Immune escape from HIV-specific antibody-dependent cellular cytotoxicity (ADCC) pressure

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Effective immunity to HIV is poorly understood. In particular, a role for antibody-dependent cellular cytotoxicity (ADCC) in controlling HIV is controversial. We hypothesized that significant pressure from HIV-specific ADCC would result in immune-escape variants. A series of ADCC epitopes in HIV-infected subjects to specific consensus strain HIV peptides were mapped using a flow cytometric assay for natural killer cell activation. We then compared the ADCC responses to the same peptide epitope derived from the concurrent HIV sequence expressed in circulating virus. In 9 of 13 subjects infected with HIV using a set of consensus HIV peptides, ADCC antibodies were unable to recognize the concurrent HIV sequence. Our studies suggest ADCC responses apply significant immune pressure on the virus. This result has implications for the induction of ADCC responses by HIV vaccines.

natural killer cells | viral evolution | neutralizing antibody

There is an urgent need to identify effective immunity to HIV and to use this information to design efficacious vaccines and immunotherapies. The recent Thailand-based RV 144 HIV vaccine trial using a canarypox prime/protein boost approach led to modest but significant protective efficacy (1). There is intense interest in the correlates of immunity in this study. CDS3 cytotoxic T-lymphocyte (CTL) responses generally are weak and infrequently are induced by this regimen, and neutralizing antibody (NAb) responses typically are directed narrowly toward the vaccine strains only. This regimen induces reasonably potent antibody-dependent cellular cytotoxicity (ADCC) antibodies (2), and such responses could be responsible in part for the efficacy observed.

ADCC antibodies often are present at very high titers in HIV-infected subjects, and many, but not all, studies have correlated ADCC responses with slower progression of HIV infection (reviewed in ref. 3). Macaque simian immunodeficiency virus (SIV) vaccine studies and passive antibody transfer studies in recent years strongly suggest a role for ADCC in assisting protective immunity (4–6).

Most HIV ADCC assays measure total responses of all ADCC antibodies to particular whole proteins such as the HIV-1 envelope protein (Env) (7). It now is clear that both CTL and NAb responses against HIV can be directed to particular epitopes that are more effective in controlling viremia than those targeting alternate epitopes (8, 9). Similarly, ADCC responses to specific epitopes may be more effective in controlling HIV replication than others. We recently developed a method to detect and map linear ADCC antibody epitopes by analyzing the ability of ADCC antibodies to activate natural killer (NK) cells in the presence of overlapping HIV peptide sets (10, 11). The HIV antibody-induced expression of degranulation markers by NK cells in this assay correlates with killing of infected cells in cytotoxicity-based ADCC assays. ADCC responses to epitopes identified by this NK cell-activation assay can recognize and lyse HIV-infected cells (12). The NK cell-activation assay allows a finer characterization of epitope-specific ADCC responses.

One measure of the pressure applied by immune responses is their ability to force viral mutations, resulting in escape from immune recognition. Both CTLs and NAb select for immune escape variants during the course of HIV-1 infection (13, 14). Some CTL immune escape variants have reduced replicative capacity (reduced “fitness”) that slows the progression to disease (15, 16). Sequencing single HIV genomes from subjects with acute HIV-1 infection reveals that multiple mutations are acquired during the first months of infection, and most align with sites of CTL or NAb escape mutations (17). However, some mutations do not map clearly to known sites of CTL or NAb escape, suggesting there may be other immune responses, such as ADCC responses, sufficiently potent to select immune escape strains.

We mapped a large series of HIV-specific ADCC epitopes in subjects infected with HIV using a set of consensus HIV peptides. We then cloned and sequenced the subjects’ HIV strains across the relevant epitopes and analyzed whether their ADCC responses were able to recognize their own virus strain. In multiple HIV-specific ADCC responses studied, we found evidence for evolution of immune escape.

Results

Mapping of HIV-Specific ADCC Epitopes. To study evolution across HIV-specific ADCC epitopes, we first mapped a large series of linear HIV-specific ADCC epitopes. We used an intracellular cytokine staining (ICS)-based ADCC assay which measures NK cell activation by ADCC antibodies in the presence of 15-mer peptide pools corresponding to whole subtype B consensus HIV-1 proteins as previously described (10, 11). Patient plasma samples giving positive responses to peptide pools spanning whole proteins were then tested against subpools of 30 peptides to define the region of the epitope more narrowly. Plasma activating >1% of NK cells in response to subpools were then mapped to individual linear 15-mer peptides (Fig. S1). This epitope-mapping exercise yielded a total of 59 epitopes in 22 subjects (Table S1).

Sequencing of Plasma HIV-1 RNA Across ADCC Epitopes. After identifying a series of ADCC epitopes mapped to consensus subtype
of ADCC response, we accessed samples taken within 2 mo of initial HIV infection, and in one subject we analyzed a sample taken 6 y after initial infection. We sequenced the earlier plasma RNA, purchased additional peptides if necessary, and evaluated ADCC responses across a titration of peptide concentrations (Fig. 3). In one subject, a virus sequence from 5 y earlier (only 6 wk after a seroconversion reaction) exactly matched the consensus B sequence to which the epitope was mapped (Fig. 3A). In two other identified ADCC epitopes, additional viral variants were present 8 y previously, within 2 mo of initial infection. Across both these epitopes, the variants identified 8 y previously had better ADCC recognition than the recent variants isolated from the subject (Fig. 3B and C). In one subject, a virus sequence taken 2 y previously but 8 y after initial infection had sequence differences but still was fully escaped (Fig. 3D). These results suggest the ADCC recognition of the consensus peptide reflects variants cross-recognized by ADCC responses induced earlier during infection and that the more recent variants have evolved to escape recognition by ADCC responses.

**Analyses of Sequences Within and Adjacent to ADCC Epitopes.** If ADCC responses specifically selected mutations within the epitope, we might expect to find more mutations within the epitope than random mutations in adjacent sequences. To address this possibility, we cloned and sequenced viral RNA across two epitopes in the C1 region of Env, which has relatively stable sequence. Changes within the epitope were more common than in adjacent sequences (Fig. S2). Because Env sequences are variable, we also analyzed changes in Env that were found only rarely in more than 300 Env clones from different subjects (kindly provided by B. Korber, Los Alamos National Laboratory, Los Alamos, NM). Changes at conserved residues were more common within the ADCC epitopes than outside the epitope.

Another possible reason for the sequence changes observed in the mapped ADCC epitopes is that the epitope was co-recogized by a T-cell response. To address this possibility, we examined T-cell responses to the ADCC epitopes mapped using the same ICS-based assay used for NK cell activation but gating instead on CD3+ T lymphocytes. We did not find any T-cell responses co-inciding with the ADCC epitopes mapped, suggesting T-cell immunity did not result in the mutations observed.

**Mapping Mutational Patterns of ADCC Escape.** The abrogation of ADCC recognition by amino acid changes in the identified linear epitopes suggests that critical amino acids mutate to escape these responses. Multiple subjects had responses mapped to the peptides comprising Env consensus B amino acids 89–103 (termed “E23”) and 33–47 (termed “E9”). To assess whether there were critical amino acid residues that resulted in loss of activity of the ADCC antibodies, we generated a panel of alanine scan mutant peptides (with alanine replacing the amino acid at each residue in turn) across both epitopes. We tested plasma from several donors to the 15 mutant peptides (five for E23, three for E9), in comparison with the consensus peptide to which the epitope was mapped (Fig. 4). For each subject, there were key residues that resulted in reduced ADCC recognition. For epitope E23 (Fig. 4A), two residues (92F and 100V) when mutated to alanine abrogated ADCC recognition of ADCC antibodies in all five subjects. However, many other residues that substantially influenced ADCC antibody function were different for each subject. For example, at position 95W, subject 1 had almost no recognition of the alanine mutant, whereas subject 5 had enhanced recognition of this mutant; at position 101E, the reverse was true. There were also amino acids critical for recognition of the E9 epitope by all subjects (Fig. 4B), but other mutations showed variable reductions in recognition across the subjects. This result suggests that ADCC antibodies targeting individual HIV-1 peptides are sufficiently different that immune escape can evolve with different patterns.
Because we sequenced across both the E9 and E23 ADCC epitopes within the C1 region of Env, we could compare sequence variation within the epitopes and the C1 amino acids between and adjacent to the epitopes. We found that mutations between the epitopes were rarer and tended to be more conservative changes than mutations within the epitopes (Fig. S2). This strongly suggests ADCC responses are applying direct immune pressure at these epitopes.

ADCC Escape from Glycoprotein 140 Proteins. Presentation of linear antibody epitopes within a whole protein can be influenced by conformational and glycosylation changes that affect antibody binding. Additionally, some antibodies recognize conformation-
dependent epitopes that are composed of amino acids that may be distal to each other in the linear sequence. To assess ADCC escape in the context of a whole Env protein, we expressed trimeric glycosylated glycoprotein 140 (gp140) proteins that contained patient-derived mutations within one or both Env ADCC epitopes located within E9 and E23, respectively. We chose these mutations because both are commonly targeted Env-specific ADCC epitopes and several subjects had ADCC responses targeting both epitopes. We compared the ability of the wild-type and mutated HIV-1\textsubscript{ADS} gp140 proteins to activate NK cells in the presence of whole plasma from one antiretroviral therapy (ART)-naive subject that targeted both epitopes (Fig. 5A). A modest loss of ADCC activity for either singly mutated protein and a more substantial loss of ADCC activity to the Env protein with both mutations were detected.

These studies evaluated NK cell activation as a marker for ADCC activity and immune escape. The generation of the protein reagents also allowed us to evaluate whether mutations result in loss of classical ADCC killing using a cell line pulsed with whole Env protein. We used a rapid fluorescent ADCC (RFADCC) killing assay in which loss of intracellular dye reflects target cell lysis (Fig. 5 B and C). Using whole plasma from subjects, we detected minor loss of ADCC killing activity in cells pulsed with the singly mutated Env proteins. With the protein mutated at both epitopes, we detected a more significant loss of ADCC killing activity. The use of a killing-based ADCC assay confirmed the immune escape identified with the ICS-based assay. The results were consistent across a range of plasma dilutions and antigen dilutions.

**Nonneutralizing ADCC Antibodies Can Force Immune Escape.** Many previously studied Env-specific ADCC antibodies also are neutralizing (4). When antibodies mediate both neutralization and ADCC functions, it is difficult to dissect the relative contribution of each function to the generation of immune escape. The Env-specific ADCC antibodies E9 and E23 described above recognize relatively conserved regions of the C1 region of Env, a region not commonly a target of characterized NAb. However, to evaluate formally whether these ADCC antibodies also are neutralizing, we purified the E9 and E23 antibodies from serum over columns (first a protein A column to purify total Ig and then peptide-coated CNBr columns to purify the specific Ab) and assessed the function of the purified antibodies in ADCC and neutralization assays. The purified fractions were composed almost entirely of Ig (Fig. 6A) and retained the ability to activate NK cells specifically in the ICS assay (Fig. 6B). Both total serum and purified total Ig from a subject with ADCC responses could mediate neutralization of the HIV-1\textsubscript{MN} and to a lesser extent HIV-1\textsubscript{SF162} strains, as expected because the total antibody response to Env generally contains NAbS targeting multiple epitopes (Fig. 6C). However, the purified E9 and E23 ADCC antibodies did not mediate neutralization of either HIV strain. This result suggests that the generation of immune escape at the E9 and E23 epitopes was a consequence of ADCC activity rather than neutralization.

**Fig. 4.** Recognition of alanine scan mutants by ADCC antibodies. A panel of mutated epitopes in which each amino acid is replaced by alanine (or lysine if originally alanine) across two commonly recognized ADCC epitopes was assayed for NK cell activation. The ratio of the ADCC response to the consensus B peptide in comparison with the alanine mutant is shown (1 represents equal recognition). (A) E9 peptide (amino acids 33–47 on consensus B HIV-1 Env). (B) E23 peptide (amino acids 93–107).

**Fig. 5.** ADCC escape from gp140 proteins. Gp140 proteins containing wild-type or mutant forms at one or both of two epitopes (E9 and E23) recognized by the same subject were assayed for ADCC responses by (A) the ICS-ADCC NK cell-activation assay (mean ± SEM of triplicates is shown) and by an RFADCC killing assay using (B) different plasma dilutions and (C) different antigen concentrations.
ADCC antibodies to Env C1 E9 and E23 epitopes are nonneutralizing. NK cells. (no. 18) Antibodies (without no. 108 7509 pseudotyped viruses. HIV-negative serum (May 3, 2011) method) did not activate a significant fraction of CD3+56+ NK cells. (C) Whole serum and purified total Ig from subject #72, but not antibodies purified against the E9 and E23 peptides, mediate neutralization of HIV-1MN and HIV-1SF162 pseudotyped viruses. HIV-negative serum (−) and the patient-derived NAb b12 acted as negative and positive neutralizing antibody controls, respectively, and vesicular stomatitis virus glycoprotein (VSVg) acted as a non-HIV Env-bearing pseudovirus control.

Fig. 6. ADCC antibodies to Env C1 E9 and E23 epitopes are nonneutralizing. (A) Antibodies to E9 and E23 Env C1 region peptides from subject #72 were purified using protein A (purifying total Ig) and E9 or E23 peptide-loaded CNBr columns (purifying the specific Ab) and were run on SDS-PAGE gels to illustrate their purity. Lane 1 shows total purified Ig. Lanes 2–4 show three fractions of E9 Abs; lanes 5–7 show three fractions of E23 Abs purified from the Ig sample shown in lane 1. (B) ICS assay for ADCC activity of E23 and E9 purified antibodies. Sera, purified Ig, or purified E9 or E23 antibodies were tested against the E9 and E23 peptide epitopes. Control DMlSO stimulation and use of an irrelevant Ab (to a Vpu ADCC epitope purified using the same method) did not activate a significant fraction of CD3+56+ NK cells. (C) Whole serum and purified total Ig from subject #72, but not antibodies purified against the E9 and E23 peptides, mediate neutralization of HIV-1MN and HIV-1SF162 pseudotyped viruses. HIV-negative serum (−) and the patient-derived NAb b12 acted as negative and positive neutralizing antibody controls, respectively, and vesicular stomatitis virus glycoprotein (VSVg) acted as a non-HIV Env-bearing pseudovirus control.

Discussion
This report provides evidence that nonneutralizing ADCC antibodies can force HIV escape mutations. By mapping a large series of ADCC linear epitopes and sequencing autologous HIV RNA across epitopes, we found multiple ADCC antibodies that no longer could recognize autologous viral sequences. Viral sequences from very early in infection were recognized more strongly by recent plasma samples, suggesting evolution of escape over time. Immune escape was identified initially using ADCC peptide epitopes and an NK cell-activation assay and subsequently confirmed using whole Env proteins and a standard killing-based assay.

The HIV genome is able to make multiple changes to avoid CTL, NAb, and antiretroviral drug pressure, and perhaps it should be no surprise that ADCC antibodies also are implicated in viral escape. Nonetheless, the potent immune pressure that can be applied by HIV-specific ADCC antibodies has been brought into sharper focus only recently. In particular, elegant macaque passive transfer studies of NAb mutated in the Fc region so they no longer engage Fc receptors and exert ADCC activity show reduced potency in preventing HIV infection (4). We show that nonneutralizing ADCC antibodies no longer recognize autologous HIV-1 strains, indicating that the selective pressure mediated by ADCC activity is sufficient to drive immunological escape. Passive transfer studies of “ADCC-only” antibodies (without neutralizing activity) will be of interest to evaluate further the potential therapeutic utility of ADCC activity.

ADCC antibodies could be responsible, in part, for the partial efficacy of the recently reported canarypox prime/protein boost vaccine efficacy trial in Thailand (1). Identifying potent ADCC responses that are difficult to escape and inducing these responses by vaccination is a priority for future studies.

This work probably substantially underestimates the number of ADCC epitopes targeted by each HIV+ subject because (i) only linear epitopes are readily mapped and dissected with our ICS technique, and (ii) only consensus B overlapping peptides were used for screening. Conformational ADCC antibodies also are likely to elicit escape, but mapping such responses and identifying escape patterns will be more difficult and will require large numbers of mutant whole Env proteins.

ADCC-forced mutations theoretically could incur some fitness cost to viral replicative capacity, similar to that observed for CTL escape variants (19). Constructing replicating viruses with ADCC-induced mutations will allow testing of this hypothesis. More potent ADCC antibodies are likely to target conserved or functional domains of viral proteins. The Env protein is highly diverse and readily escapes CTL and NAb responses with apparently minimal fitness costs (13, 20–22). It is possible that any fitness cost of ADCC escape in Env could be small. Identifying ADCC antibodies targeting conserved non-Env proteins may reveal more potent ADCC antibodies. For example the Vpu ADCC we described does not appear to undergo immune escape (Fig. 2B). We recently observed that purified ADCC antibodies to this Vpu epitope can recognize and lyse HIV-infected cells (12). Further studies on the patterns of ADCC escape should allow a better understanding of how to limit ADCC escape or force larger fitness costs. Our alanine scan data to date show that even subjects with ADCC antibodies that target the same region of Env undergo divergent patterns of immune escape, suggesting multiple pathways for immune escape at these epitopes are possible.

If escape from ADCC responses does incur a fitness cost, we predict that some ADCC-induced mutations will revert to wild type in the absence of immune pressure following onward transmission of HIV-1. This reversion is observed commonly with CTL mutations (16). Note that this reversion of ADCC-induced mutations may be transient if the recipient subsequently mounts ADCC responses to the same epitope (23). Longitudinal analyses of HIV strains in donor–recipient pairs for reversion of ADCC-induced mutations will be of substantial interest.

In summary, we provide evidence of viral escape from HIV-specific ADCC antibodies. Escape patterns are complex and evolve over time. On the one hand, this result provides evidence of the pressure applied by ADCC antibodies. On the other hand, however, it presents challenges in inducing the most effective ADCC antibodies by vaccination.
Materials and Methods

HIV-Infected Subjects. Viremic HIV-infected adults who were ART-naive (n = 80) or who had ART-resistant HIV (n = 12) were recruited from the Melbourne Sexual Health Centre and the Alfred Hospital (Australia) to donate blood samples (10–24). All subjects provided informed consent. The relevant human research ethics committee approved all studies.

HIV-1 Antigens. HIV-1 peptides (15 amino acids in length) overlapping by 11 amino acids of consensus B sub-type strain were kindly provided by the National Institutes of Health (NIH) AIDS reagent repository. To map ADCC activity across Env, we studied subpools of ∼30 Env peptides and then individual Env peptides as previously described (10). Plasmids for the expression of soluble, uncleaved Env analogs (gp140) were generated by mutating the DNA sequence corresponding to the cleavage site between gp120 and gp41 and inserting a stop codon immediately before the transmembrane domain to produce pN1-AD8-140 as previously described (25). Plasmids encoding Env gp140 proteins with specific mutations corresponding to putative ADCC escape mutants at two epitopes also were generated by PCR-based mutagenesis. All plasmids were transiently transfected into 293T cells, and gp140 was purified from the tissue culture medium using Ni-agarose.

RFADCC Assay. The RFADCC assay was used as previously described (7, 10). In brief, the CEM-Nkr-CCRS T lymphoblast cell line (kindly provided by the NIH AIDS reagent repository) was labeled with the intracellular dye carboxyfluorescein succinimidyl ester (CFSE) and the membrane dye PKH26 and then pulsed with the antigens (3 μg/mL) × 10^6 cells/μL for 2 h at room temperature, otherwise noted). Healthy donor peripheral blood mononuclear cells (PBMCs) and plasma from the HIV-infected subjects were added to the labeled CEM-Nkr-CCRS cells for 4 h. The proportion of cells that maintained membrane expression of PKH26 but had lost intracellular CFSE (i.e., lysed cells) was analyzed by flow cytometry.

ICS Assay for ADCC Activity. The ICS-based assay was used to measure HIV antibody-mediated NK cell cytokine expression and degranulation as previously described (10, 11). In brief, 200 μL of fresh whole blood or 50 μL of patient Na-heparin anticoagulated plasma together with 150 μL of healthy donor blood was incubated with either the pool of overlapping 15-mer Env peptides or gp140 Env proteins for 5 h in the presence of Brefeldin A and Monensin (Sigma). At the end of the incubation CD56−/CD69+ or CD69+ cells were subjected to spinoculation at 1,200 × g for 2 h at room temperature, and cultured for 2 d. Target cells were analyzed for EGFP expression by flow cytometry. The reported % neutralization = (1 − [virus + immune serum or antibody/virus + medium]) × 100, where infection levels observed in the presence and absence of neutralizing antibodies are presented as the mean ± SD of duplicate samples.

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