HIV-Specific Antibody Responses in HIV-Infected Patients: From a Monoclonal to a Polyclonal View

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Key Words

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Abstract

HIV infections represent a major global health threat, affecting more than 35 million individuals worldwide. High infection rates and problems associated with lifelong antiretroviral treatment emphasize the need for the development of prophylactic and therapeutic immune intervention strategies. It is conceivable that insights for the design of new immunogens capable of eliciting protective immune responses may come from the analysis of HIV-specific antibody responses in infected patients. Using sophisticated technologies, several monoclonal neutralizing antibodies were isolated from HIV-infected individuals. However, the majority of polyclonal antibody responses found in infected patients are nonneutralizing. Comprehensive analyses of the molecular targets of HIV-specific antibody responses identified that during natural infection antibodies are mainly misdirected towards gp120 epitopes outside of the CD4-binding site and against regions and proteins that are not exposed on the surface of the virus. We therefore argue that vaccines aiming to induce protective responses should include engineered immunogens, which are capable of focusing the immune response towards protective epitopes.

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Introduction

The analysis of HIV-specific antibody responses started with the discovery of the virus and advanced through several technological developments [1–3]. Figure 1 summarizes research findings made through a variety of techniques in the form of a time line. Studies providing insights into the structure of the envelope (Env) spikes also contributed to the elucidation of HIV-specific antibody responses (fig. 1). The Env spikes represent the HIV surface antigens and are constituted by three pairs of gp120 and gp41 proteins [4]. The resolution of the structure of the complete trimer was hindered mainly by the fact that the protein is highly glycosylated and carries variable regions that are structurally unstable [5–7]. Solving the structure of the deglycosylated gp120 core (i.e. deleted of variable regions) and of gp120 variants bound to human monoclonal antibodies resulted in the identification of antibody-binding sites and classification of Env epitopes [6–10]. The subsequent development of cryo-electron microscopy tomography for the first time enabled the visualization of Env spikes in their 3D form on the surface of viral particles [11, 12]. In parallel, techniques for the isolation of human monoclonal antibodies progressed and expanded the panel of available broadly neutralizing antibodies (bNAb) [13–16]. With the conclusion of the first HIV vaccine trial showing some efficacy, many stud-

Fig. 1. Time-line showing advances in the study of HIV-specific antibody responses. Selected advances regarding clinical, technical and research findings are displayed. Ab = Antibody; HAART = highly active antiretroviral treatment; Hu-M NAb = human monoclonal neutralizing antibody; EBV = Epstein-Barr virus; WB = Western blot.

Fig. 2. Characteristics of HIV-specific antibody responses. Ab = Antibody; ADCC = antibody-dependent cellular cytotoxicity; ADCDC = antibody-dependent complement-dependent cytotoxicity; ADCP = antibody-dependent cellular phagocytosis; ADNKA = antibody-dependent NK cell activation; IC = inhibitory concentration; T_0 = initial time point; T_{ref} = reference time point; VL = viral load; $\downarrow$ = decrease; $\uparrow$ = increase.

ies focused on the elucidation of the immunological mechanisms associated with the observed effect [17–19]. The finding that nonneutralizing antibodies had a protective role in vaccinated individuals also redirected research efforts towards a better understanding of antibody effector functions that do not involve direct HIV neutralization [18].

HIV-specific antibody responses are studied according to several features of the antibodies, such as function, isotype, specificity and composition (fig. 2). Useful resources are also offered by online databases containing information regarding HIV sequences and features of human monoclonal antibodies (fig. 3). Among the properties that are used to characterize antibody responses, this review focuses on the specificity and isotype/IgG subclass of HIV-specific antibodies.

The specificity of antibody response can be studied at the proteome level (i.e. which HIV proteins elicit antibody responses?) and at the epitope level (i.e. which sites of a protein are bound by the antibody?). The HIV proteome is constituted of several proteins, including Env,

Fig. 3. Online resources for the study of HIV-specific antibody responses. Ab = Antibody; bNAb = broadly neutralizing antibody; IC = inhibitory concentration; NAb = neutralizing antibody.

HIV Sequence Database: <http://www.hiv.lanl.gov/>

- HIV sequence search per clade, virus isolate, protein, etc.

HIV Molecular Immunology Database: <http://www.hiv.lanl.gov/content/immunology/>

- Ab search per name of protein, epitope, isotype, etc.
- Tables of NAb and epitopes
- Standardized protocols for the study of NAb

Broadly Neutralizing Antibodies Electronic Resource: <http://bnaber.org/>

- bNAb search per name, IC₅₀, IC₈₀ breadth, depth
- bNAb sequence characteristics (e.g. somatic mutations, putative genes)
- Links to 3D structures per bNAb

Electron Microscopy Data Bank: http://www.ebi.ac.uk/pdbe/emdb/searchResults.html?q=hiv*

- EM images of HIV particles, HIV proteins, HIV Env in complex with Abs
- EM search per name, author, resolution, etc.

structural, functional and accessory proteins [20]. In mature HIV particles, Env proteins protrude from the lipidic Env membrane, matrix proteins (p17) are associated with the inner lipid layer and capsid proteins (p24) form a conical shell enclosing the viral RNA, RNA-associated nucleocapsid multimers (p7) and other viral components [21]. Functional proteins encoded by the *pol* gene are mainly responsible for reverse transcription (reverse transcriptase + RNase, p66), integration (integrase, p31) and maturation (protease, PR). Accessory proteins are involved in protein expression (Rev and Tat), immunopathogenesis and immune evasion (Nef, Vif, Vpr, Vpu) [20].

Analyzing the type and targets of HIV-specific antibody responses may therefore help to understand why the majority of HIV-infected patients are unable to control infection despite the presence of high antibody levels. Indeed, it was recently described that HIV-1 controllers had increased antibody effector functions that were associated with certain specificities and subclass profiles [22]. The development of multiplex immunoassays, such as antigen microarrays, is currently opening the field for a broad and systematic subclass and specificity profiling that could be useful for the identification of antibody signatures associated with certain clinical or biological outcomes [23–27]. This review will summarize the technologies used to study the isotype and specificity of HIV-specific antibody responses, the time course of antibody responses arising during early infection, antibody responses found in diverse body fluids, and the molecular targets and isotype/subclass distribution of Env- and non-Env-specific antibodies found in HIV-infected patients.

Analysis of Isotypes, Functions and Specificities of HIV-Specific Antibody Responses

The specificities, functions and isotypes/subclasses of antibody responses elicited during HIV infection can be studied by several methods (table 1). In fact, besides techniques that can be applied for the study of antibody responses in terms of simple binding to the antigen/epitope, there are also techniques that allow studying certain functions of the antibodies, such as neutralization, complement activation and antibody-dependent cellular cytotoxicity [14, 28–34]. In addition, several techniques were developed to generate monoclonal antibodies and to study immune complexes (e.g. virion-bound antibodies) as well as antibody-secreting cells [14, 35–44]. For example, the neutralizing activity of antibodies and their specificity can be assessed by measuring the neutralization of different Env-pseudotyped viruses, i.e. of viruses expressing different Env proteins, including those of different HIV strains and those mutated at potential antibody-binding sites [14, 35]. This technique has the advantage of testing the antibody's neutralization effect and its binding towards the Env protein as a 'natural' molecule embedded in a viral membrane.

To analyze the specificity of antibodies, which may exert other effector functions, methods such as ELISA, immunoblotting, customized Luminex binding assays and microarrays are currently used (table 1) [27, 42, 45–48]. These assays are based on the recognition of Fc portions of antibodies present in human samples by labelled secondary antibodies (e.g. anti-human IgG). They therefore allow determinations of antibody isotypes and IgG subclasses specific for the antigens used, which can be pro-

Table 1. Methods used to study type, specificity and function of HIV antibody responses

	Method to study Ab type and specificity	Isotype/IgG-subclass	Specificity		Ref.
			proteins	peptides ¹	
Study of Ab responses	Western blotting, immunoblotting	IgG, IgG ₁₋₄ , IgM	√	√√	[47, 51, 145, 153, 156]
	ELISA	IgG, IgG ₁₋₄ , IgA, IgM	√	√√	[42, 45, 46, 48]
	Customized Luminex binding assay	IgG, IgA, IgM	√	√	[42, 49]
	Customized Luminex subclass assay	IgG, IgG ₁₋₄	√	–	[50]
	Peptide array	IgG	–	√√	[54]
	HIV microarray	IgG, IgG ₁₋₄ , IgA, IgM, IgE	√	√√	[27]
Study of neutralizing Abs	Neutralization assays with Env-pseudotyped viruses	–	Env-pseudotyped viruses		[14, 35]
Structural studies	Crystallization of monoclonal antibody in complex with antigen	–	√	√	[39, 40]
	Epitope excision/extraction and MALDI-TOF	–	Protein bound to Ab		[41]
Study of virion-bound Abs	Virus capture assay with a-huIgG or a-huIgM	IgG, IgM	–	–	[42]
	Depletion of specific Abs with Ag-beads and virus capture	–	√	–	[43]
Study of Ab-secreting B cells and secreted Abs	Integrated single-cell analysis and microengraving	IgG ₁ , IgG ₃ , IgA _{1/2} , IgM	√	√	[44]
Method to study Ab function					Ref.
Virus neutralization	Neutralization assay (reduction in luciferase reporter gene expression after single-round infection of reporter cell lines)				[35]
Virus capture	Virus capture assay; infectious virus capture assay				[28, 29, 42, 43]
Ab-dependent complement-dependent cytotoxicity	Calcein release-based antibody-dependent complement-mediated lysis assay Complement-dependent microtoxicity assay				[28–30]
ADCC	ADCC-GranToxiLux assay; rapid fluorometric ADCC assay				[31, 32]
Ab-dependent NK cell activation	Intracellular cytokine staining; NK cell degranulation				[33]
Ab-dependent cellular phagocytosis	THP-1 phagocytosis assay				[34]

Ab = Antibody; ADCC = antibody-dependent cellular cytotoxicity; Ag = antigen; Env = envelope; NK = natural killer.

¹ Performed with single proteins and peptides (√) or overlapping peptide panels (√√).

teins, peptides and carbohydrates [42, 48–50]. Mapping the position of linear epitopes on the amino acid sequence of proteins is achieved with panels of synthetic overlapping peptides [46, 48, 51–53]. However, testing the specificity of antibodies against a large number of peptides and proteins with assays such as ELISA is time-consuming and requires large amounts of the samples. This has limited epitope mapping studies and testing for multiple antigens in the past. The development of multiplex technologies such as microarrays has recently overcome these limitations. In fact, an array containing overlapping 15-mers derived from Env proteins of different HIV subtypes was used to map Env-specific IgG responses elicited during vaccine trials and during infection [54]. Because of the in-

clusion of peptides from different HIV subtypes, this array can be used to gain information about interclade cross-reactivity patterns. Furthermore, we developed an HIV microarray containing recombinant proteins of the HIV-1 clade C proteome and overlapping peptides (≥25 amino acids) covering the amino acid sequences of gp120 and gp41 [27]. This array has the advantage that it contains both proteins and peptides, allowing the measurement of antibody levels specific for both conformation-dependent and linear epitopes on one chip. In addition to the detection of human IgG, pilot experiments show that determination of IgG subclasses and isotypes is also possible with this chip [27].

Table 2. HIV-specific antibody responses in early infection

		Type	Specificity (in order of prevalence) ¹ [Ref.]	Appearance (days post-T ₀) ² [Ref.]	Half-life estimate [Ref.]
Plasma: virion-bound Abs	IgG IgM IgA	gp41 > gp120 [42] gp41 > gp120 [42] n.d.	8 d [42]
Plasma: free Abs	IgG IgM IgA	gp41, gp120, p55, p24, p17, p66, p31 > Tat, Nef [49] gp41, gp120, p66 > Nef [49] gp41, gp120, p55, p66, Nef > p31, Tat [49]	gp41, p55, p66, gp120, p17, p31: 13–53 d [42], 21–84 d [55] gp41: 13.5 d, p55: 25.5 d [49]	gp41: 48 d [49]
Genital lavage: free Abs	IgG IgM IgA	gp41, p55, p66, p31 > gp120, Nef [49] gp41 > p55, p66, Tat, Nef, gp120, p31 [49] gp41, p55 > p66, gp120, Tat, p31 [49]		gp41: 2.7 d [49]

Ab = Antibody; d = days; n.d. = not done.

¹ (HIV antigens with prevalence ≥50%) > (HIV antigens with prevalence <50%).

² Approximate time of appearance from T₀ (first day the plasma viral load ≥100 copies/ml).

Development of HIV-Specific Antibodies upon Infection: Time Course of Early Responses and Occurrence in Body Fluids

Studies based on ELISA, multiplex binding assays and virus capture assays with anti-human antibodies analyzed the development of initial antibody responses during acute HIV infection (table 2). The first antibodies were detected in plasma as virion-bound antibodies, 8 days after the plasma viral load was ≥100 copies/ml. Immune complexes contained IgG and IgM antibodies specific for gp41 and gp120 [42]. Free HIV-specific IgG was first detected in plasma at around 13–53 days and 21–84 days with the sequential appearance of gp41-, p55-, p66-, gp120-, p17- and p31-specific antibodies [42, 55]. The detection of free HIV-specific IgG and IgM in the serum of HIV-infected individuals (i.e. seropositivity) is used as a marker for infection in current HIV-diagnostic tests (i.e. ELISA- and Western blot-based tests) [56, 57]. A recent study investigating early HIV-specific antibody responses in genital lavage samples pointed out that IgG, IgM and IgA develop early upon infection and are directed towards Env, structural and functional proteins with a predominance of gp41-specific antibodies [49].

In contrast to the rapid development of HIV-specific antibodies, the first neutralization activity was shown for antibodies detected 1–2 and 3 months postinfection in HIV clade B and clade C infections, respectively [55, 58, 59]. These antibodies neutralized autologous viruses and could induce viral escape mutants [58]. bNAb that also

inhibit the infection of heterologous strains are developed by some HIV-infected patients at a later stage of infection [60, 61]. A recent study showed that bNAb were also found in HIV-infected infants at 11–28 months after infection [62]. The time course of the earliest antibody-mediated immune responses in respect to innate and T cell-mediated responses has been reviewed elsewhere [56].

Most of the information regarding HIV-specific antibodies derives from studies focusing on free antibodies found in plasma or serum samples. Some analyses, however, demonstrated that antibodies directed towards several HIV proteins and of different isotypes and IgG subclasses can also be found in body fluids, such as cerebrospinal fluids, saliva and genital secretions (table 3) [49, 63–71]. Further studies are still necessary to clarify to what extent antibodies found at these sites represent a transudate from the blood or are locally produced, and to elucidate their protective role during HIV infections [49, 63, 64, 66, 70–77].

Env Spikes as Targets for HIV-Specific Antibodies

Env proteins certainly represent major molecular targets of HIV-specific antibodies and are of importance for virus neutralization. Images obtained by cryo-electron tomography showed how gp120/gp41 trimers protrude from the viral membrane in the form of spikes (~4–35 spikes per HIV-1 virion), constituting HIV's surface antigens [11, 78]. In the study of Env-specific antibody responses, epitopes have been classified according to their

Table 3. HIV-specific antibody responses in body fluids

	Type	Specificity (in order of prevalence) [Ref.]	Trans./local production [Ref.]	Effector function
Cerebrospinal fluid	IgG	Env, Gag, Pol [63, 64]	Local [63, 64]
		IgG ₁	gp41-pep [65]	
		?		ADCC in HIV+ with CNS impairment [74] No neutralization [74, 75]
		?	Tat [66]	? Neuroprotective [66]
	Saliva	IgG	gp120, Pol [67]	Trans., ? local [72, 73]
		IgA	gp160, gp41, p24 [67–69]	
	Genital secretions	IgG	gp41 > p55, p66 [49]; gp160, p24 [70]	Trans. and local [49]
		IgG ₁ , IgG ₃	gp120 [71]	ADCC [76] Transcytosis of cell-associated virus [70]
		IgM	gp41 > p55, p66 [49]; gp160, p24 [70]	? Neutralization [77]
		IgA	gp41 > p55, p66 [49]; gp160, p24 [70]	Trans. and local [49]

ADCC = Antibody-dependent cellular cytotoxicity; CNS = central nervous system; Trans. = transudation.

Fig. 4. Types of epitopes described for Env-specific antibodies. Classification of epitope types according to the epitope's biochemistry (amino acid residues, glycans), position of residues/glycans constituting the antibody-binding site (linear, discontinuous) and requirements of protein folding or oligomerization (conformational, quaternary). Adapted from Zolla-Pazner and Cardozo [79].

different properties, such as the type and position of the molecules involved in the binding site and the requirement of the intact protein's structure for binding (fig. 4). For example, linear epitopes represent continuous stretches of amino acids and must be distinguished from discontinuous epitopes, which are constituted by portions that are distant in the protein sequence but brought together when the protein is folded. When an epitope is dependent on the structural conformation of the antibody-binding site, it is termed conformational epitope [79]. The structural requirements for antibody

binding can even regard not only the protein itself, but also depend on its oligomerization status, such as for quaternary epitopes on Env trimers [80].

Env Epitopes from a Monoclonal View

The epitopes of Env-specific antibodies have been characterized very well for human monoclonal antibodies isolated/cloned from HIV-infected individuals. Crystallization of antibody-antigen complexes enabled identifying single amino acids and glycans involved in the interaction [8–10, 81, 82]. On the other hand, neutralization

Table 4. Targets of Env-specific monoclonal antibodies

Epitope type			Epitope – involved Env domains and features ¹ [Ref.]	Epitope – immune escape strategies [Ref.]	Hu-M Abs – examples with effector function ²	Hu-M Abs – features [Ref.]
gp120 CD4-binding site	D	C R	C2, C3, C4, V5, C5 [10], target of the majority of bNAbs	Conformational masking by V1V2 [96, 97] Glycosylation [97]	b12 ^{bN,A} , CH103 ^{bN} , VRC01 ^{bN,A} , 8ANC131 ^{bN} , HJ16 ^{bN} , 3BCN60 ^{bN} , 15e ^{N,A} , F105 ^{N,A} , 448-D ^{N,A} , 1125H ^{N,A}	Long CDR H3 [101] CD4 mimicry by VH1–2 [163], short CDR L3 loops [103] CD4 mimicry by VH1–46 [164] Extensive somatic mutations [104] Enhanced flexibility [104]
gp120 CD4-induced (coreceptor binding site)	D	C R	V1–V2 (β1 β2), V3, C4 (β20 β21) [6]	Conformational masking (exposed upon gp120-CD4 binding) [6] Steric hindrance [94] V2 sulfated Tyr (173, 177) [165]	17b ^{wN,A} , 48D ^{wN,A} , 412d ^N , N12-i3 ^A , N12-il5 ^A	CCR5 mimicry by extended CDR H3 with sulfated Tyr or multiple Asp/Glu(–) [9] Multiple V _H replacement [166]
gp120 V3 loop	L	C R	306–319 [85, 86], coreceptor binding, ‘structurally conserved’ [40]	Mutation, clustered into regions [40] Conformational masking by V1V2 [97] Glycosylation [97]	447/52-D ^{N,A} , 19b ^{wN} , 694/98D ^{N,A} , 2182 ^N , 4117C ^{N,A} , 41148D ^{N,A}	Preferential VH5–51 gene usage [167]
gp120 V1V2, V3 crown and glycans	D	C Q RG	V1V2, V3 crown, V2-glyc (N160) [82]	Mutation Glycan type [100] Glu(–) 168–171 [168]	PG9 ^{bN,A} , PG16 ^N , 2909 ^N , CH01-04 ^{bN} , PGT141–145 ^{bN}	Long CDR H3, often sulfated Tyr [80, 82]
gp120 V3 and glycans	D	C RG	a) V3, V3-glyc: N301, N332 [81] b) V3, V1V2-glyc: N137, V3-glyc: N332, N301 [89] c) V3, V4, V3-glyc: N332, V4-glyc: N392, N386 [39]	Mutation [98]	a) PGT128 ^{bN} b) PGT121 ^{bN} , PGT122 ^{bN} , 10-074 ^{bN} c) PGT135 ^{bN}	Penetration of glycan shield by extended CDR H3, CDR H2 insertion [81] Multiple V _H replacement [166]
gp120 inner domain and glycans	D	C ? RG	Inner domain, C2, C3, V5, C2-glyc: N234, N276, ? gp41-residues and gp41-glyc: N637 ³ [90]	n.a.	8ANC195 ^{bN}	Heavy chain-only contact to gp120 [90] Light chain contact to gp41 ³ [90]
gp120 C5 region	L	C R	a) 491–500 [169] b) 498–504 [170] c) I489, E509 [41]	n.a.	a) 42F ^A , 43F ^A b) 670-D ^A c) 1331A ^{n.a.}	n.a.
gp120 glycan only	D	C G	C2–,C3–,V4-glyc: N295, N332, N339, N392 [8]	Mutation [99]	2G12 ^{bN,A}	Domain-swapped Fabs [8]
gp41 cluster I	L	R	579–604 [171, 172]	n.a.	246-D ^{A,E} , 4B3 ^A , 98-43 ^A , F240 ^E	n.a.
gp41 cluster II	L	C R	a) 644–663 [87] b) n.a. [87]	Conformational masking (exposed upon fusion) [88]	a) 120–16 ^A b) 98–6 ^A , 126–50 ^A	n.a.
gp41 MPER	L	C R	662–683 [122, 123, 173–175], conserved region with structural plasticity [176]	Transient exposure [177] Steric hindrance [95] Host mimicry [178] Hydrophobicity [179]	4E10 ^{bN,A} , 2F5 ^{bN,A} , Z13 ^{bN} , 10E8 ^{bN} , m66.6 ^{bN}	Binding to phospholipids [180], possible contact through CDR H3 Arg(+) [181] Long CDR H3 [102] Binding to auto-Ag [178, 179]

(–) = Negatively charged; (+) = positively charged; Ag = antigen; Hu-M Abs = human monoclonal antibodies; C = continuous epitope; D = discontinuous epitope; G = glycan epitope; glyc = glycans; L = linear epitope; N = asparagine; n.a. = not available; Q = quaternary epitope (exclusive or preferential binding to trimer); R = amino acid residues; V_H = variable heavy Ig domain.

¹ Domains as in figure 5 and amino acid residues numbered according to the HXB2 numbering scheme (www.hiv.lanl.gov).

² A = Antibody-dependent cellular cytotoxicity; E = enhancement of infection; N = neutralization; bN = broad neutralization; wN = weak neutralization; as referred in the www.hiv.lanl.gov immunology database.

³ Suggested by 3D modeling.

Table 5. gp120 peptides recognized by polyclonal antibodies of HIV-infected patients

Do-main	Peptide position ¹	Analyzed HIV-1 clade	Peptide amino acid sequence	Ab type	Cohort features and prevalences of recognition	Ref.
C1	101–112	B	VEQMHEDIISLW	IgG	RP (n = 13)	[109]
	104–121	A, B, C, D	MHEDIISLWDQSLKPCVK2	IgG	C > A, B (n = 169)	[54]
C1/V1	120–131	A, B, C, D	VKLTPLCVTLNC	IgG	C > A, B (n = 169)	[54]
	123–157	C	TPLCVTLNCNTTSHNNSSPSPMTNC	IgG, IgG ₁	Af 13% (n = 15) Eu 33% (n = 15)	[48]
V2	173–197	C	NALFYRSDIVPLEKNSSEYILINC	IgG > IgG ₁	Af 73% (n = 15) Eu 67% (n = 15)	[48]
C2	193–217	C	LINCNTSTITQACPKVSFDPIPIHY	IgG, IgG _{1,3,4}	Af 20% (n = 15) Eu 40% (n = 15)	[48]
V3	293–320	C	AIVCTRPNNTRKSIRI--GPGQVIFYT	IgG, IgG ₁₋₄ , IgA, IgM	Af 93% (n = 15) Eu 100% (n = 15)	[48]
	301–322	A, B, C, D	NNTRKSIRI--GPGQTFYAIGD2	IgG	n.a. high % (n = 169)	[54]
	303–312	B	TRKSIRIQRGPG	IgG	RP (n = 13) > SP (n = 13)	[109]
	304–318	B	RKSIRIQRGPGRAFV	IgG ₁	90% (n = 10)	[51]
V3/C3	324–338	B	GNMRQAHCNISRAKW	IgG ₁	50% (n = 10)	[51]
	315–339	C	QVFYTNEIIGNIRQAHCNISRELWN	IgG	Af 100% (n = 15) Eu 100% (n = 15)	[27]
C3	343–354	B	KQIASKLREQFG	IgG	SP (n = 13) > RP (n = 13)	[109]
	355–366	B	NNKTIIFKQSSG	IgG	RP (n = 13) > SP (n = 13)	[109]
V4	396–428	C	SNASDANNNTITLPCKIKQIINMWQ	IgG, IgG ₁	Af 20% (n = 15) Eu 20% (n = 15) Af IgG ₁ 67% (n = 15)	[48]
	415–426	B	TLPCRICKQFINM	IgG	SP (n = 13)	[109]
C5	479–511	C	WRSELYKYKVVEIKPLGIAPTKAKRRVVEREKR	IgG, IgG ₁₋₄ , IgA	Af 67% (n = 15) Eu 73% (n = 15)	[48]
	489–503	B	KIEPLGVAPTKAKR	IgG ₁	90% (n = 10)	[51]
	499–510	B	TKAKRRVVQREK	IgG	SP (n = 13) > RP (n = 13)	[109]
	503–514	B	RRVVQREKRAVG	IgG	SP (n = 13) > RP (n = 13) RRVVQREKRAVG < TKAKRRVVQREK	[109]
	497–511	B	APTKAKRRVVNREKR	Ig-IC	45% (n = 42)	[110]
	496–521	B	VAPTKAKRRVVQREKRAVGIGALFLG	Total Ig	Af 53% (n = 159) USA 39% (n = 103)	[111]
	504–516	A, B, C, D	RVVEREKRAVGIG2	IgG	n.a. high % (n = 169)	[54]

A = HIV-1 clade A; B = HIV-1 clade B; C = HIV-1 clade C; D = HIV-1 clade D; Ab = antibody; Af = African; Eu = European; Ig = immunoglobulin; Ig-IC = immunoglobulin immune complex; n = number of subjects; n.a. = not available; RP = rapid progressors; SP = slow progressors; > = higher prevalence of recognition than comparison group; < = lower prevalence of recognition than comparison group.

¹ HXB2 numbering; www.hiv.lanl.gov. ² Reactivity was tested towards peptides from different strains, but only the clade C sequence is shown here.

assays with Env-pseudotyped viruses were useful for the definition of quaternary epitopes [15, 83, 84].

Table 4 summarizes Env epitopes, their type and molecular features (e.g. gp120 and gp41 domains involved) and examples of human monoclonal antibodies targeting these epitopes. Some Env-specific antibodies recognize

protein regions involved in receptor and coreceptor binding (e.g. CD4-binding site, CD4-induced epitope and V3 loop) [6, 10, 40, 85, 86]. Some bind regions that are exposed after conformational changes necessary for fusion of the viral membrane with the target-cell membrane (e.g. gp41 cluster II) [87, 88]. Other Env-specific antibodies

bind conformational epitopes constituted by Env domains and attached glycans (e.g. V1V2, V3 crown and glycans) or by glycans alone (e.g. gp120 glycan-only) [8, 39, 81, 82, 89, 90]. When modeled on the cryo-electron tomography pictures of the Env trimer, the epitopes represent mainly independent surface patches [91–93].

Great attention has been dedicated to the elucidation of the structural determinants that permit the virus to escape antibody binding (i.e. features of the Env protein) and the ones that permit the antibody to make contacts to a highly glycosylated protein able to adopt several conformations (i.e. unique features of the antibody). Immune escape can be mediated for example by steric hindrance, masked by variable loops, mutation and glycosylation shielding amino acid residues [40, 55, 94–100]. On the other hand, several unique features have been shown to be essential for antibody neutralization. Some of these regard the antibody's complementarity-determining regions (CDRs), which are usually engaged in antigen contact. For example, long CDR H3 can mediate recognition of the CD4-binding site or of the coreceptor-binding site and penetration of the glycan shield or contact to the hydrophobic MPER (membrane proximal external region) epitope [9, 80–82, 101, 102]. Short CDR L3 loops can mediate CD4 mimicry and CDR H2 can also be involved in the penetration of the glycan shield [81, 103]. Unique mechanisms, such as heavy chain-only contact or domain-swapped Fabs (antigen-binding fragments), have been shown to characterize bNAb that bind to the gp120 inner domain and to the gp120 glycan-only epitope, respectively [8, 90]. Extensive somatic mutations seem to play a major role in the acquirement of these unique features and of high antigen affinity [104]. Details of the escape and binding mechanisms have been reviewed elsewhere in relation to the structure of the epitopes [92].

However, monoclonal antibodies only represent single antibodies occurring in a polyclonal antibody response and, therefore, do not reflect the polyclonal antibody response in HIV-infected patients. An elegant study by Kwong and colleagues [105] showed that for sera with broadly neutralizing activity the Env specificities could be mapped using clusters of HIV-1 isolate-neutralization patterns identified for human monoclonal antibodies, but bNAb are very rare during natural HIV infection (e.g. 1.7–3.7% [106], 4–11% [107], 6% [108]).

gp120 and gp41 Linear Epitopes Recognized by the Polyclonal Antibodies of Patients

Despite the high glycosylation rate of the Env, HIV-infected patients mount strong polyclonal antibody re-

sponses against linear epitopes constituted by Env peptides. Most of the information regarding linear epitopes derives from early reports using HIV subtype B synthetic peptides to define the specificity of IgG responses in HIV-infected patients [51, 109–116]. Recently, Gottardo et al. [54] used a panel of short peptides from different clades to map the binding sites of IgG antibodies. We obtained similar results with a peptide array of 25-mer peptides from HIV-1 clade C [27, 48].

Tables 5 and 6 summarize the results obtained from gp120 and gp41 peptide-based mapping studies for which information regarding peptide amino acid sequences, cohorts and antibody prevalence were available [27, 48, 51, 54, 109–116]. The amino acid sequences common to peptides recognized by specific antibodies are listed in tables 5 and 6, and are displayed in relation to gp120 and gp41 domains in figure 5. The gp120 peptides that were most frequently recognized shared the following sequences: 304-RKSIRIxGPG-314, 325-GNxRQAHCNISRxW-338 and 489-(E/K)I(K/E)PLGxAPTKAKR-503. They were located in the V3 loop, a region downstream of V3, and in the C5 region, respectively. Importantly, these major epitopes were located outside the gp120 CD4-binding site. For gp41, frequently recognized sequences were: 562-GIKQLQARV-580, 583-(V/I)ERYLKDQQL-592 and 593-LG(I/L)WGCSGKLIC-604. These sequences cluster in the region 562–604, which starts within the first heptad repeat involved in the membrane fusion process [117–119]. It is interesting that HIV-specific antibodies also target the gp41 C-terminal region [i.e. 843-IPRRIRQG(F/L)E-852], which is part of the gp41 cytoplasmic domain [120, 121].

Figure 5 also shows the position of linear epitopes targeted by human monoclonal antibodies. Linear epitopes recognized by monoclonal antibodies often overlap with the major targets of the polyclonal responses. However, a difference is found in the gp41 MPER: polyclonal peptide-specific responses cluster to the N-terminal part of the domain (which is also recognized by human monoclonal antibody 2F5), but they do not recognize the more membrane-proximal region bound by neutralizing monoclonal antibodies Z13 and 4E10 (fig. 5; table 6) [48, 54, 109, 122, 123].

IgG Subclass Distribution of Env-Specific Antibodies during HIV Infection

Relatively few studies have investigated the IgG subclass and isotype distribution of antibody responses in HIV-infected patients. Env-specific antibodies were found to be dominated by strong IgG₁ responses and in

Table 6. gp41 peptides recognized by polyclonal antibodies of HIV-infected patients

Do-main	Peptide position ¹	Analyzed HIV-1 clade	Peptide amino acid sequence	Ab type	Cohort features, prevalences of recognition	Ref.
HR-1	536–548	A, B, C, D	TLTVQARQLLSGI	IgG	n.a. high % (n = 169)	[54]
	566–580	A, B, C, D	LQLTVWGKQLQARV ²	IgG	n.a. high % (n = 169)	[54]
	572–596	C	GKQLQARVLAIERYLKDQQLLGLW	IgG, IgG ₁ >> A, M	Af 80% (n = 15) Eu 87% (n = 15)	[48]
	582–613	B	AVERYLKDQQLLGIWGCSGKLICTTAVPWNAS	IgG ₁ >> IgG ₂ > IgG ₄	n.a. (n = 94)	[112]
	576–592	B	LQARILAVERYLKDQQL	IgG ₁	27% (n = 58)	[113]
	578–599	B	RILAVERYLKDQQLLGIWGCS	IgG ₁	55% (n = 58)	[113]
	578–599	B	RILAVERYLKDQQLLGIWGCS	IgG	88% (n = 68)	[114]
	583–594	B	VERYLKDQQLLG	IgG	SP (n = 13) > RP (n = 13)	[109]
	589–610	A, B, C, D	DQQLLGIWGCSGKLICTTAVPW ²	IgG	n.a.	[54]
	592–616	C	LLGLWGCSGKLICTTAVHWNSSWSN	IgG, IgG ₁ >> IgG ₄	Af 93% (n = 15) Eu 100% (n = 15)	[48]
	593–604	B	LGIWGCSGKLIC	Total Ig	Af 50% (n = 159) USA 91% (n = 103)	[111]
	593–604	n.a.	LGLWGCSGKLIC	n.a.	100% (n = 22)	[115]
	598–604	n.a.	CSGKLIC	n.a.	48% (n = 44)	[115]
	599–610	B	SGKLICTTAVPW	IgG	SP (n = 13) > RP (n = 13)	[109]
	597–618	B	GCSGKLICTTAVPWNASWSNKS	IgG ₁ >> IgG ₂ >>> IgG _{3,4}	IgG ₁ 100% (n = 58) IgG ₂ 26% (n = 58)	[113]
	609–623	n.a.	PWNASWSNKSLEQIW	IgG, IgM	Eu 28% (n = 193)	[116]
	612–636	C	SSWSNKSQDYIWGNMTWMQWDREIN	IgG, IgG ₁	Af 13% (n = 15) Eu 53% (n = 15)	[48]
HR-2	632–656	C	DREINNYTDIIYTLLEESQSQQEKN	IgG, IgG ₁ >> IgG ₃	Af 20% (n = 15) Eu 53% (n = 15)	[48]
	647–659	B	EESQNQQEKNEQE	IgG	10% (n = 62)	[114]
MPER	652–676	C	QQEKNEKDLLALDSWNNLWNWFSIT	IgG, IgG ₁	Af 33% (n = 15) Eu 47% (n = 15)	[48]
	655–671	A, B, C, D	KNEKDLLALDSWKNLWN ²	IgG	B, C > A (n = 169)	[54]
	655–666	B	KNEQEELLELDKW	IgG	SP (n = 13)	[109]
	659–670		ELLELDKWASLW		RP (n = 13)	
LLP3	787e–809	C	GLQTGWQALKYLGSLVQYWGLELKK	IgG, IgG ₁	Af 27% (n = 15) Eu 27% (n = 15)	[48]
LLP1	825–856	C	GTDRLIEGLQGIGIRAIYNIPRRIRQGFEAALL	IgG, IgG ₁	Af 20% (n = 15) Eu 40% (n = 15)	[48]
	841–856	B	GTDRLIEGLQGIGIRAIRHIPRRIRQGLERILL	IgG	61% (n = 62)	[114]
	839–853	B	GTDRLIEGLQGIARHIPRRIRQGLER	Total Ig	Af 26% (n = 159) USA 12% (n = 103)	[111]

A = HIV-1 clade A; B = HIV-1 clade B; C = HIV-1 clade C; Ab = antibody; Af = African; Eu = European; Ig = immunoglobulin; n = number of subjects; n.a. = not available; RP = rapid progressors; SP = slow progressors; > = higher prevalence of Ab recognition than comparison group; < = lower prevalence of Ab recognition than comparison group.

¹ HXB2 numbering scheme with numbers indicating amino acid sequence position and letters indicating inserted amino acids compared to the HXB2 sequence, www.hiv.lanl.gov.

² Reactivity was tested towards peptides from different strains, but only the clade C sequence is shown here.

cidated. Higher gp120-specific IgG₁ titers and gp120-specific IgG₃ prevalence has been observed in HIV controllers compared to progressors [45]. Interestingly, antibody responses directed to gp41 may be correlated both with progression or control, depending on the subclass and epitope specificity. For example, IgG₁ levels directed to linear epitopes in the gp41 immunodominant region 578–622 are higher during chronic infection and progression [127]. On the contrary, there is evidence that gp41-specific IgG₂ may be associated with nonprogression [128–130]. In fact,

it is quite possible that patients with reactivities in IgG subclasses which exhibit less complement activation and/or ADCC experience less killing of their immune cells and therefore have a better prognosis [131]. Analyses of large-sample cohorts for which clinical information is available would be very useful to elucidate if individual profiles of antibody isotype/subclass recognition of Env epitopes are associated with certain courses of disease, symptoms or response to medication.

Table 7. Polyclonal antibodies specific for non-Env proteins

	IgG prevalence ¹		IgG-subclass and isotype distribution		Ab response during the course of infection [Ref.]				IgG association with progression or nonprogression [Ref.]			
	%	Ref.	Ig	Ref.	Ig	↓	↑	constant	mixed	P	NP	No
MA (p17)	39–100	[27, 48, 143–145]	IgG ₁ > IgG ₄ , IgG ₂ , IgG ₃ , IgA > IgM	[48, 51]	IgG			[27, 48, 143]	↑32 m, ↓ [144]		[144, 145]	
CA (p24)	65–100	[27, 48, 143, 144]	IgG ₁ > IgG ₄ , IgG ₂ , IgG ₃ , IgA > IgM	[48, 51]	IgG	[143]		[27, 48]	↑33 m, ↓ [144]		[45, 144]	
NC (p7)	0–60	[27, 48]	IgG ₁ > IgG ₃	[51]	IgG			[27, 48]				n.a.
PR	40–93	[27, 48, 146, 152, 160]	IgG ₁ > IgG ₄ , IgG ₂ > IgG ₃ , IgA	[48]	IgG	[152]		[27, 48]	–/↓/↑ [160]		[152, 160]	
IN (p31)	60–100	[27, 48, 126, 143, 146]	IgG ₁ > IgG ₄ , IgG ₃ > IgA, IgM	[48, 126]	IgG IgG ₁ IgG ₃			[27, 48] [126] [126]			[146]	
RT (p66)	79–97	[27, 48, 126, 143]	IgG ₁ > IgG ₄ > IgG ₃ , IgA, IgM	[48, 126]	IgG IgG ₁ IgG ₃		[126]	[48, 143] [126]				n.a.
Nef	13–66	[27, 48, 152, 158]	IgG ₁ > IgG ₄ > IgG ₂ , IgA, IgM	[48]	IgG	[152]		[27, 48]			[152]	
Tat ²	0–83	[27, 48, 147–149]	IgG ₁ > IgG ₃ , IgG ₄ , IgA	[48]	IgG IgM		[149]	[27, 48]			[46, 147, 148]	[45]
Rev ²	12–73	[27, 150–152]	n.a.		IgG	[150]	[151]	[27]				n.a.
Vif	18–81	[27, 153, 154]	n.a.		IgG		[153, 154]	[27]				n.a.
Vpr	13–53	[27, 155]	n.a.		IgG			[27]			[46]	[155]
Vpu ²	26–93	[27, 155–157]	IgG ₁ , IgG ₂ , IgG ₃ , IgG ₄	[156]	IgG	[157]		[27]	–/↓/↑ [156]			[155]

↓ = Decrease of Ab levels or prevalence; ↑ = increase of Ab levels or prevalence; –/↓/↑ = constant, decreasing or increasing Ab levels or prevalence observed in the same study; Ab = antibody; m = months; n.a. = not available; P = progression (defined as progression to AIDS = low CD4 or high viral load); NP = nonprogression; No = no association.

¹ IgG prevalence range observed in cohorts of HIV-infected individuals. ² Not expressed in the virion, but only in the infected cell.

Antibody Responses Directed towards Structural, Functional and Accessory Proteins

Besides antibodies recognizing Env surface antigens, HIV-infected individuals also develop antibodies targeting structural (p17, p24, p7), functional (p66, PR, p31) and accessory proteins (Nef, Tat, Rev, Vif, Vpr, Vpu). These proteins are not present on the surface of the virion – certain of them are not even found within the virion, but instead only in infected cells (e.g. Tat, Rev, Vpu) – but they can be exposed to the immune system upon the destruction of infected cells [20]. Additionally, some proteins are found on the membrane of infected cells (i.e. p24, p17, Nef, Tat and Vpu) [132–136] and some are even secreted from infected cells and can be found in serum and cerebrospinal fluid (i.e. p24, p7, Nef, Tat and Vpr) [135, 137–140]. The relevance of HIV reservoirs in eliciting immune responses still has to be clarified; however, persistence of viral antigens has been shown to cause sub-

sequent waves of immune stimulation during chronic infections [141, 142]. Table 7 summarizes the prevalence and isotype/subclass distribution of antibody responses against non-Env proteins. The most frequently recognized targets are structural p17, p24 and *pol*-derived p31 and p66 (table 7) [27, 48, 126, 143–146]. In fact, diagnostic tests, such as confirmatory Western blot, are based on the additional detection of p17, p24 and p31-specific IgG (e.g. InnoLIA, Innogenetics, Ghent, Belgium). Interestingly, specific antibodies are also elicited against accessory proteins with a variable IgG prevalence (table 7) [27, 48, 147–158].

The isotype/subclass distribution of antibodies recognizing non-Env proteins is less well characterized (table 7). However, we recently analyzed reactivity profiles using a panel of HIV-1 clade C recombinant proteins [48]. Strong IgG₁ responses were accompanied mainly by IgG₄ responses. Additionally, IgG₂ responses were frequently detected against p24 and PR, and IgG₃ responses

against p24 and p31 [48]. The mechanisms that lie behind the differential production of antibody subclasses for the individual proteins still have to be understood. Interestingly, a new mechanism of HIV evasion from antibody responses has recently been described that affects only single antibody types [159]. IgG₂ and IgA class switch was attenuated by intercellular shuttling of the accessory protein Nef from infected macrophages to B cells. In patients, the mechanism affected only single specificities. In fact, it has been reported that individuals naturally infected with virus variants lacking Nef had higher gp120-specific IgA₂, p24-specific IgG₂, IgA₁ and IgA₂, and Tat-specific IgG₂ and IgA₂ responses [159].

Studies using follow-up serum samples of HIV-infected patients provided some insights into the course of antibody responses over time (table 7). Interestingly, protein- and peptide-specific antibody responses showed mainly constant patterns during the course of infection [27, 48, 126, 143]. However, during acute infection antibodies targeting p17 and p24 increased in the first 32 and 33 months, respectively, and then decreased during progression [144]. An important question in this regard is whether the presence/titer of antibodies specific for individual proteins is predictive of a certain clinical course of the disease. For example, it has been shown that p17, p24 and PR-specific antibodies were associated with nonprogression or a stable clinical status [45, 144, 145, 152, 160]. On the other hand, p31-specific antibodies were found to be associated with a worse prognosis, and results obtained for Tat- and Vpr-specific responses were controversial (table 7) [45, 46, 146–148, 155]. The use of antibody reactivity patterns as prognostic markers has not yet been established for the clinical practice. Analyzed cohorts differed in many aspects, such as the infection route and definition of clinical staging, as well as in the sensitivity of immunoassays and antigens used. It would be very interesting to investigate whether a

set of antibody reactivities with prognostic value could be identified analyzing antibody responses directed towards Env as well as non-Env proteins in parallel, and including the dissection of isotypes and subclasses.

Concluding Remarks

Several neutralizing human monoclonal HIV-specific antibodies have been identified and their characterization has contributed to the elucidation of the structural determinants of antibody-antigen interactions. However, such antibodies occur only in a minority of HIV-infected patients and represent a small fraction of the patients' polyclonal HIV-specific antibody responses. Studies analyzing the specificities of the polyclonal antibody responses in HIV-infected patients have shown that the antibodies which develop during natural infection are misdirected towards epitopes that are outside of the gp120 CD4-binding site, and against proteins inside the virion or on virus-infected cells, and therefore cannot block the binding of the virus to CD4 cells. Since a considerable proportion of the Env-specific antibodies is directed against certain peptides, it may be possible to use such peptides for diagnostic tests to find out if they can eventually be used to discriminate infection with certain strains. Furthermore, engineering HIV vaccines to redirect the antibody response towards the CD4-binding sites in order to obtain effective therapeutic and prophylactic vaccines for HIV must be considered.

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