ($\times 10^6$/l†)		
	Group No.	first	final	OI*	Treatment	entry	endpoint	Antigen p24‡
Stable PGL	1	III	III	—		590	603	+
	2	III	III	—		818	640	+
	3	III	III	—		710	412	+
	4	III	III	—		900	1116	−
	5	III	III	—		571	737	−
	6	III	III	—		661	371	−
	7	III	III	—		555	1218	−
	8	III	III	—		895	676	−
	9	III	III	—		399	300	−
AIDS	10	III	IV C-1, D	Pcp, KS		325	247	−
	11	III	IV C-1	Pcp		430	208	−
	12	III	IV C-2	Thrush	AZT	175	147	+
	13	III	IV C-1	Tox.		164	18	+
	14	III	IV C-2	Thrush	AZT	233	34	−
	15	III	IV C-1	Pcp		95	60	−
	16	III	IV C-1	Pcp		127	30	+
	17	III	IV C-2	Thrush	AZT	486	282	+
	18	III	IV C-1	CE		383	323	−
	19	III	IV C-1	CMV		197	22	+
	20	IVA	IV C-1	Pcp		517	114	+

* *Pneumocystis carinii* pneumonia (Pcp), Kaposi sarcoma (KS), Toxoplasmosis (Tox) Candida esophagitis (CE), generalized cytomegalovirus infection (CMV).

† Normal range 340-1610 × 10^6/l.

‡ p24-antigen detected at least once during the observation period.

Western blot for IgG subclass analysis

HIV diagnostic kits with antigen-coated strips (Biotech-du Pont, Rockville, MD) were used. Undiluted serum samples were rocked overnight with the strips, washed three times for 5 min in phosphate-buffered saline with 0·05% Tween 20. MoAb anti-IgG1 and 3 (Oxoid) diluted 1:500 and 1:200 respectively in blotting buffer (PBS, 0·05% Tween 20 and 5% dried milk powder or 0·5% BSA) were added for 120 min. After washing, biotinylated anti-mouse IgG (Amersham, London, UK) diluted 1:500 was added for 60 minutes. The streptavidin-biotinylated horseradish peroxidase complex (Amersham) diluted 1:400 was added, and after washing the strips were developed in 4-chloro-1-naphthol for 30 min. The reaction was stopped by rinsing several times with deionized water. Only clearly visible bands were evaluated. All assays were done together with a reference control serum.

Peptide ELISA

Synthesized peptides that together represent the gag regions p17 and p24 of the HIV genome were used as antigen in ELISA assays. The peptides were chemically synthetisized by Johnsson & Johnsson, Biotechnology Center, La Jolla, CA and based on the HTLV-III sequence of Ratner *et al.* (1985). They consist of 15 aminoacids (a.a.) each with overlapping sequences of 10 a.a. There were 27 peptides representing p17 and 45 peptides representing p24. Microplates (96-well, Nunc, Denmark) were

Table 2. Subclass IgG titres in stable PGL patients and PGL patients progressing to OI

	IgG subclass			
	1	2	3	4
Entry samples				
Stable PGL	9	2	4	3
($n=9$)	71 000	325	240	210
	(30–120 000)	(100–550)	(100–360)	(100–280)
PGL→OI	11	1	2	2
($n=11$)	73 000	350	485	770
	(50–110 000)		(270–700)	(540–1000)
End point samples				
Stable PGL	9	2	4	3
($n=9$)	88 000	330	270	400
	(32–120 000)	(100–560)	(100–400)	(100–700)
PGL→OI	11	1	2	2
($n=11$)	65 000	350	135	640
	(20–90 000)		(100–170)	(280–1000)

* Titres evaluated at whole HIV-1 viral lysate (Organon).

Results are expressed as the number of patients and ELISA titres (mean titre with range in parentheses).

coated with 100 μl of 10 μg/ml peptide solutions in 0·05 sodium carbonate buffer, pH 9·5. After adsorption for 18 h at room temperature, the plates could be stored for 8–12 weeks at 4° C before use. The ELISA method of IgG subclass detection on these plates was similar to the one described above. Absorbance values of >3 s.d. above the mean values of negative sera were considered positive.

RESULTS

Subclass ELISA on HIV viral lysate

Anti-HIV IgG antibodies of subclasses 1–4 were first analysed on HIV viral lysate plates. All patients (100%) had a strongly reactive IgG1 (Table 2). Even in the group of patients who developed AIDS the IgG1 anti-HIV titres remained high. IgG3 occurred in one or more samples in six patients (30%). IgG2 and 4 occurred in 15% and 25% respectively. Two of the patients with stable PGL showed IgG reactivity in all subclasses. In the group that developed OI there was a decrease of the individual titres of all subclasses in 55% compared to 14% in the patients with stable PGL. IgG4 showed a surprisingly high frequency in the viral lysate ELISA, but has, together with IgG2, shown poor reactivity to peptides (unpublished data) and was therefore not further evaluated here. The IgG4 reactivity may partly be explained by its binding to goat immunoglobulins (Jeffries *et al.*, 1986), which was used in the Organon assay.

HIV antigen ELISA

The presence of HIV antigen was measured by a HIV-p24 assay and is shown in Table 1. Serum antigen was detected at least once during the observation period in three of the stable PGL patients, and in six of the patients progressing to OI. Although the presence of p24 seemed to be more frequently detected (data not shown) in the OI group, no significant difference (Chi-square 0·247; $P=0·62$) between the two groups could be seen in the present study.

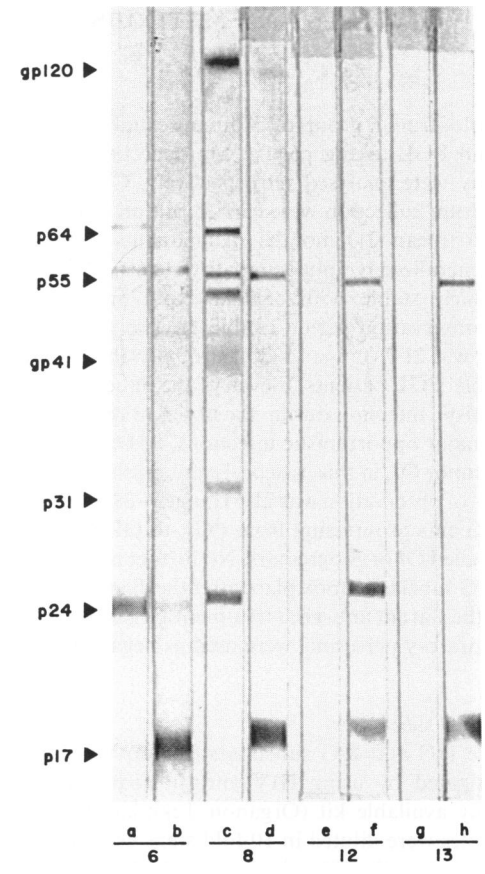

Fig. 1. Western blot of IgG subclasses 1 and 3 and end point of the study. Patients nos. 6 and 8 are stable PGL, while 12 and 13 had developed OI. a, c, e and g represent IgG1, and b, d, f and h IgG3.

Subclass Western blot

Subclass-specific IgG reactivity was analysed by Western blot (Figs 1 and 2). Western blot of entry sera were similar for both groups of patients. IgG1 antibody was seen in all individuals and was directed to at least two HIV-specific proteins. Generally IgG1 showed a varied pattern to all HIV proteins which was in accordance with Western blot for total IgG (data not shown). At end point analysis, however, some differences were identified as seen in Fig. 2, which shows the added reactivity at end point of the study. There was a general decrease of IgG1 reactivity against all HIV proteins in the OI group, and a few of these patients lost all IgG1 reactivity in Western blot as seen in Fig. 1, stripe 12e and 13g. IgG3 was almost entirely directed to gag proteins in both groups (p17, p24 and their precursor p55), but some reactivity to other HIV-proteins could still be seen in the stable group (Fig. 2). When comparing IgG1 and 3 reactivities to core proteins p17 and p24, IgG3 was seen as the dominating subclass to p17, while both IgG1 and IgG3 reacted with p24. Three patients in the OI group showed a reactivity against p17 and p24 restricted to IgG3 by Western blot during this study, and in five of the remaining eight individuals with both IgG1 and 3 response to p17 and p24, IgG3 reactivity remained at a

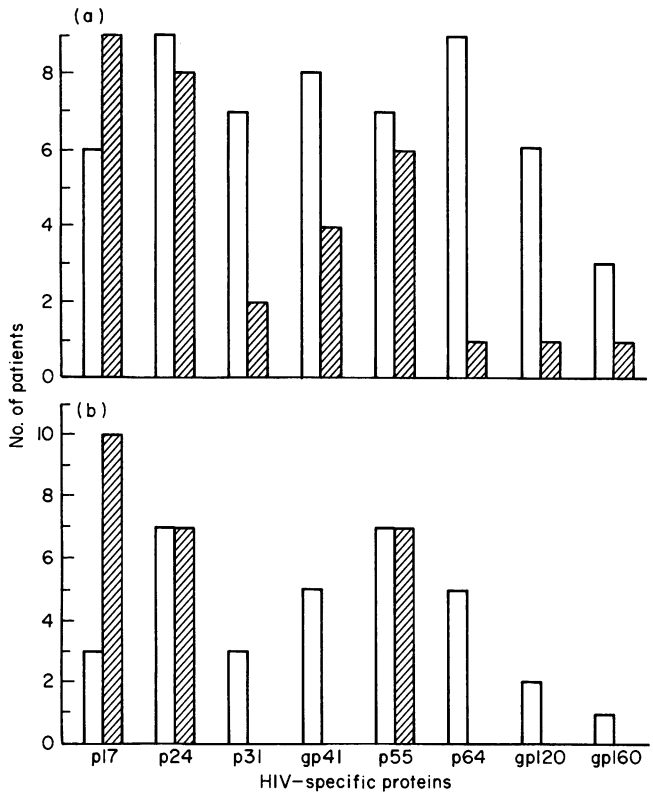

Fig. 2. IgG subclass Western blot patterns at end point of study for nine patients with stable PGL (a), and for 11 patients progressing to OI (b). The numbers of patients reactive to each HIV protein in the two groups are shown. IgG1 (□); IgG3 (■).

time when IgG1 had decreased considerably. Assays with undiluted serum were necessary for detection of IgG3 in these patients, indicating low IgG3 titres.

Peptide ELISA

We investigated whether the IgG1 and 3 reactivities to core proteins p17 and p24 were directed to specific regions and if so, if there was any difference between the subclasses investigated. Short synthetic peptides representing the p17 and p24 gag regions were used as antigen in ELISA assays. The reactivities of end point sera from both groups to each peptide are shown in Fig. 3.

IgG1 reactivity directed to several epitopes was distributed over the entire amino acid sequence. The variation of reactivity to the different peptides between patients was considerable. Even in the same patient variation of reactivity was seen between different samples. For IgG3 the reactivity according to divergence, absorbance values, number of positive reactions and epitopes was similar to IgG1. The peptides eliciting the most frequent responses of both IgG1 and IgG3 were nos. 10, a.a. 18–32 (KIRLRPGGKKKYKLK) and 22, a.a. 78–92 (LYNTVATLYCVHQRI) in the p17 region, and nos. 56, a.a. 248–262 (GWMTNNPPIPVGEIY) and 63, a.a. 283–297 (LDIRQGPKEPFRDYV) in the p24 region. We found only one p24 peptide, no 48, a.a. 208–222 (EAAEWDRVHPVHAGP) with an IgG1 response only, and two p24 peptides, nos. 37, a.a. 153–167 (NAWVKVVEEKAFSPE) and 40, a.a. 168–182 (VIPMFSALSEGATPQ) with a reactivity restricted to IgG3. There was a frequency of reactivity of 35% for each of these peptides. The most IgG3 reactive region of p24 was found around peptides 62–64, a.a. 278–302, where 55% of the patients responded. The

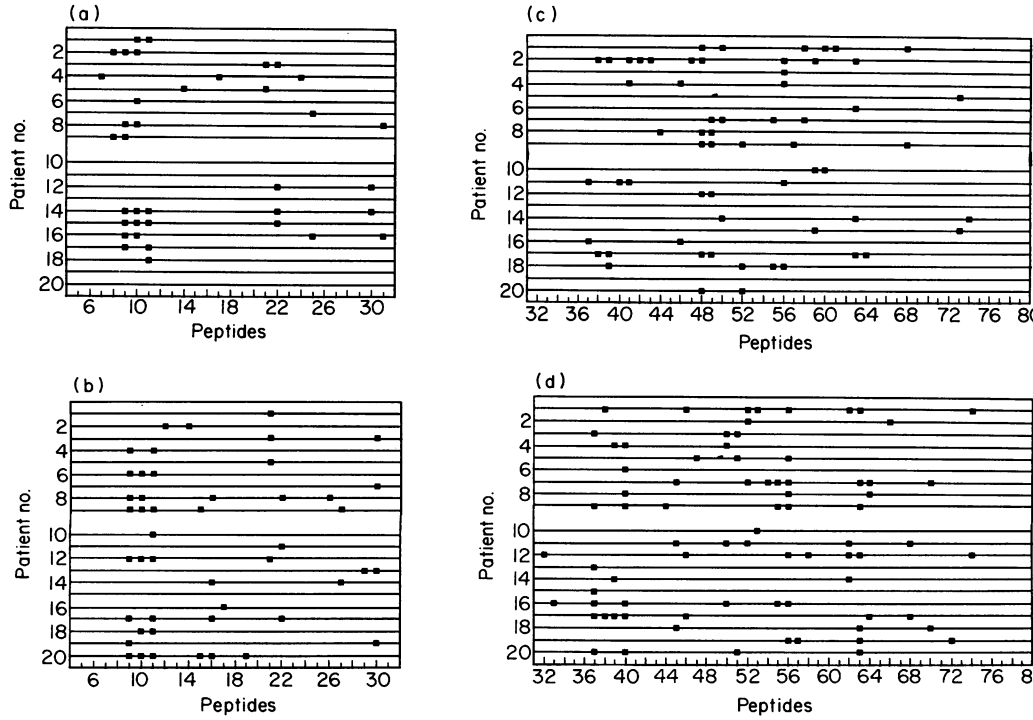

Fig. 3. IgG subclass 1 (a) and 3 (b) reactivity in individuals with stable PGL (1–9), and OI (10–20), to peptides representing p17 (a, b) and p24 (c, d) at end point of study.

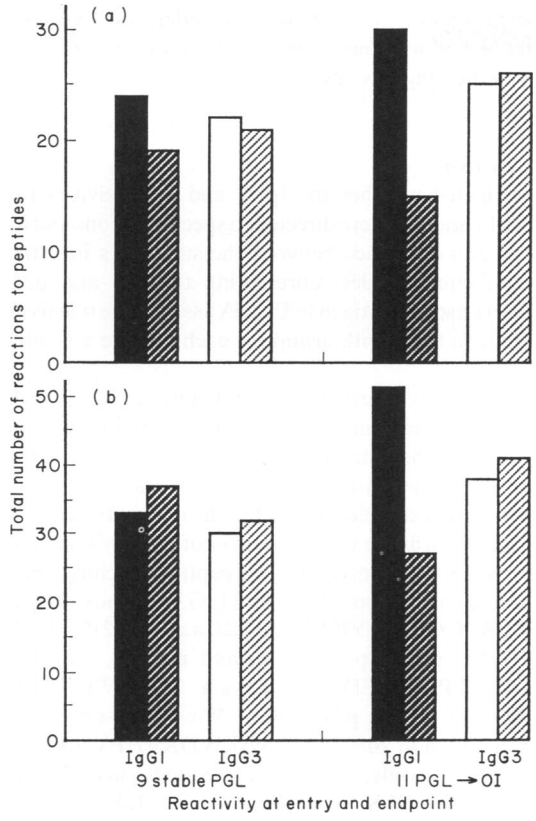

Fig. 4. Total added reactivity to p17 (a) and p24 (b) peptides in the stable PGL and PGL-OI groups at entry and end point of the study.

common sequence of these three peptides was GKPEP. In the aminoterminal part of p17 (peptides nos. 9–11, a.a. 13–37) one well defined region was found that elicited high reactivity of both IgG1 and 3. Seventy-five per cent of the patients showed IgG1 and/or IgG3 response to at least one of the peptides. The sequence represented in all three peptides was PGGKK. There was no particular difference between the response of IgG1 and IgG3 to this region, except that this was the only region where the accumulated absorbance values were twice as high for IgG1 compared with IgG3. No such difference was seen anywhere else on p17 or p24.

There was no obvious single peptide to which reactivity appeared or disappeared with progression. The over-all reactivity of patients in the PGL and OI groups to core proteins was compared by calculating the total numbers of positive peptide reactions to all peptides representing p17 (27 peptides) and p24 (45 peptides). A decreased divergence of antibody reactivity to both p17 and p24 peptides in the group of patients who developed OI was seen. This was most obvious with IgG1, while IgG3 reamined almost at the same level.

DISCUSSION

The main humoral anti-HIV responses are in the IgG subclasses 1 and 3, previously demonstrated by Sundqvist *et al.* (1986) and Mergener *et al.* (1987). IgG1 has been shown to be the dominant anti-HIV IgG subclass, and was found here in high titres in all patient samples. IgG3 was detected by ELISA in six out of 20 patients on one or more occasions. With peptide ELISA IgG3 antibodies directed to gag peptides were found in all 20 patients. This result was also confirmed by Western blot where reactivity to core proteins was seen in 20/20 patients. The discrepancy between ELISA results on one hand and peptide and Western blot assays on the other, might be explained by the fact that IgG3 was mainly directed to core proteins, p17, p24 and their precursor p55. Those antigens may be underrepresentated in the Organon Elisa kits, which are coated with virus lysates. Alternatively, epitopes not accessible on the native antigen may be exposed by a peptide, and reactive regions of p17 and p24 are made available by the SDS treatment of antigens preceding Western blotting.

The use of small peptides as antigens permitted a more thorough analysis of these phenomena. The IgG1 response was strongly focused to a region (peptides 9–11) on the p17 sequence. This region is rich in charged a.a. (lysine and arginine) and also contains proline as a potential structure-forming element. In other studies this region has given frequencies of seroreactivity up to 80–90% (own unpublished observations). A site with IgG1 but not IgG3 reactivity was found on the p24 peptide 48, while the reverse was true for peptides 37 and 40 on p24. More experience is needed to evaluate whether these sites are truly subclass-specific.

It has been suggested by Lange *et al.* (1986) and Weber *et al.* (1987) that anti-p24 antibodies are significantly associated with lack of disease progression. They found no association between anti-gp41 and anti-gp120 titres and clinical outcome. McDougal *et al.* (1987) found that loss of titres to p24 was a part of a generalized loss of titre to viral proteins except for the preservation of anti-gp41. The decline of anti-p24 titres could be an effect of defective B cell function, excesssive viral antigen production forming immune complexes or a combination of these factors (Goudsmit *et al.*, 1987). We found that IgG3 directed to p17 and p24 could be detected in AIDS patients after IgG1 anti-gag response was lost. This was seen in Western blot for IgG subclasses or ELISA assays using short synthetic peptides representing these regions as antigens. IgG3 in these patients occurred in small amounts and might not have been detected by standard total IgG assays. Klasse & Blomberg (1987) have recently shown a high relative antibody reactivity to p17 in IgG3 and an association between antibodies to p17 and asymptomatic stage of disease. Our results on IgG3 agree with this. The disease progression in our study was mainly associated with decrease of IgG1 to both p17 and p24 peptides, while IgG3 reactivity did not decrease. The reason why IgG3 does not follow the major IgG1 decrease of antibodies is not known. An explanation may be that the clearance of complexes mainly consisting of IgG1 is more efficient, depending on higher affinity of IgG1 than IgG3 to p24, or that IgG1 forms larger complexes more easily cleared. It is also not known why IgG3 is mainly directed to gag proteins. Low IgG3 reactivity could be seen to a few restricted peptides representing gp120 and gp41 regions by using peptide ELISAs (T. Mathiesen, personal communication and own unpublished observations). The divergence of reactivity to the gag peptides of both IgG1 and 3 was considerable, especially concerning the peptides representing p24. Altogether the peptide ELISAs seemed to be more sensitive in detecting anti-gag antibodies than either ELISA or Western blot. This was true, especially for IgG1 against p17 where 75% (15/20) of the patients responded in peptide ELISA, compared to 45% (9/20) with Western blot.

We have seen that HIV-specific IgG3 is mainly directed to core proteins and occurs more frequently than expected earlier in all stages of HIV-1 infection. This may indicate reactivations of HIV in the patients (Linde, 1987). Recently we have shown (Ljunggren *et al.*, 1988) that HIV-specific antibody-dependent cellular cytotoxicity (ADCC) (Ljunggren *et al.*, 1987) is mediated by IgG1, and that no effect was seen by IgG3. In other viral diseases IgG3 shows a high neutralizing activity (Mathiesen *et al.*, 1988; Gilljam & Wahren, 1989) and is directed to envelope proteins. The progressivity of HIV disease may indicate that IgG3 to envelope should be important for neutralization here too.

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