Modeling Partially Effective HIV Vaccines In Vitro

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Significant public health benefits could be realized with human immunodeficiency virus (HIV) vaccines that are incompletely effective. However, standard assays of experimental HIV vaccine immunogenicity may not correlate with antiviral effectiveness and cannot identify subtle effects. We developed an in vitro challenge assay (IVCA) that measures the net antiviral effect in whole peripheral blood mononuclear cells (PBMCs) to any titered HIV isolate. We then modeled partially effective postvaccination immune status 4 ways: use of PBMCs from highly exposed, uninfected individuals; depletion and partial reconstitution of autologous CD8+ cells from PBMCs from HIV-positive long-term nonprogressors; partial blocking of infection with chemokines; and variation in challenge virus dose. IVCA could detect as little as 3-fold differences in the challenge titer (30, 10, and 3 50% tissue-culture infective doses) or odds ratio of HIV infection. This robust and simple assay should be useful in determining which HIV vaccine candidates are suitable for field trials of efficacy.

The development of an HIV vaccine is hindered, in part, by the absence of acquired HIV-specific immune responses that are clearly responsible for protection. Even the more modest goal of suppression of viremia by preexisting vaccine-induced immunity is hampered by the absence of clear causal relationships between aviremia in some HIV-positive infected individuals and the immune responses they exhibit. Nevertheless, important evidence for the beneficial effects of neutralizing antibodies (Abs) and CD8+ cell-mediated responses has emerged from the simian immunodeficiency virus (SIV) macaque model, in which Abs (administered before or during the acute phase of infection) and CD8+ T cells (experimentally depleted) have both been demonstrated to have potent antiviral effects [1–4].

In humans, CD8+ cells have been associated with reduction in peak viremia during early infection [5], maintenance of virus suppression in chronically infected individuals [6], and a lack of infection in some highly exposed uninfected (EU) individuals [7]. A protective role for cytotoxic T lymphocytes (CTLs) has been deduced from transient suppression of HIV after the adoptive transfer of in vitro–expanded HIV-specific CD8+ cells [8]. Although neutralizing IgG drives the evolution of escape mutations in the env region [9], antibody has not been proved to suppress viremia in established infection. IgA with neutralizing activity has been associated with lack of infection in some EU individuals, who should hold clues to acquired protective immunity [10, 11]. However, results of HIV-specific CTL activity, noncytotoxic activity, mucosal Abs, and chemokine production have sometimes varied among studies of EU individuals and have rarely been assayed in parallel [7, 12–15].

Moreover, any of these responses may be a correlate, rather than the cause, of protection due to other unknown host factors; in any case, none has been shown to reliably predict resistance against infection among prospectively followed EU individuals or progression from nonprogression among HIV-positive individuals. Similarly, HIV-specific CD4+ T cell proliferation has been associated with low levels of viremia in long-term nonprogressors (LTNPs) [16] but is not accepted as the cause of sustained suppression of virus. Finally, Ab-dependent cell-mediated cytotoxicity has been demonstrated in vitro for both naturally infected [17] and
vaccinated individuals [18], but its role in vivo remains undetermined.

Problems in evaluating candidate vaccines with respect to functionally relevant immunogenicity make it difficult to set criteria for moving products forward into efficacy trials. We describe an in vitro challenge assay (IVCA) that uses peripheral blood mononuclear cells (PBMCs) to address or circumvent many of these problems. Antiviral immunity in the IVCA, defined as resistance to exogenous HIV challenge, is seen (in order of decreasing strength) in PBMCs from HIV-positive individuals with low or undetectable levels of viremia, HIV-positive individuals who have been aviremic for several months during antiretroviral therapy (ART), EU individuals, HIV-2–infected Senegalese persons, and (transiently) in a small proportion of normal uninfected volunteers after 3 or 4 vaccinations with canarypox-vectored HIV vaccines [19–21]. The resistance of PBMCs was largely CD8+-cell dependent but showed additional levels of complexity among HIV-2–positive and EU samples.

Our previously published studies used IVCA in a format most suitable for determining differences in resistance among experimental groups by comparing the proportions of resistant: susceptible replicate cultures at single or cumulative time points [21]. For maximal utility in vaccine evaluation, we have now modified the IVCA to score individual volunteers as susceptible or resistant to challenge infection at individual time points. Also, because the first effective vaccines are likely to provide virus-suppressive, rather than sterilizing, immunity for many recipients, we modified IVCA for quantitation of intermediate levels of resistance, and used it in 4 different models of partial immune protection.

SUBJECTS, MATERIALS, AND METHODS

EU, LTNP, and healthy donors. EU volunteers were recruited in Baltimore and included persons with a high probability of exposure to HIV while remaining negative for serum HIV antibody according to the results of EIA or Western blot. Inclusion criteria for high-risk exposure were a ≥2-year period of shared injection drug use and/or unprotected sex with many (>10) high-risk or known HIV–1–infected partners during the previous year. Of 18 EU individuals, 2 were identified as being heterozygous for the CCR5Δ32 allele. LTNP’s were HIV–1–positive subjects infected for >10 years who, in the absence of ART, had maintained virus loads <1000 RNA copies/mL and CD4+ cell counts >500 cells/mm^3 [20]. Healthy donors were adults in good health, negative for HIV according to the results of EIA, and had no known risk factors for exposure. Cells from healthy donors used for comparison with those for EU individuals were collected and stored during the same time period. Informed consent was obtained from all subjects. The study conformed to human experimentation guidelines of the US Department of Health and Human Services and was approved by the Johns Hopkins University institutional review board.

PBMC isolation and cryopreservation. PBMCs were isolated using standard ficoll-hypaque gradient centrifugation. Samples were cryopreserved in 90% fetal bovine serum (FBS) and 10% dimethyl sulfoxide (DMSO) by stepwise temperature reduction and were stored in liquid nitrogen. At the time of assay, samples were thawed rapidly, washed free of DMSO, resuspended at 1–2 × 10^6 PBMCs/mL, and left overnight at 37°C in complete medium (RPMI 1640, 10% FBS, 2 mmol/L L-glutamine, 100 mg/mL streptomycin, and 100 U/mL penicillin).

Viruses and virus titration. HIV-1_BNL is a clade B R5 virus grown in monocyte-derived human macrophages (Advanced Biotechnologies). HIV-1_P15 is a clade B R5 primary isolate from an HIV-positive individual in the Baltimore-Washington area (kindly provided by Dr. David Montefiori, Duke University Medical Center, Durham, NC). Viruses were titered in cryopreserved PBMCs from 20 low-risk seronegative control subjects, and the selected TCID_{50} (3, 10, or 30) was confirmed in cryopreserved, thawed PBMCs from a similar group of low-risk individuals. Virus titers were calculated using the Reed-Muench method [22]. Challenge doses refer to cryopreserved, thawed, PBMC TCID_{50}. Culture conditions (other than TCID_{50}) were held constant.

IVCA. Thawed cells were incubated overnight and examined for viability by trypan blue exclusion; only those with ≥90% viability were used. This criterion was met in 95% of thawed samples. PBMCs were resuspended in fresh complete media that contained 5 ng/mL anti–CD3 monoclonal antibody (OKT3; Ortho Diagnostics) and 2 U/mL interleukin (IL)–2 (Boehringer Mannheim Biochemicals) at a concentration of 1 × 10^6 cells/mL. Cells were plated in 1-mL replicates in 48-well tissue culture plates and incubated. Lymphoblast stimulation was assessed by microscopic examination. We have determined the time for maximal proliferation to be ~90 h after the initial OKT3 stimulation under these culture conditions. Virus containing the appropriate PBMC TCID_{50} was added to individual cultures at a volume of 100 μL. When cell numbers permitted (e.g., for normal donors), challenge was performed on octuplicate cultures. Plates were incubated for 1 h at 37°C, and cells were washed free of unbound virus. PBMCs were then resuspended in 1 mL of 50% fresh complete medium with IL-2 and 50% medium from the initial 3 days of stimulation (conditioned medium). Supernatants were harvested on days 3, 7, and 10, and cultures were refed with complete medium plus IL-2. Culture supernatants from challenge days 0, 7, and 10, were measured for p24 antigen by use of an EIA (Frederick Cancer Research Center, National Cancer Institute, Bethesda, MD). Supernatant p24 levels <40 pg/mL were considered to be negative, whereas positive cultures were quantitated across
a range of 40–400,000 pg/mL. Residual unbound virus in day 0 supernatant was always negative according to EIA results.

**CD8** T cell depletion and CD8** cell add-back for in vitro challenge.** Immunomagnetic-bead CD8 depletions and separation of CD8 cells from beads were performed according to the manufacturer’s procedures (Dynal). The CD8** cell–depleted PBMCs contained <1% residual CD8** T cells, according to the results of immunofluorescence staining. Autologous CD8** cells were added back to replicate CD8-depleted PBMC cultures, to a final concentration of 2%, 5%, or 10% of total cells. Virus challenge was the same as for whole PBMCs and was performed in parallel.

**Addition of β chemokines.** Cultures were set up as for standard virus challenge. To parallel cultures, RANTES and macrophage inhibitory protein–1β were added at a concentration of 100 ng/mL on days 0 and 3 after challenge.

**Data analysis and presentation.** For each inoculum titer, the number of positive wells and the mean p24 values of positive wells were determined for day 10 and plotted on the x- and y-axes, respectively. Data from control samples challenged at matching doses were used to define a boundary, represented graphically by the upper right quadrant, that contained all data points in the upper 95% for both parameters at a given challenge dose. Shift from within the quadrant (containing ≥90% of controls) to outside the quadrant along either axis (or both) was defined as indicating statistical significance. For some experiments, data are expressed in standard scatter plots or bar graphs. Data comparing EU and control groups were analyzed using simple logistic-regression analysis, accounting for dependence among donor replicates, to yield an odds ratio (OR) of resistance [21]. Differences in proportions of individuals were assessed using Pearson’s χ² test.

**RESULTS**

**Model 1: EU PBMC resistance after in vitro challenge.** Groups and cohorts of EU individuals may always include a proportion of individuals who have avoided infection by chance rather than by true resistance to infection. Therefore, the degree of resistance for EU individuals can not be determined from in vivo data alone. Limited prospective reports have indicated incomplete resistance at the group [23] and individual levels, yet some of these subjects have gone on to become HIV-positive after stopping and then resuming high-risk behavior [24]. This suggests varying levels of not fully durable immunity, which is an excellent model of postimmunization status with a partially effective vaccine. We previously reported the relative resistance of EU versus control groups using the old-format IVCA at 10 TCID₅₀ [21]. Since then, our expanded IVCA studies have revealed ~10-fold variation encompassing >90% of low-risk control PBMCs tested (n = 100) with respect to challenge susceptibility (data not shown). Thus, ~10% of control samples show infection rates typical of 3 TCID₅₀ when challenged with a pretitered dose of 10 TCID₅₀, whereas an approximately equal number of controls respond to a pretitered 3 TCID₅₀ inoculum as if it were 10 TCID₅₀. To detect subtle but significant levels of resistance to infection in the face of this 10-fold variation among controls, we studied samples from EU individuals at 3 and 30 TCID₅₀.

As shown in table 1, high-risk EU individuals appeared to be mostly resistant to the low-dose (3 TCID₅₀) challenge. Even though some control donors also had no virus growth (as expected from stochastic considerations), the difference in positive cultures between EU and control groups (14% vs. 61%) was highly significant (P < .001; OR, 11.3; 95% confidence interval, 4.3–29.7). Measurements of p24 showed a trend of lower values among the 8 positive EU cultures, but the statistical significance of this trend was not determined, because the great majority of control values were off the scale for positive using the assay kit available at that time.

An unselected subset (n = 11) of the EU donor PBMCs was retested in parallel with 11 normal control donor PBMCs for resistance to 30 TCID₅₀ HIV-1₁₅. No EU sample exhibited full resistance (i.e., all replicates tested negative for p24), and

<table>
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<tr>
<th>Samples</th>
<th>Positive/total no. of cultures (%)</th>
<th>p24, mean ± SE, pg/mL</th>
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<tbody>
<tr>
<td>Unexposed controls (n = 11)</td>
<td>44/44 (100)</td>
<td>109,234 ± 12,395</td>
</tr>
<tr>
<td>EU (n = 11)</td>
<td>40/44 (91)</td>
<td>53,061 ± 20,866</td>
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<tr>
<td>More susceptibleb (n = 5)</td>
<td>20/20 (100)</td>
<td>114,805 ± 24,052</td>
</tr>
<tr>
<td>More resistantb (n = 6)</td>
<td>20/24 (83)</td>
<td>1607 ± 590</td>
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</table>

a P = .03, EU vs. control samples.
b Categories were identified post hoc.
there was little difference in the proportion of susceptible cultures from control and EU donors. However, a comparison of mean p24 levels revealed a significant difference between the 2 groups ($P = .03$; table 2 and figure 1) caused by suppressed levels of virus replication in 6 of 11 EU samples. The other 5 EU samples, as a group, were resistant to challenge with 3 TCID$_{50}$ (12% cultures positive) but lost in vitro resistance at 30 TCID$_{50}$, possibly because of a threshold effect.

**Model 2: normal PBMCs challenged at 3 titered doses.** If IVCA can distinguish among small differences in virus challenge dose, then even moderate degrees of effective induced immunity might be detectable. After challenge with 30 TCID$_{50}$, all day-10 replicates from 59 control samples were positive, with a relatively narrow range of individual mean p24 pg/mL values (4.1–5.4 log) and low variation among individual donor replicates (median SD, 0.1 log; 90th percentile SD, 0.3 log). At 10 and 3 TCID$_{50}$, 96% and 67% of wells were positive, respectively, whereas individual donor replicate variation among positive wells increased (median SDs of 0.35 log and 0.62 log, respectively) but remained quite low. For >80% of donors, lower challenge doses gave fewer positive replicates and/or lower mean p24 values of positive wells. Even among the most susceptible 20% of donors (those with 8/8 replicates positive for infection after 3 TCID$_{50}$ challenge), the group trend was toward lower mean p24 values at lower challenge doses. Thus, with a decreasing challenge dose, the dimensions of the 95% normal susceptibility quadrant increased progressively along both axes. Among all donors as a group, challenge susceptibility (the proportion of points within the 95% boundary) was significantly different ($P < .001$) at 30 versus 3, 30 versus 10, and 10 versus 3 TCID$_{50}$ virus inocula (figure 2 and table 3).

**Model 3: normal PBMCs treated with β chemokines.** Elevated levels of β chemokines are associated with protection in EU individuals and improved outcome in some HIV-positive patients [15, 25]. We used β chemokines as representatives of any soluble immune factor(s) mediating resistance, modeling a postimmunization state by adding incompletely blocking levels to normal control cultures. There was a consistent significant decrease in mean p24 production and/or proportion of positive wells for all 5 donors at all doses of challenge virus. At 30 TCID$_{50}$, the effect of chemokines was mostly seen as lower p24, whereas, at 3 TCID$_{50}$, the decrease occurred primarily in the number of positive replicates, which suggests a threshold level of resistance that prevents detectable virus replication had been reached in some cultures. Figure 3 shows the decrease in virus replication in the presence of the R5 chemokines after challenge with the 3 doses of virus.

**Model 4: incremental reconstitution with autologous LTNP CD8$^+$ cells.** We have previously described the dependence of LTNP challenge resistance on CD8$^+$ cells via lytic and nonlytic mechanisms [19]. Similarly, diverse HIV-specific CD8$^+$ cell mechanisms may be induced after vaccination. We modeled vaccine-induced, HIV-specific CD8$^+$ cell activity with autologous add-back experiments, starting with whole PBMCs from 2 LTNP who had virus loads below the detection limit of the assay. These cultures completely resisted challenge with 30 TCID$_{50}$ of HIV-1$_{R5}$. When depleted of CD8$^+$ cells ($<1\%$ CD8$^+$ by flow criteria), the remaining PBMCs supported virus growth, as expected. Cultures produced a mean p24 of $\sim 10$ ng/mL at day 10.

Incremental reconstitution with 2%, 5%, and 10% autologous CD8$^+$ cells resulted in the progressive suppression of virus production. Because CD8$^+$ cells made up $\sim 30\%$ of total undepleted PBMCs in this donor, IVCA was sensitive to reconstitution with <10% of the total CD8$^+$ antiviral activity present in this individual’s PBMCs. The greatest incremental effect was seen going from 2% to 5% total cell reconstitution with CD8$^+$ cells (>90% suppression vs. fully depleted), with additional, but still incomplete, suppression of virus obtained at 10% CD8$^+$ cells in culture (figure 4).

**DISCUSSION**

In the absence of a single reference standard of protective natural immunity, most vaccine developers believe that a combination of neutralizing Abs and CD8$^+$ cell–mediated immunity would be required for an optimal vaccine response. Although current candidate vaccines have fallen short of the desired strength and durability of induced cellular responses, even in...
Figure 2. In vitro virus growth after challenge with 3 (white circles; n = 47), 10 (white triangles; n = 52), and 30 (black circles; n = 59) TCID$_{50}$ of HIV-1BaL among control donors. Each was tested in 8 replicate cultures. The partial boxes delimit the upper 95% (above the 5th percentile) of values of each axis at 3 (dotted line), 10 (dashed line), and 30 (solid line) TCID$_{50}$. The proportion of donors within the box of a particular dose was significantly different from that at the other doses. Thus, the assay discriminated between 3-fold differences in virus titer and, as a corollary, would be able to discriminate between 3-fold differences in effective immunity.

Table 3. Criteria for healthy control donor susceptibility at various TCID$_{50}$ levels.

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<tr>
<th>TCID$_{50}$</th>
<th>5th percentile$^a$ at each TCID$_{50}$</th>
<th>Susceptible donors$^b$</th>
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<tr>
<td></td>
<td>No. of positive wells</td>
<td>Mean log p24 of positive wells, pg/mL</td>
</tr>
<tr>
<td>30</td>
<td>8</td>
<td>4.35</td>
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<td>10</td>
<td>6</td>
<td>3.67</td>
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<td>3</td>
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$^a$ The 5th percentile determines the cutoff point for the upper 95th percentile.

$^b$ Susceptible donors (the fraction of normal donors above the 5th percentile) were defined by those within upper 95th percentile by both criteria: no. of positive wells and mean log p24 of positive wells.

completely effective HIV vaccines could have a significant effect on the worldwide epidemic [26]. The technical aspects of various assays are not completely standardized and are subject to additional variation based on strain-specific antigen differences and the availability of reagents. Furthermore, the functional relevance of each response being assayed (e.g., CTL precursors, interferon-γ production, tetramer staining, lymphoproliferation, and chemokine secretion) remains controversial.

In its current quantitative format, IVCA is sufficiently sensitive to detect relative resistance to infection in 4 different models of partial vaccine efficacy: (1) cells from EU donors, (2) 3-fold variation in virus inocula at relatively low TCID$_{50}$, (3) partial blocking with CCR5-binding chemokines, and (4) partial restoration of HIV-positive LTNP CD8$^+$ cells. Moreover, the use of multiple challenge doses allowed the detection of an apparent threshold effect among some EU individuals. These findings suggest that IVCA would identify (1) vaccines capable of inducing partial efficacy at the population level or reduced viremia in a proportion of those infected, (2) induced lytic and nonlytic mechanisms of biologically significant protection, and (3) vaccines inducing one-tenth the in vitro antiviral CD8$^+$ cell activity induced by natural infection, regardless of actual effectiveness.

Although SIV macaque vaccine models have provided some examples of sterilizing immunity, most studies have reported a reduction in peak and steady-state viremia levels [27, 28]. In contrast to experimental primate models, human exposures to HIV may (typically) represent fractional ID$_{50}$ values, because
Figure 3. Effect of β chemokines (RANTES and macrophage inhibitory protein–1β, each at 100 ng/mL) on in vitro virus growth after challenge with 30 (solid line), 10 (dashed line), or 3 (dotted line) TCID$_{50}$ HIV-1 BaL among samples from 5 control donors. (At 3 TCID$_{50}$, only 4 donors were tested, because cells were not available from 1 donor). The model of chemokines at levels lower than required for blocking represents any soluble anti-HIV factor. At all 3 doses of virus, the assay was sensitive to detect significant reductions (shift outside the box) in virus growth after the addition of chemokines. Black circles, with β chemokines; white circles, without β chemokines.

the probability of infection is generally <1% per exposure [29]. In the context of postvaccination HIV exposure, individuals are likely to fall along a spectrum—from sterilizing immunity to suppressed viremia to complete lack of effect—depending on host genetic background, prior exposure, and ongoing risk. Therefore, IVCA’s ability to detect (at low challenge doses) even partial protective immunity caused by various combinations of factors is a particularly desirable feature that is unavailable with other assays.

Furthermore, the stochastic behavior of the assay at low chal-
short-term culture, and EIA are all simple and readily trans-

Figure 4. The effect of incremental reconstitution of CD8 cells in CD8-
depleted cultures from long-term nonprogressors (LTNP) on in vitro virus
growth after challenge with 30 TCID<sub>50</sub> HIV-1BaL. These LTNP were aviremic according to the results of standard virus load assays; furthermore, autologous whole peripheral blood mononuclear cell (PBMC) cultures tested negative for p24 even after maximal phytohemagglutinin stimu-
lation. Measurable p24 was that of the challenge virus. The experiment was repeated once, and cells were available for duplicate cultures only. Thus, a statistical analysis was not possible. But, as proof of principle, this showed that the assay was sensitive to detect the effect produced by small numbers of effector CD8 cells.

challenge doses is unique among available challenge models (typi-
cally designed to generate 100% infection in control groups). It seems reasonable to speculate that vaccines that cause a re-
duction in the IVCA mean p24 culture levels but no reduction in proportions of positive wells would reduce steady-state vi-
remia, as opposed to providing sterilizing immunity. On the other hand, vaccines that cause significant numbers of wells to remain undetectable for virus growth might have a better chance of conferring true sterilizing immunity. Partially effec-
tive vaccines will probably cause significant shifts along both axes (i.e., a reduction in numbers of positive wells and in the mean p24 level of positive wells). Resistance may be better revealed by one challenge dose than another, depending on the mechanism and individual donor variation. For example, the lowest dose is probably the better approximation of in vivo exposure, but decreased numbers of positive wells in control samples at 3 TCID<sub>50</sub> can work against the statistical detection of biologically significant effects that, in turn, are revealed at higher challenge doses.

The newly designed IVCA retains its applicability to vaccine trials in developing regions of the world. PBMC separation, short-term culture, and EIA are all simple and readily trans-

References


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