

# Research Letters

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## HIV-1 natural viral suppressors: control of viral replication in the absence of therapy

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**The objective of this study was to identify and define a cohort of HIV-1-infected individuals with the ability to suppress HIV-1 replication naturally (patients with undetectable viral loads for at least 2 years without antiretroviral drugs). Proviral DNA ranged from one to 74 copies/10<sup>6</sup> peripheral blood mononuclear cells, with the mean value lower by 1–2 logs than long-term non-progressors. This ability to control HIV-1 viral replication naturally has led us to refer to them as natural viral suppressors.**

Over the past 10 years numerous studies have been undertaken on HIV-1 long-term non-progressors (LTNP), and although some new insight has been gained from studying this cohort, such studies have been marked by contradictory results, and ultimately key immunoregulatory mechanisms involved with the control of HIV-1 replication have not been elucidated [1–14]. The term ‘long-term non-progressor’ refers to HIV-1-positive patients who, depending on the study, have had CD4 cell counts greater than 500–600 cells/ $\mu$ l for more than 7–10 years in the absence of therapy [1–3,15,16]. It is estimated that up to 5–10% of patients with HIV infection can be classified as LTNP [17,18]. Importantly, nowhere in the definition of LTNP is viral load considered, and in most series the HIV-1 viral loads are heterogeneous, ranging from 10<sup>2</sup> to 10<sup>4</sup> copies/ml [1–3,15]. However, in most LTNP series at least one or more patients have had undetectable HIV-1 viral loads, and recently several case reports and small studies have focussed on this group of patients [19–21]. We have established a sizeable cohort of patients who demonstrate the natural ability to suppress HIV-1 replication *in vivo* to a degree only seen with HAART therapy, and refer to them as ‘natural viral suppressors’ (NVS).

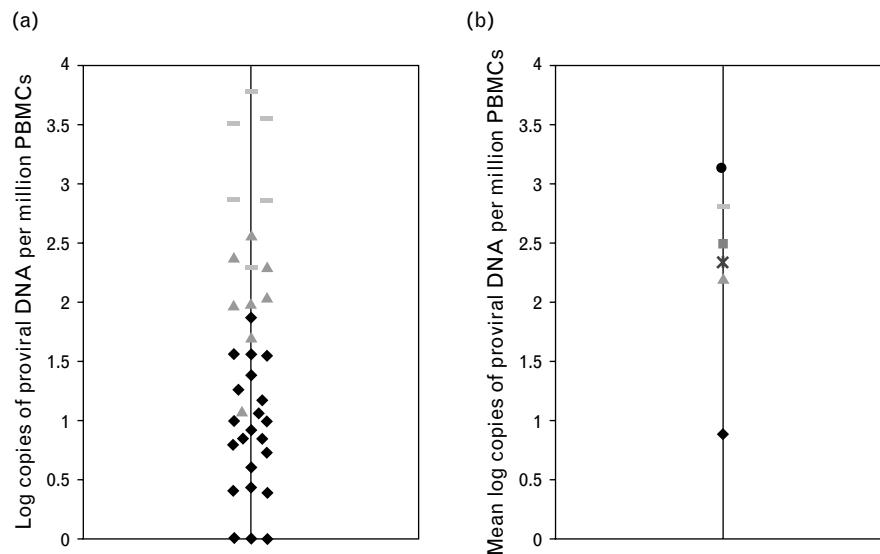
In order to be classified as a NVS, volunteers were required to have confirmed HIV-1 infection by both Western blot and the detection of proviral DNA in peripheral blood mononuclear cells (PBMC). Furthermore, natural viral suppression was defined as a minimum of four viral load determination of less than 400 copies/ml for at least a 2-year period in the absence of the use of antiretroviral therapy. One viral blip or viral load greater than 400 copies/ml during the above time period

was permitted if the subsequent viral loads were less than 400 copies/ml. NVS entry criteria also required volunteers to be antiretroviral naive, although no more than 2 weeks of antiretroviral drug use was permitted as was short-term use for pregnancy prophylaxis.

DNA was extracted from PBMC by use of the QIAgen mini-blood kit (Qiagen, Valencia, California, USA). Real-time polymerase chain reaction was performed using the SyberGreen kit (Qiagen). An aliquot of 10  $\mu$ g of PBMC DNA was amplified using SK145/SK431 targeting the *gag* region; 25  $\mu$ l SyberGreen mix was added to 5.1  $\mu$ l of each 2.5  $\mu$ mol primer and DNA. Polymerase chain reaction conditions were as follows: DNA from PBMC was denatured at 95°C for 15 min, then amplified for 40 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1.3 min. The limit of detection of this assay was one copy per 1.5  $\times$  10<sup>6</sup> cells as determined by the amplification of serial dilutions of DNA prepared from the ACH-2 cell line. Controls consisted of HIV-1 infected individuals off therapy with HIV-1 viral loads greater than 100 000 copies/ml (progressors) and those with HIV-1 viral loads of 500–15 000 copies/ml (low viral loads).

So far, 22 HIV-1 NVS have been enrolled in the prospective cohort. These individuals have a median of 12 years of HIV infection, 5.5 years of HIV-1 viral suppression, and a CD4 cell count of 887 cells/ml. As a group, these patients have the lowest reported proviral DNA levels of any HIV-infected patients, with a range from one to 74 copies of proviral DNA per 10<sup>6</sup> PBMC. The mean proviral DNA copy number was lower than both the controls we used in this study, HIV progressors and individuals with low HIV-1 viral loads (Fig. 1a). The latter group consisted of HIV-infected individuals who had viral loads of 500–15 000 copies/ml, viral loads that are comparable with the majority of LTNP. Because some individuals in this group did not meet the classic definition of LTNP, we compared the mean HIV proviral load of the NVS cohort with five published LTNP cohorts [1,2,22–24], and the NVS cohort was consistently 1–2 logs lower than that described in LTNP (Fig. 1b), again confirming that individuals classified as NVS did in fact demonstrate more effective control of *in-vivo* HIV-1 replication as reflected by quantification of the HIV-DNA proviral copy number.

Interestingly, there has been a report of several patients who had both undetectable proviral DNA and HIV-1 viral loads [20]; however, this was probably caused by limitations of the assay (such as the lack of sensitivity of the assay, which only went down to 10 copies/10<sup>6</sup> PBMC) or



**Fig. 1. HIV-1 proviral copy numbers per  $10^6$  cells.** (a) Progressors are individuals with HIV-1 viral loads greater than 100000 copies/ml. Low viral loads refer to individuals with viral loads of 500–15000 copies/ml.  $\blacklozenge$  Natural viral suppressors;  $\blacktriangle$  low viral loads;  $-$  progressors. (b) Mean HIV-1 proviral copy numbers/ $10^6$  cells of the natural viral suppressor cohort compared with published data of long-term non-progressor (LTNP) cohorts [1,2,22–24].  $\blacklozenge$  Natural viral suppressors;  $\blacktriangle$  LTNP1;  $-$  LTNP2;  $\blacksquare$  LTNP3;  $\bullet$  LTNP4;  $\times$  LTNP5. PBMC, Peripheral blood mononuclear cell.

a false positive Western blot. In our own experience, using assays that tested one microgram of DNA (corresponding to approximately 150 000 PBMC or a limit of detection of 7 copies/ $10^6$  PBMC), only 25% of the individuals tested positive for proviral DNA (data not shown). The requirement of having a positive proviral DNA is thus important to ensure individuals are truly infected with HIV, especially as some members of the cohort have repeatedly been tested for HIV by their primary physicians, and several were even told they did not have HIV after viral load testing was negative.

Patients with HIV-1 infection who are able to suppress HIV infection naturally in the absence of antiretroviral therapy represent one unique extreme of HIV-infected patients. In the past, the focus has been on LTNP who, as a cohort, have demonstrated a variable ability to suppress HIV-1 replication. This may be a reason for the contradictory findings seen in many LTNP studies and the fact that the key effectors responsible for HIV-1 suppression have yet to be defined. We focused on those patients who have demonstrated the natural ability to suppress HIV-1 virus replication consistently, regardless of CD4 cell counts. This difference, although subtle in terminology, is nonetheless important, especially considering that LTNP comprise up to 10% of the HIV-infected population, whereas NVS have a prevalence of 1.5% in our clinic population.

The study of NVS, which represent a more homogeneous group than LTNP with regard to HIV-1 suppression, should lead to a better understanding of the immunoregulatory mechanisms involved in HIV-1 viral suppress-

sion. Although it is too early to postulate on the factor or factors that make this group unique, there are probably multiple mechanisms involved in the effective in-vivo regulation seen in HIV-1 NVS.

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### Full fusion competence rescue of the enfuvirtide resistant HIV-1 gp41 genotype (43D) by a prevalent polymorphism (137K)

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We followed genotypic changes in HIV-1 gp41 during enfuvirtide therapy in a group of antiretroviral-experienced patients. The study confirmed the development of a resistance mutation (N43D), but revealed an interesting compensatory mutation (E137K), which was either present in the pretreatment virus or developed during therapy. In a functional assay of viral envelope fusogenicity we found that the 43D genotype had a 1.4 log<sub>10</sub> decrease in infectivity, which could be restored by the introduction of 137K.

During treatment of HIV-1 the occurrence of drug-resistant variants is a major obstacle for the long-term success of therapy. It has been established that genotypic resistance mutations are a strong independent predictor of protease inhibitor treatment outcome [1]. Studies have tried to determine the clinical importance of one or more resistance mutations [2]. An inverse correlation between the number of resistance mutations in genes targeted by antiretroviral drugs and viral fitness has been reported [3]. This suggests that continued treatment despite resistance may confer an advantage in patients with limited options in treatment [2].

Enfuvirtide is the first drug to target the HIV-1 viral envelope (Env) protein [4]. The Env protein consists of two functional subunits; the surface subunit gp120 and the transmembrane subunit gp41. Upon receptor attachment, large conformational changes in the gp41 subunit initiate and complete the membrane fusion step. Gp41 contains well-conserved heptad repeat domains (HR1 and HR2) [5]. During fusion, HR1 helices form a trimeric coiled-coil structure upon which the HR2 helices fold back, thereby forcing the membranes into close proximity. Enfuvirtide blocks this step by competitively mimicking the viral interactions between HR1 and HR2 [6].

Genotypic changes in HR1, especially positions 36V/D, 38A/S and 43D/S, have a pronounced impact on enfuvirtide susceptibility [7–9]. To assay the fitness of enfuvirtide-resistant variants we sought to detect the genotypic changes in HR1 as well as HR2.

Nine highly antiretroviral-experienced patients were enrolled in the study. One missed follow-up and was

withdrawn. From eight patients HIV-1 plasma RNA was isolated and amplified by reverse transcriptase–polymerase chain reaction. Of these, we obtained a sequence from five patients. These patients were sampled at the subsequent visits to the outpatient clinic. Informed consent was obtained from all patients. The study was approved by the Ethics Committee at the County of Aarhus, Denmark.

All five patients in the study experienced a temporary decline in plasma HIV-RNA when enfuvirtide was introduced to the antiretroviral regimen. Patient 1 had a viral load decrease of  $1.8 \log_{10}$  initially but rebounded at day 21. Simultaneously, 43D was detected and remained throughout the observation period. At day 48, a 137K mutation appeared in conjunction with 43D. Sequencing of HIV-RNA from patient 2 revealed a N43S mutation after more than 23 months of enfuvirtide therapy. Patients 6 and 7 harboured resistance mutations V38A (day 14) and G36D (day 66), respectively. Patient 9 experienced a decrease ( $2.5 \log_{10}$ ) in viral load upon enfuvirtide initiation, but at day 101 a complete rebound had occurred. Sequence analysis confirmed the interesting mutation constellation 43D–137K. Of interest is the presence of 137K as a natural viral polymorphism in patients 7 and 9 supported by the Los Alamos sequence database [10].

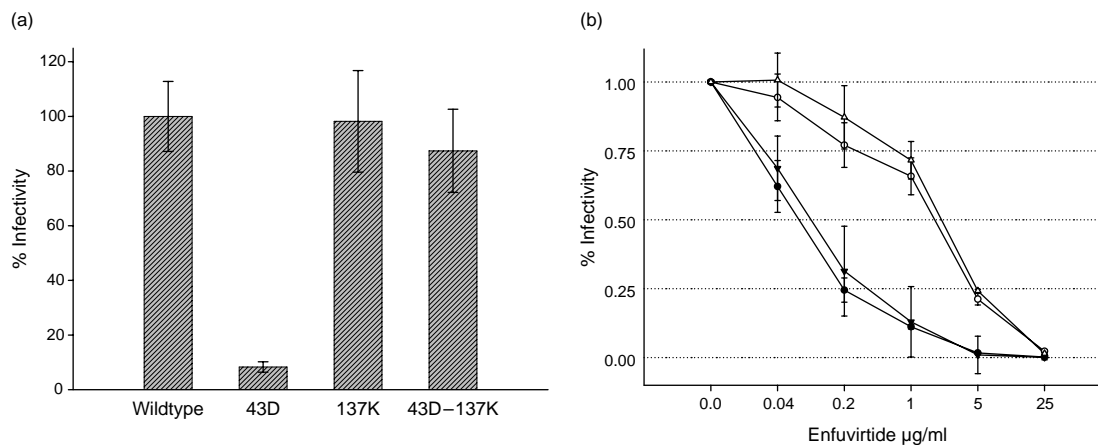
The enfuvirtide-resistant genotype 43D, the single HR2 mutant 137K, and the double mutant 43D–137K were generated in a bicistronic HIV-1 envelope vector (M. Tolstrup, in preparation). MLV HIV-1 envelope pseudotyped viruses were generated by transient transfection of 293 T cells. Titre was measured using flow cytometry.

The wild type, the single 137K, and the double mutant 43D–137K had comparable infectivity with  $5.3 (\pm 0.13)$ ,  $5.2 (\pm 0.08)$  and  $5.1 (\pm 0.15) \log_{10}$  IU/ml, respectively. The 43D single mutants had an infectivity of  $3.9 (\pm 0.01) \log_{10}$  IU/ml, which was found to be significantly less than wild type ( $P < 0.0001$ , Student's *t*-test). Figure 1 (a) shows the relative infectivity. The decreased infectivity (8% of wild type) of the 43D mutant corresponds to a fitness reduction of 92%, whereas the double mutant 43D–137K and single 137K display infectivity comparable to wild type (87 and 98%, respectively).

The inhibitory effect of enfuvirtide on the four genotypes is depicted in Fig. 1(b). The  $IC_{50}$  values for the single 43D and double 43D–137K genotypes are significantly larger than wild type, with 1.85 and 2.1  $\mu\text{g}/\text{ml}$  ( $P < 0.001$ , Student's *t*-test), respectively. This corresponds to an approximately 30-fold increase in resistance.

To investigate further the cause of the observed infectivity differences between the genotypes, we investigated the level of HIV-1 Env in the producer cells. First, we verified an equal amount of transcribed RNA between the genotypes. Second, we measured the cell surface expression of the Env protein by flow cytometry. Both assays revealed identical levels across the four genotypes.

In this study we found that in patients treated with enfuvirtide the occurrence of 43D in HIV-1 Env gp41 results in an approximately 30-fold increased resistance and reduces the viral fitness by 92%. Importantly, a complete rescue of viral fitness was caused by the 137K genotype that either developed during treatment or was present before treatment. This results in a dominant viral



**Fig. 1. Relative infectivity of the four genotypes and inhibitory effect of enfuvirtide.** (a) Infectivity of the four genotypes as measured in a single-round assay is displayed as the percentage compared with wild type; the average of four independent experiments. The infectivity of the 43D genotype was statistically significant from all others at  $P < 0.001$ . No statistically significant difference was found between any of the remaining three genotypes. (b) Enfuvirtide inhibition of the four genotypes depicted as percentage infectivity compared with no enfuvirtide. Enfuvirtide  $IC_{50}$  values were obtained by fitting a linear regression line to the inhibitory curve. Student's *t*-test was applied to detect difference relative to wild type.  $IC_{50}$  values; wild type (0.066  $\mu\text{g}/\text{ml}$ ), 137K (0.085  $\mu\text{g}/\text{ml}$ ), 43D (1.85  $\mu\text{g}/\text{ml}$ ), 43D–137K (2.1  $\mu\text{g}/\text{ml}$ ); the average of three independent experiments. —●— Wild type; —○— 43D; —▼— 137K; —△— 43D–137K.

population with wild-type fitness and low susceptibility to enfuvirtide.

These data suggest that the negatively charged aspartate is unfavourable in the formation of the coiled-coil structure and structural rearrangements during fusion. This is the first report to test the 43D single mutant because other studies have used primary isolates that have been obscured by the compensatory mutation 137K [7,11]. Considering the clear effect on fusion competence of introducing 137K on top of 43D presented here, we therefore hypothesize that the constraint on forming the fusion-competent coiled-coil lies in the charge repulsion observed in the 43D–137E genotype. Structure modeling predictions recently indicated that mutations in residues 135–145 in HR2 could improve the stability of the coiled-coil [12]. Our finding of a compensatory mutation in HR2 substantiates this. It is therefore highly plausible that other enfuvirtide-resistant variants with mutations at position 36V/E and 38A/E could interact with residues in HR2 in an analogous manner.

This furthers the speculation on the clinical implications for the presence of the 137K/Q polymorphism. It is evident that viruses with 137K will have a shorter evolutionary pathway to develop an enfuvirtide-resistant phenotype combined with wild-type fitness. We summarized 63 enfuvirtide patients reported in seven studies [7,8,11,13–15]. The results indicated a disproportion of the 43D resistance mutation based on HR2 genotype [74% (14/19) of 43D carry mutation at 137;  $P=0.014$  Fisher's exact test]. Along this line it has previously been suggested that the pre-existence of baseline fitness compensatory mutations could influence virological response because of rapid resistance evolution [3]. It will therefore be of interest to investigate the time to virological failure in enfuvirtide-treated patients depending on the HR2 genotypic profile.

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## Durable neutralizing antibodies after remote smallpox vaccination among adults with and without HIV infection

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**The only US licensed vaccine with established efficacy against smallpox, Dryvax, is contraindicated for HIV patients. Detectable smallpox-neutralizing antibodies are still present among US adults. This study compared vaccinia-neutralizing antibody titers between 20 HIV-infected and**

**20 uninfected veterans matched for age and military entry. Vaccinia-neutralizing antibodies were detected in 95% HIV-infected and 100% uninfected veterans; 40% HIV-infected and 70% uninfected adults had protective titers. Therefore, after robust vaccination, neutralizing antibodies are maintained for prolonged times despite CD4 cell depletion.**

The only US licensed smallpox vaccine with established efficacy is a live attenuated vaccinia vaccine, Dryvax [1]. Routine smallpox vaccination within the United States stopped in 1971 for the general population and in 1990 for the military, and then was re-instituted in 2002 for selected military and government personnel. Protection from lethal infection among those remotely vaccinated has been shown [2]. Neutralizing antibody has been detected for up to 75 years in some individuals vaccinated with one to two doses [3].

Veterans who served in the military before 1991 received at least two Dryvax vaccinations: first during their childhood before 1972 and second upon entry into the military. The durability of adult antibody responses among those who acquired HIV infection after smallpox immunization is, however, unknown. Because of concerns for progressive vaccinia among patients with compromised cell-mediated immunity [4], HIV infection is currently a contraindication for Dryvax vaccination [5] except in the event of an exposure during a smallpox emergency [6]. In this pilot study, we compared differences in neutralization antibody titers between HIV-infected and uninfected veterans who had previously been immunized.

Veterans with active military service during 1980–1990 were recruited; any individuals revaccinated after 1991 were excluded. After obtaining informed consent, 20 HIV-infected and 20 uninfected control veterans were matched for age within 5 years and entry into active military service. Each veteran was evaluated for a history of smallpox vaccination and the presence of a vaccination scar as a marker of successful primary vaccination. For HIV-infected individuals, the year of HIV seroconversion, CD4 cell count, HIV-RNA level, and the use of antiretroviral therapy were collected. For control subjects, medical records were reviewed to confirm the absence of HIV diagnosis, or immunocompromising disease(s) or treatment(s). No HIV-infected or control subject had any documented history of hyper or hypogammaglobulinemia or significant protein loss from renal or gastrointestinal sources; three control subjects with diabetes mellitus had evidence of trace albuminuria.

Serum was collected from each subject and stored at  $-80^{\circ}\text{C}$  until assayed in batches for specific neutralizing antibody titers [7]. The lower limit of detection for the vaccinia neutralizing antibody was 1:20, with antibody

titers of 1:100 or greater considered in the protective range based on a monkey pox challenge in primates [8]. Measles IgG was chosen as a control vaccine against live attenuated virus [9]. Measles IgG titers, as measured by a commercial enzyme immunoassay (LabCorp, Burlington, North Carolina, USA) had the lower limit of detection of 0.91; titers greater than 1.09 were considered immune.

Categorical data were analysed using Fisher's exact test and continuous data using Student's *t*-test. The proportions of subjects with protective/immune smallpox and measles antibody titers were compared between the HIV-infected and control groups.

All subjects had evidence of previous smallpox vaccination scars. Twelve patients were receiving HAART consisting of two or more nucleoside/nucleotide reverse transcriptase inhibitors and either non-nucleoside reverse transcriptase inhibitors or protease inhibitors, or both. One patient received nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors and protease inhibitors concomitantly; none received an HIV fusion/entry inhibitor.

Table 1 summarizes the clinical characteristics and antibody data for all subjects. Although matched within 5 years of age, a difference of 2.8 years in mean age was seen between the two groups, but the year of entry into the military and thus revaccination was similar for both groups. Measles antibody titers were detected in 17 out of 20 HIV-infected and 20 out of 20 uninfected individuals. Importantly, 19 out of 20 HIV-infected and 20 out of 20 control veterans had detectable vaccinia-neutralizing antibodies. Although a higher proportion of control subjects had vaccinia-neutralizing antibody titers greater than 1:100 compared with HIV-infected subjects (14/20 versus 8/20), this difference did not reach statistical significance.

This study demonstrated that veterans vaccinated first as children and then upon military entry maintained durable vaccinia-neutralizing antibody titers, including those with HIV. The majority of these HIV-infected individuals also retained significant measles titers.

The long-term persistence of humoral immunity after measles [10] and smallpox [6] vaccinations have been reported for immunocompetent hosts. The measles-specific IgG titers, detected in 85% of our HIV-infected subjects, were comparable to the published report of detectable antibody titers in 95% of remotely measles-vaccinated HIV-infected adults, with no differences between those with CD4 cell counts greater than 400 cells/ $\mu\text{l}$  compared with those with more advanced HIV [11].

Our data demonstrate the durability of virus-specific neutralizing antibodies after vaccinia vaccinations, not

**Table 1. Summary of clinical and antibody data for 20 HIV-infected and 20 control subjects.**

Parameter	HIV-infected subjects	Control subjects	<i>P</i>
Age, years	39.0 ± 3.9	41.8 ± 4.1	0.03 <sup>b</sup>
Sex, female/male	0/20	2/18	0.49 <sup>c</sup>
Entry into military, year	1984 [1981–1990]	1984 [1981–1990]	1.00 <sup>b</sup>
Vaccination scar present	20/20 (100%)	20/20 (100%)	1.00 <sup>c</sup>
Median years of known HIV	6 [0.3–18]		
Median CD4 cell count, cells/μl	241 [0–904]		
Median CD4 percentage	13% [0–36%]		
Median CD8 cell count, cells/μl	840 [119–2412]		
Median CD8 percentage	55% [29–77%]		
HIV RNA, <sup>a</sup> log <sub>10</sub> copies/ml	4.45 ± 0.91		
Undetectable HIV RNA	3/20 (15%)		
HIV RNA > 500 000 copies/ml	2/20 (10%)		
Receiving HAART	12/20 (60%)		
Vaccinia-neutralizing antibody	19/20 (95%)	20/20 (100%)	1.00 <sup>c</sup>
Mean reciprocal titer	103 ± 91	145 ± 98	0.18 <sup>b</sup>
Median reciprocal titer	60 [ $< 20$ –329]	125 [20–378]	
Vaccinia titer ≥ 1:100	8/20 (40%)	14/20 (70%)	0.11 <sup>c</sup>
Measles antibody	17/20 (85%)	20/20 (100%)	0.23 <sup>c</sup>
Mean measles titer	1.97 ± 0.57	2.27 ± 0.59	0.13 <sup>b</sup>
Median measles titer	1.90 [ $< 0.91$ –3.05]	2.30 [1.19–3.46]	
Measles titer > 1.09	17/20 (85%)	20/20 (100%)	0.23 <sup>c</sup>

Results are expressed as mean ± SD, [range] and (%).

<sup>a</sup>For those with detectable circulating viremia in the range of 75–500 000 copies/ml.

<sup>b</sup>Difference in means was tested by two-tailed Student's *t*-test.

<sup>c</sup>Difference in proportions was tested by two-tailed Fisher's exact test.

only among the immunocompetent, but also in adults with acquired HIV infection and a variable loss of CD4 cells and function. Among control subjects born before 1969, 74% had vaccinia-neutralizing antibody titers greater than 1:100. This rate is similar to that reported for the Japanese general population, in which approximately 80% of individuals born before 1969 had detectable neutralizing antibodies after routine smallpox vaccinations [12].

Vaccinia-neutralizing antibodies have been pivotal in preventing smallpox disease and death [2]. Smallpox immunity and recovery from disease were shown to be B and T-cell dependent. In studies of B-cell immunity, smallpox-specific B cells were found to be maintained at a frequency of approximately 0.1% total circulating IgG-positive B cells in excess of 50 years after vaccination, and correlated with circulating antibody levels [7,13]. Similarly, long-term vaccinia-specific memory T cells were 0.01–0.15% of the total CD4 cells in 14 out of 16 adults [14]. The previous studies, however, could not determine whether the maintenance of antibody titers was dependent on long-term propagation of vaccinia-specific helper T cells.

Our findings of significant titers of vaccinia-neutralizing antibodies even in HIV-infected individuals with critical CD4 cell loss (10/20 with  $< 200$  CD4 cells/μl) suggest that CD4 T-cell-independent mechanisms contribute to the long-term maintenance of antibody titers. Several reports documented the persistence of long-lived bone-marrow plasma cells [15–17]. Human memory B cells were shown to undergo activation and differentiation into

plasma cells in response to polyclonal stimuli, such as bacterial-derived agonists of Toll-like receptors. Whereas antigen boosting leads to a transient increase in specific antibody levels, the periodical polyclonal activation of memory B cells, even in a T-cell-independent fashion, may contribute to the maintenance of lifelong serological memory [18].

Importantly, the durable vaccinia neutralization antibody titers in remotely vaccinated and subsequently HIV-infected adults should offer some protection against the risk of fatal disease, if smallpox is used as a bioterrorism weapon.

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This study was reviewed in accordance with the ethical standards on human experimentation and with the Helsinki Declaration of 1975 and its revision in 2000, and was approved by the Institutional Review Board and Research and Development Committee of the VA Medical Center.

The views expressed in this article are those of the authors and do not necessarily represent the views of the Department of Veterans Affairs or the Food and Drug Administration.

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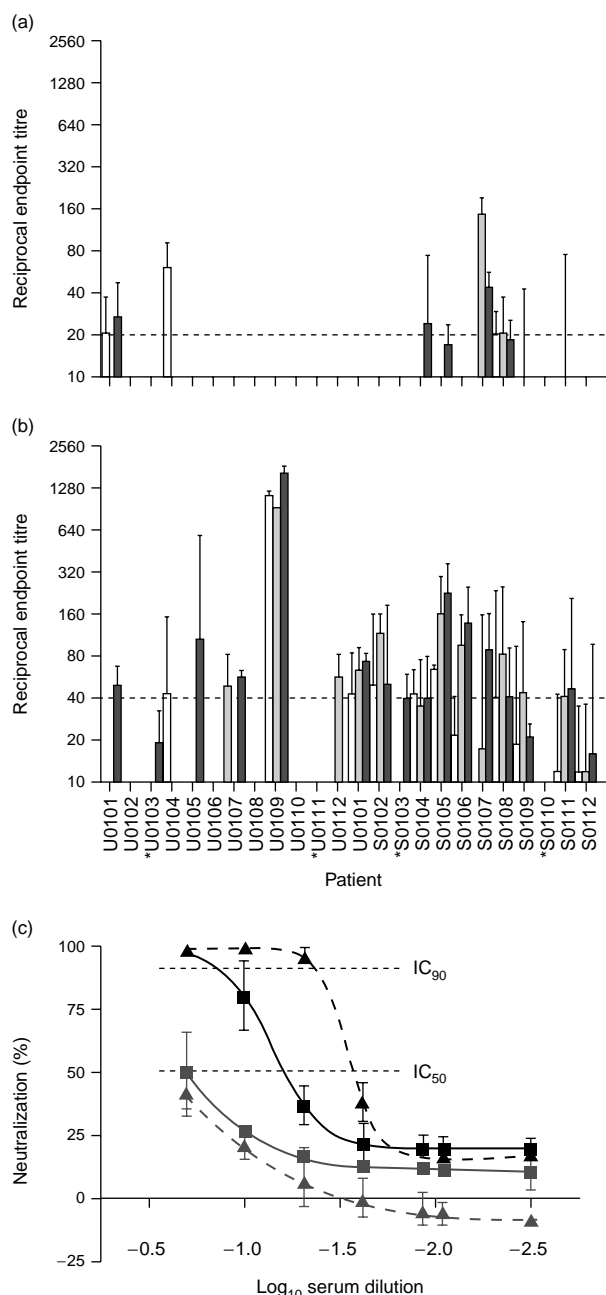
## A functional human IgM response to HIV-1 Env after immunization with NYVAC HIV C

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**Env-specific IgG and IgM were detected in 25 and 60%, respectively, of volunteers immunized with NYVAC expressing clade C gp120. The serum sample with the highest IgM titre but undetectable IgG neutralized the homologous isolate with a reciprocal IC<sub>90</sub> titre of 7.8 in the absence of complement, and 24.4 in the presence of complement (P=0.0003). These results suggest that vaccine-induced, Env-specific IgM may have antiviral activity and should be subjected to further investigation.**

It is generally accepted that a protective, prophylactic HIV-1 vaccine will probably have to elicit broadly neutralizing antibodies. Several candidate immunogens designed to do this have been evaluated in clinical trials [1]. Broad neutralization has not been achieved, however, and the current cohort of vaccine candidates being tested in clinical trials are designed primarily to induce T-cell responses. Although the gold standard measure of the antiviral activity of antibodies against HIV-1 Env is virus neutralization *in vitro*, it has been suggested that other antibody functions including antibody-dependent complement-mediated inactivation (ADCMI) [2], antibody-dependent cellular cytotoxicity [3,4], and IgG-FcγR-dependent endocytosis and degradation [5] can contribute to antiviral activity. Such antibody-associated activities have yet to be shown to correlate with protection, warranting further investigation.

The European Union consortium EuroVacc has developed and tested vaccine candidates based on the synthetic HIV-1 construct HIV C developed by Sanofi Pasteur. HIV C is codon-optimized to express a fusion protein Gag-Pol-Nef and is secreted gp120 from the clade C primary isolate HIV-1<sub>97CN54</sub>, which is highly relevant to



**Fig. 1. Env-specific IgG and IgM responses in EV01 volunteers and the antiviral activity of serum U0109 week 24 in the presence of fresh or heat-inactivated normal human serum.** (a) Serum IgG and (b) serum IgM responses to gp140<sub>97CN54</sub> were determined by enzyme-linked immunosorbent assay. Bars represent the median titre and range from three to four replicates. The dashed lines indicate the cutoff points above which sera are considered positive. \* Indicates the four volunteers who received placebo immunizations of vehicle alone. (c) The antiviral activity of patient U0109 week 24 (WK24) serum diluted in a fixed 10% (v/v) concentration of fresh or heat-inactivated normal human serum (HI NHS) is compared with the antiviral activity of dilutions of fresh or HI NHS alone. Note that the U0109 combinations plateau out around the level of neutralization achieved by 10% fresh or HI

the epidemic in southeast Asia [6]. This construct was used to produce the recombinant poxvirus vector NYVAC HIV C that has been tested in two phase I clinical trials in Europe [7,8]. As part of the first trial, EV01, we determined the serum IgG and IgM responses to HIV-1<sub>97CN54</sub> Env at weeks 4, 8 and 24 after one or two doses of NYVAC HIV C, given at weeks 0 and 4. Env-specific IgG and IgM responses were measured by enzyme-linked immunosorbent assay (ELISA). ELISA plates (Greiner Bio-One; Stonehouse, UK) coated with 50 ng/well gp140<sub>97CN54</sub> were washed in phosphate-buffered saline (PBS) and blocked for 2 h with 200 µl/well of 2% (w/v) dried milk in PBS. Serial dilutions of volunteer serum prepared in PBS/1% bovine serum albumin (Sigma, Poole, UK) were added to the washed plates for 1 h. After further washing, 50 µl/well of anti-human IgG-horseradish peroxidase or anti-human IgM-horseradish peroxidase (Jackson ImmunoResearch Europe, Soham, UK) was added at 0.8 µg/ml for 1 h before washing and the addition of ultra-tetramethylbenzidine ELISA development reagent (PerBio Science, Cramlington, UK). The reaction was stopped and the optical density (OD) read at 450 nm. The OD of the prebled serum was subtracted from the OD of each postvaccination serum, and the assay cutoff was determined from the mean absorbance plus 2SD of wells incubated without serum. Sigmoidal dose-response curves were fitted to the data using GraphPad Prism (version 4.01) [9]. The reciprocal of the dilution at which the curve bisected the assay cutoff was reported as the endpoint titre. The assays were repeated on three to four independent occasions.

Weak or borderline IgG responses (endpoint titre ≥ 20) were observed in 25% of vaccinees (Fig. 1a) and none of the 12 sera samples from the four placebo recipients. Placebo recipient S0103 had an IgM titre greater than 20 at week 24. To interpret the IgM-titration data in a parsimonious manner, sera were only scored positive if they had a median IgM titre of 40 or greater, and furthermore, only if they had a biologically plausible IgM profile. For example, IgM responses detected at week 24 without evidence of responses at earlier timepoints were discounted. Either of these criteria excluded all four placebo recipients, whereas 12 out of 20 NYVAC HIV C recipients (60%) scored positive with median titres ranging from 41 to 1600, with most showing consistent responses across timepoints (Fig. 1b).

Apart from studies with neutralizing monoclonal antibodies switched to the IgM isotype [10,11], little is

**Fig. 1. (continued)**

NHS in which they were diluted. Error bars show the SE. The horizontal dashed lines indicate the IC<sub>90</sub> and IC<sub>50</sub> neutralization thresholds. □ Week 4; ▒ week 8; ■ week 24; —■— HI NHS; —▲— C NHS; —■— U0109 WK24 + 10% HI NHS; —▲— U0109 WK24 + 10% fresh NHS.

known of the anti-HIV-1 activity of Env-specific IgM. It has been suggested that IgM contributes to ADCMI mediated by IgG [2]. It has also been shown that serum IgM has both enhancing and inhibiting actions on the cell–cell spread of HIV-1 [12]. As IgM is the most potent class of antibody at activating the complement cascade, we hypothesized that Env-specific IgM, if present, might mediate ADCMI. We therefore analysed antiviral activity in the presence of fresh normal human serum (NHS) as a source of complement, or heat-inactivated (HI) NHS as a control. Neutralization and ADCMI were determined using a previously described assay [2], except that here phytohaemagglutinin/IL-2 stimulated peripheral blood mononuclear cells (PBMC) were used as target cells and 100 TCID<sub>50</sub> of PBMC-derived HIV-1<sub>97CN54</sub> was preincubated with serial dilutions of fresh or HI NHS or a combination of a fixed concentration (10%) of fresh or HI NHS with serial dilutions of vaccinee serum. After 1 h incubation of serum and virus at 37°C, PBMC were added to each well for 24 h, after which the cells were washed and resuspended in fresh medium. The medium was exchanged on days 4 and 7, and the p24 concentration determined on day 10 by p24 ELISA, as described elsewhere [13]. The percentage neutralization was determined by comparison with control wells incubated with virus alone. The results of four replicates were analysed by fitting sigmoidal dose–response curves using GraphPad Prism.

One vaccinee, U0109, had no detectable IgG, but made a particularly strong IgM response to Env that was detected across all timepoints and that peaked at 1/1600 at week 24. We tested this serum for neutralization of the autologous isolate HIV-1<sub>97CN54</sub> (Fig. 1c). Fresh and HI NHS alone had weak intrinsic antiviral activity. By contrast, the combination of U0109 week 24 serum and HI NHS achieved IC<sub>90</sub> at a titre of 7.8 (total serum concentration 22.8%), whereas the combination with fresh NHS achieved IC<sub>90</sub> at a titre of 24.4 (total serum concentration 14.1%). This represents weak but significant neutralization in the absence of complement ( $P=0.0354$ , one-tailed  $t$ -test) and a 3.1-fold enhancement of neutralization in the presence of complement ( $P=0.0003$ , one-tailed  $t$ -test). This is encouraging given that virus cultivated in PBMC incorporates the full range of complement protection factors and is especially resistant to ADCMI [14].

In conclusion, we report for the first time the detection of Env-specific IgM responses in volunteers receiving an experimental vaccine immunogen, NYVAC HIV C expressing gp120. Serum from one volunteer exhibited IgM-associated neutralizing activity with an IC<sub>90</sub> that was significantly enhanced in the presence of fresh NHS as a source of complement. This work suggests that vaccine-induced, Env-specific IgM responses can have significant antiviral activity, and indicates that such responses should be evaluated in future studies.

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### Frequency of CCR5 variants among rural populations with low HIV-1 prevalence in Cameroon

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**Among rural populations in Cameroon, HIV-1 prevalence is low and the genetic diversity broad. An unusual population-level genetic background may modulate this pattern of HIV infection. We examined HIV-1 prevalence, CCR5 $\Delta$ 32 and CCR5 promoter –2459 G genotype frequency among 1390 rural inhabitants. No individual was identified with the CCR5 $\Delta$ 32 allele, but homozygotes for the CCR5 promoter variant –2459G (27.5%) were relatively common. A seroprevalence of 3.1% of HIV-1 was reported.**

Within small isolated human populations that become infected with new viruses, many factors contribute to disease outbreak. Of these, the receptors that these viruses use to gain entry into cells play a major role in modulating the spread of infection in the population. Notable are the primate lentiviruses, HIV and SIV, which use CCR5 to enter primate cells efficiently. In central Africa where Cameroon is located, the diversity of HIV-1 is broad. We and others have identified many rare HIV-1 variants from remote populations in the Cameroon rainforest [1]. These findings suggest that the virus has been in central Africa longer than elsewhere in Africa, although the prevalence is relatively lower than in eastern and southern Africa. In 2004, an HIV prevalence of 4.9 and 3.0% was reported in urban and rural populations in Cameroon, respectively (National Institute of Statistics, Cameroon, [www.statistics-cameroon.org](http://www.statistics-cameroon.org)).

The use of CCR5 predominates in the initial HIV infection. Therefore, polymorphisms in this gene that affect its expression and function may modulate the susceptibility of human cells to HIV-1 and SIV. Of these, the 32 base pair deletion in the coding region of CCR5 (CCR5 $\Delta$ 32) and CCR5 promoter –2459 A-to-G single nucleotide polymorphism (SNP) (CCR5 –2459G) have been reported among Caucasian [2] and less so among

African populations. CCR5 $\Delta$ 32 homozygotes show relative resistance to R5 HIV-1 in contrast to the CCR5 –2549G/G genotype, which showed no association with transmission among women in Kenya [2,3]. In a cohort of exposed seronegative Caucasian men, however, individuals with the genotype combination CCR5 $\Delta$ 32/wt-CCR5 –2459 A/G showed an advantage in resisting sexual HIV-1 transmission. In addition, haplotypes CCR5 $\Delta$ 32/CCR5 –2459A, (which are in complete disequilibrium), and CCR5wt/CCR5 –2459G also exert a protective effect against HIV-1 transmission [4].

HIV-1, which drives a global epidemic, is probably of zoonotic origin. The epizooty of *Pan troglodytes troglodytes* and SIV in central Africa, the frequent exposure of humans to non-human primate body fluids over long periods, and the identification of HIV-1 group N in Cameroon, provide substantial evidence of cross-species transmission of primate retroviruses in this region. The identification of HIV-1 group N variants further suggests a single transfer event of SIV from *Pt. troglodytes* (SIVcpz) to humans [5]. It is no surprise that other simian retroviruses were identified in our study population, which practices hunting for subsistence [6]. Although the prevalence of HIV-1 group N in Cameroon is low, the close relatedness of Env from HIV-1 group N and SIVcpz [5] provide evidence that HIV-1 molecular epidemiology may be modulated in this region by the prevalence of SIV and the frequency of exposure of humans to feral chimpanzees. Ethnographically divergent populations may show a different population-level response to these viral infections. Therefore, in an attempt to understand other factors that modulate HIV-1 infection patterns among rural populations in Cameroon, we searched for any unusual population-level genetic differences in the HIV co-receptor, CCR5, which may account for the prevalence of HIV-1 in this region.

A total of 1390 inhabitants from seven villages located in the rainforest (Ndikinimeki, Bangourein, Massangan, Nyabessang, Moloundou, Lomie and Manyemen) were randomly selected and tested for HIV-1 (enzyme-linked immunosorbent assay; Ortho Diagnostics, Raritan, New Jersey, USA, and Western blot assay, HIV Blot 2.2; Genelabs, Singapore). A multiplex polymerase chain reaction strategy followed by enzymatic digestion of polymerase chain reaction products were used for the simultaneous determination of the CCR5 $\Delta$ 32, CCR5 –2459G genotypes [7]. The POPGEN 1.0 software was used to calculate allele frequency, genotype frequency and heterozygosity.

Here, we report an HIV-1 seroprevalence of 3.1%, with the lowest in Nyabessang (1.9%) and the highest in Manyemen (5.5%), compared with 5.8% from other rural populations in southern Cameroon. The CCR5 $\Delta$ 32 allele, which is rare among black Africans, was not

**Table 1. Frequency of CCR5 –2459G genotypes.**

	Genotype frequency Total no. (%)		Observed heterozygosity
	Homozygous	Heterozygous	
Study population (n = 1365)	376 (27.5)	477 (34.9)	0.35
HIV-1-positive group (n = 43)	13 (30.2)	13 (30.2)	0.30

identified in this population. From Cameroon in central Africa, Senegal in west Africa, and South Africa, a frequency of the CCR5 $\Delta$ 32 allele of less than 0.1, 1.4 and 0.1%, respectively, has been reported compared with 14% among Europeans. The CCR5 –2459G and CCR5 –2459A alleles are common in Caucasian populations. We report an allele frequency of 45.0% for CCR5 –2459G, a genotype frequency for CCR5 –2459G/G and CCR5 –2459A/G of 27.5 and 34.9%, respectively, and a heterozygosity of 0.35% in the study population. Within the HIV-1-positive group, both the CCR5 –2459A/G and CCR5 –2459G/G genotypes were each present at a frequency of 30.2% (Table 1).

These results suggest that the low seroprevalence of HIV-1 in these communities is not caused by the CCR5 $\Delta$ 32, CCR5 –2459A/G genotypes, or a combination of these. It is probable that other biological factors and the lifestyle of these individuals contribute substantially to this pattern of HIV prevalence. The potential effects of the diverse genetic variants of HIV-1 on the evolution of the CCR5 gene among different ethnic groups in central Africa, where the risk of the cross-species transmission of primate retroviruses is high among those who hunt and handle non-human primates, remain in question. It is possible that the AIDS epidemic will result in the selection of certain polymorphisms in the CCR5 gene or other AIDS restriction genes, as appears to be the case for HLA-B alleles in HIV-1 infection [8].

The views and opinions expressed herein do not necessarily reflect those of the United States Department of Defense, nor does mention of trade names, commercial products, or organizations imply endorsement by the US government.

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