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Neutralization of genetically diverse HIV-1 strains by IgA antibodies to the gp120 CD4 binding site from long-term survivors of HIV infection

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Abstract

Objective—To identify an HIV epitope suitable for vaccine development.

Design—Diverse HIV-1 strains express few structurally constant regions on their surface vulnerable to neutralizing antibodies. The mostly-conserved CD4 binding site (CD4BS) of gp120 is essential for host cell binding and infection by the virus. Antibodies that recognize the CD4BS are rare, and one component of the CD4BS, the 421–433 peptide region, expresses B cell superantigenic character, a property predicted to impair the anti-CD4BS adaptive immune response.

Methods—IgA samples purified from the plasma of subjects with HIV infection were analyzed for the ability to bind synthetic mimetics containing the 416–433 gp120 region and full-length gp120. Infection of peripheral blood mononuclear cells by clinical HIV isolates was measured by p24 ELISA.

Results—IgA preparations from 3 subjects with subtype B infection for 19–21 years neutralized heterologous, coreceptor CCR5-dependent subtype A, B, C, D and AE strains with exceptional potency. The IgAs displayed specific binding of a synthetic 416–433 peptide mimetics dependent on recognition of the CD4 binding residues located in this region. Immunoabsorption, affinity chromatography and mutation procedures indicated that HIV neutralization occurred by IgA recognition of the CD4BS.

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Conclusions—These observations identify the 421–433 peptide region as a vulnerable HIV site to which survivors of infection can produce powerful neutralizing antibodies. This indicates that the human immune system can bypass restrictions on the adaptive B cell response to the CD4BS, opening the route to targeting the 421–433 region for attaining control of HIV infection.

Keywords

Neutralizing antibodies; CD4 binding site; HIV diversity; B cell superantigen; HIV vaccine

Introduction

The HIV-1 pandemic has been caused by viral strains expressing structurally divergent versions of the coat protein gp120. The infection and previously tested vaccine candidates induce robust synthesis of antibodies to immunodominant gp120 regions, but these regions are mutable, and the antibodies display only strain-specific neutralization. Vaccine trials intended to induce antibody and cytotoxic T cell responses did not reduce the risk of HIV infection in previous clinical trials [1,2], and the recent RV144 vaccine trial also illustrates the difficulties in inducing effective immune responses to the virus [3]. Consensus has developed that effective HIV vaccination will require: (a) identification of an epitope expressed in a sufficiently constant form by genetically diverse HIV strains found across the world; and (b) the induction of a robust immune response to such an epitope.

Antibodies from HIV infected subjects have been studied extensively for the ability to neutralize the virus [4–11]. Rare monoclonal antibodies from infected subjects neutralize HIV strains that are genetically divergent from the autologous virus [10,12]. Only a minority of sera from HIV-infected subjects express this capability, suggesting that production of broadly neutralizing antibodies to the conserved HIV epitope is immunologically disfavored [13–15]. Moreover, previously described monoclonal and polyclonal serum antibodies usually neutralize only a restricted set of group M primary HIV isolates when tested using the natural host cells, human T cells in primary culture. Very few structurally conserved epitopes that support broad neutralization by antibodies have been identified. These are the membrane proximal external region of gp41 [7], a carbohydrate-dependent epitope of gp120 [6] and a conformational epitope located in the CD4 binding site (CD4BS) of gp120 [16]. Binding to host CD4 receptors is obligatory for HIV infection of T cells and macrophages. The CD4BS is a large conformational determinant of discrete gp120 regions brought into spatial proximity by virtue of the 3-dimensional protein folding pattern [17–20]. Rare antibodies recognize the native CD4BS conformational state and neutralize the virus [13,15] but other anti-CD4BS antibodies display little or no neutralizing activity [4,21,22].

Crystallography and mutagenesis studies indicate that the 421–433 peptide region provides essential amino acids forming the CD4BS [17–20]. The sequence of residues 421–433 is mostly conserved in group M HIV-1 strains. This region is also distinguished by its B cell superantigen character [23,24]. A minority of preimmune antibodies produced without exposure to HIV bind the 421–433 epitope of gp120 [23–25] and proceed to catalyze the hydrolysis of gp120 [26,27]. The preimmune antibodies may furnish a limited level of innate protection against infection, but there are no reports of broadly neutralizing antibodies to the 421–433 epitope induced by HIV infection. An impaired adaptive immune response to the epitope is consistent with its superantigenic character. Superantigens bind antibodies expressed as B cell receptors by atypical interactions at conserved antibody framework regions [28–30]. Unlike conventional antigens, they do not stimulate efficient synthesis of class-switched antibodies [31–33].

In the present study, we searched for neutralizing IgA to the 421–433 CD4BS region in three hemophilia A patients with prolonged HIV infection contracted by transfusion of contaminated blood products. We focused on IgA class antibodies, as IgA from noninfected humans previously showed low-level HIV neutralizing activity superior to IgG from the same subjects [27]. We report neutralization of diverse HIV strains with chemokine coreceptor CCR5-dependency by the IgA attributable to recognition of the 421–433 region. The exceptionally potent and broad antibody neutralizing activity identifies this epitope as a major vulnerability of the virus suitable for targeting by an HIV vaccine. Our studies do not address the relationships between antibody production, immune system maturity, infection route and disease progression. Future studies are necessary to investigate these points.

Methods

Patients

Peripheral blood was collected from 3 long-term survivors infected with HIV for 19–21 years who had not developed AIDS (LTS_{19–21} donors 2857, 2866, 2886). The Methods section and Fig. S1 of the Supplemental Digital Content describe patient clinical histories, including CD4⁺ T cell counts, viral loads and anti-retroviral therapy. Samples from non-infected subjects were from the Gulf Coast Blood Bank, Houston, Texas.

IgA was purified from peripheral blood samples by affinity chromatography using immobilized anti-human α chain antibody [27,34; see also Methods section, Supplemental Digital Content]. IgA from donor 2866 (0.6 nmol) was subjected to 2 rounds of immunoadsorption with E-416–433b (3.7 nmol peptide equivalents) or control BSA (0.6 nmol, equivalent to the BSA content of E-416–433b; 18 h, 37°C) in 10 mM sodium phosphate pH 7.4, 137 mM NaCl, 2.7 mM KCl (PBS, 0.73 ml). Immune complexes were removed using anti-BSA antibody immobilized on agarose in the same buffer (0.2 ml settled gel, Sigma; 1 hour rotary shaking). The unbound IgA in the supernatant (0.8 ml) was assayed for E-416–433b binding and HIV neutralization. The epitope-specific antibodies were prepared by binding of IgA pooled from the 3 LTS_{19–21} donors (2 mg IgA; 12 nmol) to E-416–433 conjugated to agarose (35 nmol peptide equivalents/50 μ l settled gel in 1 ml; 20 h with rotary shaking). Using a spin column, unbound IgA was removed with PBS (2 ml), non-covalently bound IgA was eluted with 0.1 M glycine, pH 2.7 (0.4 ml; neutralized immediately with 0.025 ml 1 M Tris-HCl pH 9.0) and covalently bound IgA was eluted by treatment with 20 mM pyridine 2-aldoxime methiodide (0.4 ml; Sigma; 17h) followed by 0.1 M glycine, pH 2.7. IgA dialyzed against PBS prior to measuring E-416–433b binding and HIV neutralization activities.

ELISA was conducted to determine binding of antibodies, control proteins or soluble CD4 (sCD4, 4 domains; Protein Sciences Corporation) to immobilized E-416–433b (70 ng peptide equivalents/well; ref 35] or full-length recombinant gp120 (40 ng/well; subtype B, strain MN; Immunodiagnostic Inc; ref 36, see also Methods section, Supplemental Digital Content). Binding of soluble CD4 (sCD4, 4 domains; Protein Sciences Corporation) was determined similarly using rabbit anti-CD4 serum for detection (1:1000, NIH AIDS Research and Reference Reagent Program). Covalent E-416–433a binding by IgA was determined by denaturing electrophoresis followed by staining with peroxidase-conjugated streptavidin [37]. IC₅₀ values correspond to concentrations required to attain 50% inhibition of binding.

Clinical HIV isolates for neutralization assays were from The NIH AIDS Research and Reference Reagent Program. The strains were selected to represent multiple virus subtypes, availability of their gp120 sequence, and our ability to grow usable virus stocks at titer greater than 10^{2.25}–10³ TCID₅₀/ml in human peripheral blood mononuclear cells (PBMCs).

Virus infection of PHA-activated PBMCs (pool of 8–12 healthy human donors) was measured in Carl Hanson's laboratory in PBS or varying IgA concentrations in PBS (4 independent cultures/IgA concentration) using p24 enzymeimmunoassay kits [38]. Additional assays were done in David Montefiori's laboratory by essentially the same method except that the cultures were in duplicate and a mixture of PBMCs from two human donors served as the host cells [39]. Neutralization was determined by computing the reduction of p24 concentrations relative to infection in the presence of diluent instead of IgA. IgA concentrations yielding 50% neutralization (IC₅₀) were obtained from fits of the data to the equation: % neutralization = $100 / (1 + 10^{(\log IC_{50} - \log [IgA]) \cdot \text{Hillslope}})$ (GraphPad Prism; see Methods section, Supplemental Digital Content for intra- and inter-assay variability analysis).

Peptides

The electrophilic mimetics of gp120 residues contain the sequence of residues 416–433 (LPSRIKQIINMWQEVGKA) and covalently reactive phosphonate diester groups placed at Lys side chains (E-416–433a and E-416–433b conjugated to bovine serum albumin). Ala-containing mutant 416–433 peptides, non-electrophilic NE-416–433 peptide and sequence shuffled mutant 416–433 peptide (*Sh*416–433, GQKSWEIPAKNRLIMVIQ) were from Sigma-Aldrich. Synthesis, purification and analytical details are in Methods section, Supplemental Digital Content.

Results

IgA binding activity

We described previously weak HIV neutralization by IgA isolated from the blood of humans without HIV infection attributable to recognition of the 421–433 gp120 region (preimmune antibodies; ref 27). Two subsets of preimmune antibodies to gp120 have been documented, one that binds the 421–433 region reversibly by traditional non-covalent means [23,24], and another that degrades gp120 by a serine protease-like mechanism after completing the initial non-covalent binding step [27]. Here, we used a single binding assay to detect both antibody subsets in IgA preparations from 3 patients 19–21 years after diagnosis of HIV infection (long-term survivors, LTS_{19–21} patients). The assay employs mimetics of the 416–433 peptide region containing electrophilic phosphonate groups (E-416–433a, E-416–433b; Fig. 1a). The peptide component of such mimetics binds antibodies non-covalently in coordination with covalent binding of the phosphonate to antibody nucleophilic sites responsible for proteolytic activity [26,37]. Low affinity peptide analogs of the 421–433 region have been reported to bind CD4 previously [40,41].

E-416–433b was bound by all 3 LTS_{19–21} IgA preparations at levels greater than IgA from humans without HIV infection determined by ELISA (Fig. 1b). The binding was also detected by denaturing electrophoresis, indicating formation of covalent immune complexes by a subset of the antibodies (*Inset*, Fig. 1b). Specificity of IgA pooled from the 3 LTS_{19–21} donors for the 416–433 mimetic was evident from the representative competitive inhibition studies in Fig. 1c. IgA from the 3 donors tested individually displayed similar reactivity with various reagents shown in this Figure. IgA binding to immobilized E-416–433b was inhibited competitively by two soluble versions of the mimetic, E-416–433a and its non-electrophilic 416–433 peptide counterpart (NE-416–433), but not the control shuffled sequence *Sh*416–433 peptide. The binding of IgA to E-416–433 was inhibited by gp120 but not by irrelevant proteins (*e.g.*, ovalbumin), indicating conformational mimicry of the corresponding 416–433 region of the full-length protein (Fig. 1c). NE-416–433 and gp120 are devoid of artificial electrophilic groups and do not bind covalently to antibodies. Therefore, their ability to inhibit the binding to E-416–433b indicates specific non-covalent

recognition of the 416–433 epitope by the IgA. The importance of non-covalent IgA recognition of the epitope was also evident from maintenance of the E-416–433b binding activity in the presence of a small molecule electrophilic phosphonate hapten with structure identical to the phosphonate group of E-416–433 (Fig. S2a, Supplemental Digital Content). The hapten blocks covalent antibody binding by reacting irreversibly with antibody nucleophiles but does not impair non-covalent antibody binding [42].

The IgA displayed binding to immobilized gp120 that was partially inhibited by E-416–433a and NE-416–433 but not the shuffled *Sh*416–433 peptide, suggesting that the epitope-specific antibodies constitute a significant subset of the overall gp120-binding IgAs (Fig. 1d). In addition, soluble CD4 (sCD4) but not irrelevant proteins inhibited IgA binding to E-416–433b and full-length gp120 (Fig. 1c, 1e), consistent with IgA recognition of the CD4BS. The ability of sCD4 to bind E-416–433b specifically and saturably was verified in direct binding assays (Fig. S2b, S2c, Supplemental Digital Content). The shorter E-421–433a and E-421–433b epitope mimetics inhibited E-416–433b binding by sCD4 (Fig. S2d, Supplemental Digital Content), consistent with the essential role of the 421–433 peptide region in CD4 binding [17–20].

The importance of individual amino acids for non-covalent IgA recognition was evaluated from the competitive inhibition of E-416–433b binding by peptides containing Ala instead of the wildtype residues (Table 1). Peptides with Ala replacements at Ile420, Ile424 and Trp427 displayed 6.5–34.3 fold reduced inhibitory potency (IC₅₀) compared to the wildtype peptide. Ala replacements at 7 additional positions resulted in reduced IC₅₀ by at least 2-fold (416, 419, 421, 423, 425, 430 and 432. Three of these residues are CD4-contacting residues from crystal studies (Asn425, Trp427, Val430; refs 17,18) and two additional residues are thought to contribute in CD4 binding from site-directed mutagenesis studies (Lys421, Lys432; ref 20). Taken together, the data indicate specific CD4BS recognition by IgA from the LTS_{19–21} patients.

A few neutralizing antibodies to epitopes within or close to the CD4BS have been described. We studied the recognition of E-416–433b by 3 monoclonal antibodies with well-defined specificity: Monoclonal IgG b12 is described to bind an epitope overlapping the CD4BS [16]. Monoclonal IgG 17b and IgG 48d bind a conformational gp120 epitope exposed upon sCD4 binding [43]. These antibodies failed to bind E-416–433b (Fig. S3, Supplemental Digital Content).

IgA neutralizing activity

All 3 LTS_{19–21} patients were infected with subtype B viral strains as determined by the sequences of the autologous virion gp120 gene (Table S1, Supplemental Digital Content). HIV infection induces predominantly antibodies to the mutable gp120 epitopes with limited or no capacity to neutralize genetically heterologous HIV strains. We initially tested neutralization of a heterologous subtype C HIV strain to preferentially detect IgAs that neutralize the virus by recognizing the conserved HIV epitopes. The variable domain sequences of the autologous subtype B strains and heterologous subtype C test strain are substantially divergent (*e.g.*, the V3 domain epitope shown in Fig. S4a, Supplemental Digital Content). As predicted, the heterologous subtype C strain was not neutralized by mouse antibodies raised by immunization with a subtype B gp120 (Fig. S4b, Supplemental Digital Content). In contrast, purified plasma IgA from all 3 LTS_{19–21} patients neutralized the heterologous subtype C strain (Fig. 2 and Table 2). We reported previously the weak HIV neutralizing activity of IgA from humans without HIV infection, evident only after extended IgA-virus incubations (24 h; ref 27). At the shorter incubations times used here (1 hour), the neutralizing activity of purified IgAs from non-infected subjects was negligible. There was no loss of host PBMC viability cultured with the LTS_{19–21} IgA preparations

without HIV, determined as in ref 38. We also confirmed that the neutralizing activity of purified LTS₁₉₋₂₁ IgA cannot be attributed to endotoxin (Fig. S4c, Supplemental Digital Content), a contaminant that can induce release of chemokines with the potential of inhibiting HIV infectivity [44]. Like the purified IgAs, unfractionated plasma from LTS₁₉₋₂₁ patients also neutralized the virus (*e.g.*, LTS₁₉₋₂₁ donor 2857 serum dilutions yielding 50% neutralization for subtype B strains QH0692 and PAVO, respectively, were 1:250 and 1:1735; for subtype C strains 97ZA009 and Du156, respectively, 1:8132 and 1:192).

Eighteen genetically diverse, coreceptor CCR5-dependent clinical HIV isolates drawn from subtypes A, B, C, D and AE were neutralized by each of the 3 LTS₁₉₋₂₁ IgA preparations (Table 2). Neutralization of subtype C and B strains occurred with exceptional potency (sub-microgram/ml range). For comparison, Table 2 shows the neutralizing activity of a reference monoclonal antibody tested in parallel, IgG b12.

To determine if HIV neutralization is due to CD4BS recognition, the LTS₁₉₋₂₁ IgA was absorbed with the E-416–433b albumin conjugate or control albumin alone. The non-bound IgA displayed 82% reduced E-416–433b binding, indicating removal of the epitope-specific IgA. The immunoadsorbed IgA displayed 91% reduced subtype C virus neutralizing activity compared to the starting IgA preparation (computed from IC₅₀ values, Fig. 3a). In a separate experiment, we eluted the epitope-specific IgAs bound to immobilized E-416–433-agarose for neutralization tests. Two types of epitope-specific IgAs were tested, the non-covalently bound fraction recovered by acid elution and the covalently bound fraction eluted after pyridine 2-aldoxime methiodide cleavage of the phosphonate-antibody bonds. Our previous studies indicate that covalent binding of electrophilic peptides such as E-416–433 is predictive of the specific proteolytic activity of antibodies [37]. Enrichment of the epitope-specific IgA fractions eluted from the affinity column was evident from increased values of E-416–433b binding per unit IgA mass (Fig. S5a, Supplemental Digital Content). The non-covalently and covalently bound IgA fractions eluted from the column also displayed increased HIV neutralizing activity per unit IgA mass compared to the starting IgA preparation loaded on the column (respectively, by 38-fold and 1166-fold reduction of IC₅₀; Fig. 3b). Therefore, the neutralizing activity can be attributed to specific IgAs that recognize the 416–433 epitope.

The 416–433 epitope sequence of diverse HIV strains is largely but not fully conserved (Table S2, Supplemental Digital Content). We evaluated the relationship between individual epitope mutations and IgA neutralizing potency using the consensus subtype C epitope sequence as reference (Table 2; LPCRIKQIINMWQEVGKA). Only the R419→K, V430→A and R432→K/Q mutations were individually associated with decreased neutralization regardless of the presence of other mutations (Fig. 3c; respectively, $P < 0.0001$, < 0.0001 and $= 0.025/0.006$). Moreover, simultaneous mutations at positions 419, 430 and 432 were associated with decreased neutralization compared to the individual mutations (Fig. S5b, Supplemental Digital Content). Table 1 indicates that Ala mutations at these positions also reduced IgA binding to the epitope peptide. Residues 430 and 432 are important for CD4 binding to gp120 [20]. The data confirm our conclusion that neutralization is attributable to IgAs that recognize the CD4BS.

Discussion

As binding to host CD4 receptors is required for infection, diverse coreceptor CCR5-dependent HIV strains maintain the CD4BS in a mostly constant structure. We identified IgA class antibodies specific for the CD4BS 416–433 epitope produced by survivors of prolonged HIV infection that neutralized heterologous viral strains with exceptional potency. A conclusive determination of IgA specificity was possible through use of an

electrophilic 416–433 peptide that mimics the CD4 binding function of the native CD4BS expressed by HIV. The corresponding region of full-length gp120 is expressed in a sterically accessible form on the protein surface [17,18,45]. IgA recognition of the CD4BS was confirmed by competitive inhibition of IgA binding to the electrophilic peptide and gp120 by CD4. Furthermore, mutation studies indicated the shared binding specificity of CD4 and the IgAs. Immunoabsorption and epitope-specific chromatography procedures indicated the 416–433 region as the major neutralizing epitope recognized by the IgA. This is corroborated by the observed association between certain epitope mutations and altered neutralizing potency. Consistent with recognition of the conserved CD4BS region, the IgAs neutralized genetically diverse HIV strains. The IgAs contain subsets of antibodies that bind gp120 non-covalently and antibodies that proceed to hydrolyze gp120 after completing the non-covalent binding step [27]. Our binding assays using the electrophilic 416–433 peptide mimetic detect both types of antibodies. We did not measure the contributions of these activities in virus neutralization. Non-covalent CD4BS binding alone is sufficient to neutralize the virus, and gp120 hydrolysis holds the potential of enhancing the neutralizing potency [26,27].

Adaptive synthesis of anti-CD4BS neutralizing antibodies in the natural course of infection is widely acknowledged as an immunologically disfavored process [13,15]. This is consistent with the superantigenic character of the 421–433 region of the CD4BS. The epitope is recognized weakly by the framework regions (FRs) of antibodies produced without HIV infection that bind gp120 reversibly [29,30] and catalyze its degradation [26,27]. Superantigen binding to the B cell receptor FRs is thought to stimulate the cells non-productively, and premature apoptosis occurs [31,32], consistent with infrequent production of neutralizing antibodies to the CD4BS. Serum antibodies to the 421–433 CD4BS epitope are also increased in the autoimmune disease systemic lupus erythematosus [46], and recombinant antibodies to this epitope from lupus patients neutralize HIV [38]. HIV infection and lupus are rarely coexistent. We had assumed that the lupus antibodies are produced due to dysfunctional autoimmune reactivity. The studies reported here indicate that HIV infection itself stimulates production of powerful neutralizing antibodies to the CD4BS despite its superantigenic character. IgA concentrations in human blood are about 2 mg/ml [47]. Depending on the HIV-1 strain, the 50% effective concentrations of IgAs from the long-term survivors in Table 2 were 75–800,000 fold lower than the physiological IgA concentrations. HIV neutralization by unfractionated plasma from the 3 patients was also evident. We studied only 3 patients with very prolonged infection, and all 3 were positive for the neutralizing IgA. Also, our studies were limited to IgA from patients who contracted HIV by transfusion of contaminated blood products as children. Future research is needed to determine the time course of infection-induced production of neutralizing IgAs, whether the neutralizing response is restricted to IgA class antibodies and whether the response can be induced by sexually transmitted infection occurring after the immune system has reached full maturity. Similarly, our findings do not necessarily imply that anti-CD4BS IgA production impedes HIV disease progression. Detailed longitudinal studies on a larger cohort of patients that take into account other host and viral factors known to influence disease progression are necessary to address these points.

HIV presents few epitopes suitable for vaccine targeting, reflecting an unusual capacity to evade traditional protective immunity mechanisms. The essential role of the 421–433 epitope in CD4BS binding and its sequence conservation qualify the epitope as a vaccine target. However, the superantigenic character of the epitope poses a challenge. The immune response to microbial infection is a stochastic process relying on certain high probability B lymphocyte differentiation events. The process entails antigen binding to the antibody complementarity determining regions (CDRs), driving adaptive selection of somatically diversified B cell receptors with the highest antigen binding affinity. Understanding how B

cells of the LTS_{19–21} patients described here bypass restrictions on production of neutralizing anti-CD4BS antibodies may provide insight to designing a vaccine that induces similar antibodies. The potential bypass mechanisms are: (a) The B cells may slowly produce antibodies that bind the CD4BS *via* their CDRs with no utilization of the pre-existing CD4BS binding site located in the FRs; and (b) Cellular down-regulation due to CD4BS binding to the antibody FR site may be effectively counteracted by a favorable differentiation signal generated upon simultaneous engagement of another epitope on the same gp120 molecule by the CDRs. The latter bypass mechanism is supported by our recent report of antibodies with binary epitope reactivity directed to the 421–433 CD4BS epitope and another spatially distinct gp120 epitope induced by immunization of mice with an electrophilic mimetic of full-length gp120 [48]. Importantly, targeting of the 421–433 epitope is the first vaccine approach that induces the synthesis of antibodies with ability to neutralize genetically diverse HIV strains.

The inability to replicate the native structure of the CD4BS has held back development of a candidate vaccine that induces neutralizing antibodies to genetically diverse HIV strains. A conformational epitope recognized by monoclonal antibody b12 directed to the CD4BS was previously proposed as an HIV vaccine target [49]. Accurate mimicry of conformational epitopes, however, is difficult, and efforts to develop a vaccine candidate mimicking the epitope recognized by antibody b12 have not been fruitful. The linear character of the 421–433 region should facilitate development of immunogens for induction of neutralizing antibody synthesis. Mimicry of the native 421–433 region of HIV by the E-416–433 probe is evident from its recognition by CD4 and neutralizing antibodies from infected subjects. Findings of potent and broad HIV neutralization by human antibodies to the 421–433 region described in the present study and the prospect of inducing accelerated synthesis of similar antibodies using experimental immunogens offer encouragement that an effective HIV vaccine directed to the CD4BS can be developed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Specific IgA binding to 416–433 CD4BS epitope

(a) Structures of 416–433 epitope mimetics used to determine IgA binding activity. E denotes the phosphonate group located at Lys residue side chains. NE-416–433 denotes the non-electrophilic synthetic peptide, and *Sh*416–433, the shuffled sequence control peptide.

(b) Binding of E-416-433b immobilized on ELISA plates (70 ng/well) by IgA preparations from long-term survivors (LTS₁₉₋₂₁; donors 2857, 2866 and 2886) and pooled IgA from non-infected donors (■; n=13 donors). Values are means±s.d. of 2 replicates corrected for non-specific binding to wells coated with albumin alone. IgA preparations from 3 individual non10 infected donors displayed binding comparable to the pooled IgA sample shown in the figure (A490 range at 100 µg IgA/ml, 0.20–0.27; donors 1940, 1941 and 1942). *Inset*, Specific covalent binding of E-416-433a by LTS₁₉₋₂₁ IgA determined by reducing SDS-electrophoresis of boiled reaction mixtures. Shown are reactions of pooled LTS₁₉₋₂₁ IgA (70 µg/ml) incubated with 10 µM E-416-433a (lane 1) or control irrelevant E-VIP (lane 2) for 1h and stained with streptavidin-peroxidase. IgA heavy chains and light chains complexed to E-416-433a are evident, respectively, as the band at 60 kD and the doublet band at 25 kD (labeled H and L, respectively).

(c) Competitive inhibition of IgA binding to immobilized E-416-433b by various soluble proteins and peptides: sCD4 (▲), full-length gp120 (subtype B strain MN, ▼), control ovalbumin (□), E-416-433a (●), non-electrophilic peptide 416–433 (NE-416-433, ■), and control *sh*416-433 peptide (○). IgA from LTS₁₉₋₂₁ donor IgA 2857. Assay conditions as in Panel B. Residual binding computed as percent of binding in the absence of soluble competitors (A490 0.71±0.02).

(d) Competitive inhibition of IgA binding to immobilized E-416-433b by various soluble proteins and peptides: sCD4 (▲), full-length gp120 (subtype B strain MN, ▼), control ovalbumin (□), E-416-433a (●), non-electrophilic peptide 416–433 (NE-416-433, ■), and control *sh*416-433 peptide (○). IgA from LTS₁₉₋₂₁ donor IgA 2857. Assay conditions as in Panel B. Residual binding computed as percent of binding in the absence of soluble competitors (A490 0.71±0.02).

(e) Competitive inhibition of IgA binding to immobilized E-416-433b by various soluble proteins and peptides: sCD4 (▲), full-length gp120 (subtype B strain MN, ▼), control ovalbumin (□), E-416-433a (●), non-electrophilic peptide 416–433 (NE-416-433, ■), and control *sh*416-433 peptide (○). IgA from LTS₁₉₋₂₁ donor IgA 2857. Assay conditions as in Panel B. Residual binding computed as percent of binding in the absence of soluble competitors (A490 0.71±0.02).

Competitive inhibition of IgA binding to immobilized gp120 by E-416–433a and NE-416–433. gp120, 40 ng/well; IgA 100 μ g/ml (pooled from LTS_{19–21} donors 2857, 2866 and 2886). Residual binding computed as percent of binding in the absence of soluble competitor polypeptide (A_{490} 0.68 $\pm$ 0.01). (e) Competitive inhibition of IgA binding to immobilized gp120 by sCD4. gp120, 40ng/well; IgA 100 μ g/ml (pooled from LTS_{19–21} donors 2857, 2866 and 2886). Residual binding computed as percent of binding in the absence of soluble competitor protein (A_{490} 0.40 $\pm$ 0.02). sCD4 (●); ovalbumin (○). IgA from all 3 LTS_{19–21} donors also displayed binding to immobilized gp120 when tested individually (A_{490} 0.30–0.33).

Fig. 2. Heterologous clade C HIV neutralization by IgA from donors with clade B infection
 Examples of concentration-dependent IgA neutralization of a subtype C strain (ZA009). IgA purified from 3 long-term survivors of infection [LTS₁₉₋₂₁ IgA preparations 2857 (●), 2866 (●) and 2886 (●)] and pooled IgA from non-infected subjects (●). Host, PBMCs. Values are means $\pm$ s.e.m. of 4 independent culture replicates.

Fig. 3. HIV neutralization by specific IgAs to 416–433 epitope

(a) Reduced neutralizing activity of IgA (LTS_{19–21} donor 2866) immunoabsorbed with E-416–433b. The IgA was allowed to bind E-416–433b (●) or control albumin (●), the immune complexes were captured on anti-albumin antibody conjugated to agarose column, and neutralization of subtype C ZAO09 strain by the unbound IgA fraction was measured using PBMC hosts. Values are means±s.e.m. of 4 cultures. Dashed lines, 95% confidence limit. P value computed versus IgA immunoabsorbed with control albumin, Student's *t*-test. Confirmation that the epitope-reactive IgAs were immunoabsorbed was by ELISA using immobilized E-416–433b as in Fig. 1b (A490 values for IgA binding activity without immunoabsorption were 0.88±0.01, and after immunoabsorption with E-416–433b or albumin alone, respectively, 0.17±0.01 and 0.92±0.01). (b) Improved neutralizing activity of epitope-specific IgA fractions. Pooled IgA (LTS_{19–21} donors 2857, 2866 and 2886, ●) was fractionated on E-416–433 conjugated to agarose, unbound IgA was removed, and the non-covalently bound fraction (■) was recovered by elution with a pH 2.7 buffer. Covalently bound IgA (○) was recovered by treatment of the gel with pyridine 2-aldoxime methiodide and elution with the pH 2.7 buffer. HIV neutralization was measured as in Panel A. Values are means±s.e.m. of 4 replicates. Enriched E-416–433b binding by the epitope-specific IgA fractions are reported in Fig. S5a of the Supplemental Digital Content. (c) Association of 416–433 epitope mutations with reduced IgA neutralizing potency. IC₅₀ values of the three LTS_{19–21} IgA preparations in Table 2 for strains without and with mutations at individual amino acid positions were compared using the 2-tailed Student's *t*-test. Mutations were identified by comparing the 17 HIV strains listed in Table S2 of the Supplemental Digital Content with consensus subtype C epitope sequence as subtype C strains were neutralized most potently by the IgAs. (●) IC₅₀ for strains with the indicated consensus clade C residue. (●) IC₅₀ for strains with the indicated mutation.

Table 1

Amino acid requirements for non-covalent IgA recognition of the 416–433 epitope.

Mutation	IC50_{mutant}/IC50_{wildtype}
Leu416:Ala	3.87
Pro417:Ala	<1.50
Cys418:Ala	<1.50
Arg419:Ala	2.69
Ile420:Ala	6.48
Lys421:Ala	2.7
Gln422:Ala	<1.50
Ile423:Ala	2.63
Ile424:Ala	34.30
Asn425:Ala	2.13
Met426:Ala	<1.50
Trp427:Ala	6.79
Gln428:Ala	<1.50
Glu429:Ala	<1.50
Val430:Ala	2.60
Lys432:Ala	2.14

Binding of IgA (pooled from LTS 19–21 donors 2857, 2866 and 2886) to immobilized E-416–433b was measured in the absence and presence of increasing concentrations of the wildtype 416–433 peptide and mutant peptides containing Ala substitutions at individual positions (0.4–50 µg/ml) as in Fig. 1c. IC50 values were determined from plots of residual IgA binding expressed as % of binding without competitor peptide *versus* competitor peptide concentration fitted to the equation: residual binding (%) = 100/(1+10(Log[competitor peptide]–Log[IC50])). Ratios of (IC50_{mutant peptide}/IC50_{wildtype peptide})>2.0 indicate attenuated non-covalent binding of the mutants to IgA compared to the wildtype peptide. See Fig. 1c. for example of competitive inhibition of IgA binding by the wildtype peptide. Yellow boxes, residues important for IgA binding that are also important for CD4 binding by gp120 as determined by crystallography (PDB 2B4C; ref 17) or site-directed mutagenesis [19,20]. Green boxes, residues important for IgA binding but not known to play a role in gp120-CD4 binding.

Table 2
Neutralization of genetically diverse HIV strains by LTS₁₉₋₂₁ IgA preparations.

Subtype	Strain	IC50, µg/ml			
		IgA 2857	IgA 2866	IgA 2886	IgG b12
C	98Du123	0.086	0.053	0.39	>100
	99Du156	0.13	0.04	0.39	>100
	98Du172	0.0025	0.0037	0.066	9.93
	98Du422	0.026	0.023	0.19	3.62
	98TZ017	0.1	0.044	1.67	82
B	97ZA009	0.067	0.18	0.11	>100
	92BR021	0.38	2.43	1.85	0.57
	SF162	0.16	0.18	0.0031	0.72
	96PAVO	0.002	0.034	0.2	29.7
	94QHO692	0.83	0.094	0.52	1.32
A	92RW008	0.41	12.97	0.016	0.14
	92RW024	1.36	0.13	1.26	0.023
	97USSN54	6.15	10.4	4.44	0.26
D	93UG082	6.45	13.74	3.75	4.83
	94UG114	4.87	1.9	0.91	2.36
	92UG035	6.65	2.7	0.96	4.83
CRF_01_AE	90CM235	10.39	26.05	5.71	2.44
	92TH005	15.54	9.22	10.98	0.73
Geometric Mean [95% confidence]		0.40 [0.10–1.55]	0.45 [0.11–1.76]	0.53 [1.01–1.68]	

IC50 values for IgA preparations from LTS₁₉₋₂₁ donors and reference monoclonal antibody b12 were determined from dose response curves using the indicated clinical HIV isolates and PBMC hosts. All strains tested are coreceptor CCR5-dependent. Data for strains Du123, Du156, Du172, Du422, PAVO and QH0692 from the Montefiori laboratory. Remaining data from Hanson laboratory. The range of IgA concentrations tested was 0.001–85 µg/ml. See Fig. 2 for an example curve of IgA neutralizing activity.