Extensive evaluation of a seronegative participant in an HIV-1 vaccine trial as a result of false-positive PCR

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Summary

Background In the USA, more than 2000 volunteers have received one or more experimental HIV-1 vaccines in phase I and II clinical trials, and there have been breakthrough HIV-1 infections among participants receiving vaccine and placebo. Serological diagnosis of new HIV-1 infections in vaccine-trial participants will become increasingly complicated as more viral components are used in vaccines. Recognition of this problem has led to a reliance on viral-genome measurement to distinguish vaccine-induced immunity from HIV-1 infection. Currently, quantitative RNA measurement is expensive, prone to contamination, and reliable only in laboratories certified by manufacturers or that have quality-control programmes.

Methods A high-risk vaccinee presented after an acute febrile episode and was tested for HIV-1 infection by reverse transcriptase (RT) PCR of viral RNA. Further extensive tests were required to clarify the HIV-1 infection and immune status of the vaccinee, including repeat RT-PCR, nested DNA PCR, western blot, lymphoproliferation assay, cytotoxic-T-cell lysis, CD8-depleted co-culture, and HIV-1 challenge culture.

Findings Initial testing of plasma by RNA RT-PCR was reported as positive. This result was not confirmed by viral cultures, nested DNA PCR, western blot, or EIA. Additional RNA RT-PCR assays gave positive results from earlier occasions in the vaccine trial. Eventually, testing of all previously reactive samples by RNA RT-PCR was repeated in a quality-controlled laboratory, and confirmed the negative HIV-1 status of the individual.

Interpretation This case report exemplifies the difficulties with use of viral-genome measurement as a screening test to diagnose HIV-1 infection, particularly in individuals who have ever participated in HIV-1 vaccine trials. Monitoring of large numbers of phase III vaccinees by RNA RT-PCR will not be feasible. The design of efficacy trials for new vaccines should be in parallel with development of antibody-based diagnostic tests that are capable of differentiating between immunisation and true HIV-1 infection.

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See Commentary page 230

Introduction

Roughly 2200 individuals have participated in trials of experimental HIV-1 vaccines in the USA, and further enrolment, through the AIDS Vaccine Evaluation Group and the community-based HIVNET programmes, is envisioned for the immediate future. Current HIV-1 vaccine strategies include the use of several gene products in an effort to generate the highest possible concentrations of both neutralising antibodies and CD8-positive cytotoxic T cells. A major drawback of this approach is the resultant difficulty in distinguishing between HIV-1 infection and vaccine-induced seroconversion. Earlier experimental vaccines, based exclusively on all or part of the gp120 envelope protein, elicited immune responses that could be distinguished from infection by the absence of gp41 and other bands on western blot, as well as by non-reactivity in several EIA screening assays that did not contain gp120 epitopes. Vaccines based solely on parts of HIV-1 gag also produce patterns on western blot that are easily differentiated from HIV-1 infection, but, in the case of peptide vaccines, have failed to react in screening EIAs.

Serum from recipients of newer candidate vaccines containing gag, pol, and env genes or gene products may react with inexpensive, commercially licensed screening assays as well as with confirmatory western blot. This assumption has led to an anticipated reliance, in phase I and II HIV-1 vaccine trials, on assays of plasma viral RNA by reverse transcriptase DNA PCR (RT-PCR) and branched chain DNA (bDNA) methods. These tests are too expensive (more than US$100 per test) to be practicable for monitoring HIV-1 vaccine trials in many developing countries. Moreover, in all studies of plasma viraemia using these techniques, there is a significant proportion of symptom-free, HIV-1 infected individuals who have no detectable HIV-1 in plasma. As well as concern about sensitivity, doubt is raised by our findings about the specificity of measurement of plasma HIV-1 RNA as the gold standard for screening and confirmation of infection, particularly in a previously vaccinated person. We present the extensive tests required to clarify the HIV-1 status of a high-risk volunteer who had previously participated in a trial of an HIV-1-envelope-based experimental vaccine.

Patients and methods

 Volunteer 2018BP was enrolled in the “high-risk-volunteer” category of vaccine protocol 201, a phase II trial of recombinant HIV-1 gag, rgp120 (Genentech Inc, South San Francisco, CA, USA) or HIV-1 gag, rgp120 (Biocene Corp, Emeryville, CA, USA). Between day 0 (first vaccination) and day 546, this volunteer completed a course of four immunisations with 600 μg MN rgp120 in alum, and was followed up every 3 months thereafter. The volunteer’s risk behaviours for HIV-1 infection included injection drug use and sexual intercourse, with one or more known infected or high-risk partners. 2018BP’s primary sexual partner was diagnosed HIV-1 seropositive on day 654.
lymphoproliferation were assayed according to published NC, USA); baculovirus-produced p24 HIV-1MN and HIV-1 IIIB test antigen (20 µg/mL; CASTA, Greer Laboratories, Lenoir, NC, USA); and yeast-produced recombinant HIV-1 gp120 (both 1 µg/mL; Chiron Laboratories, Emeryville, CA, USA).

Viral co-culture was done in a highly sensitive CD8-depleted culture system: 5×10^6 CD8-depleted PBMC from volunteer 2018BP were mixed with 5×10^5 phytohaemagglutinin-stimulated CD8-depleted PBMC from an uninfected allogeneic donor. Immunomagnetic bead CD8-deplete depletions (Dynal, Great Neck, NY, USA) produced cultures with less than 1% CD8 cells according to flow cytometric immunofluorescence staining. Cultures were maintained for 4 weeks with weekly refeeding of media and allogeneic CD8-depleted phytohaemagglutinin blasts. Resistance to exogenous HIV-1 was assessed by means of in-vitro challenge cultures: PBMC (10^6 per mL) were stimulated with 5 ng/mL α-CD3 monoclonal antibody OKT3 (Ortho Diagnostics, Schenectady, NY, USA) and 2 IU/mL interleukin-2 (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA). Half of the PBMC (5×10^6) were challenged with 3×5 PBMC-titred TCID_{50} (50% tissue culture infectious dose) of HIV-1_{Ad} (Advanced Biotechnologies Inc, Columbia, MD, USA) in 200 µL RPMI 1640 and 10% fetal calf serum for 1 h. The other half were left unchallenged (but otherwise treated identically) for assessment of endogenous virus growth. PBMC were then washed to remove unbound virus and plated at 5×10^6 per well in 2 mL made up of 50% fresh RPMI 1640, 10% fetal calf serum (complete medium), and interleukin-2, with 50% complete media from previous 48 h of incubation. Supernatants were harvested and cultures refed with complete medium and interleukin-2 on days 3, 7, 10, and 14. Cultures were monitored for HIV-1 production by p24 EIA (Organon Teknika, Durham, NC, USA).

Standard serological methods were used to identify an HLA class I haplotype of: A30, A34; B7, B63; Bw4, Bw6. Class II HLA and TAP haplotypes were not investigated.

**Results**

On initial testing, serum samples from days 715, 831, 838, and 874 after the first vaccination were all negative for HIV-1-reactive antibodies by screening EIA. Western blot analysis was also negative except for vaccine-induced gp160 bands. At the same time, blood was sent for plasma RNA RT-PCR. One plasma sample (day 832) was positive, and a plasma sample from the following week (day 838) was negative (<200 copies/mL). CD8-depleted co-cultures established to recover virus remained negative. After several

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Shaded sections results available at day 838.

Laboratory results for 2018BP.
unsuccessful attempts to confirm positive infection status by either serology or culture, the volunteer’s laboratory results were as shown in the shaded portion of the table, and the patient’s HIV-1 infection status was still unknown. 2018BP was not told that HIV-1 infection was being considered at that time.

Discussion among participating investigators led to a retrospective batch analysis of 13 separate samples encompassing all visits during 2018BP’s participation in AIDS Vaccine Evaluation Group protocol 201. RNA RT-PCR results from this analysis are indicated in the table as first RNA RT-PCR. Although two closely spaced samples (days 547 and 559) were negative, the cluster of four positive serum samples during an 11-month period (days 252–587), including one result of about $10^{10}$ copies/mL, and a fifth low-positive test result almost a year later, convinced some investigators that this reflected true HIV-1 infection rather than a cluster of false-positive results.

In contrast to these RT-PCR results, nested cellular DNA PCR amplification tests remained negative. Fresh and cryopreserved cell pellets from days 170, 715, 821, 838, and 874 were tested for the presence of proviral DNA by nested PCR amplification. With repeated sampling we have always obtained a nested DNA PCR signal from HIV-1-infected individuals with extremely low or undetectable plasma viraemia. In the case of 2018BP, for two independent DNA PCR assays on five occasions with runs totalling 200 000 cells per occasion, all PBMC samples were negative for HIV-1 DNA. In addition, tests for the presence of HIV-1 in CD8-depleted phytohaemagglutinin-stimulated allogeneic co-cultures were repeatedly negative—a very unusual result in our laboratory with samples from individuals with detectable HIV-1 RNA.

We searched for evidence of HIV-1-specific immune responses to examine the possibility that infection had occurred but had been completely cleared. HIV-1-specific cytotoxic T-cell activity was measured with Epstein-Barr-virus-transformed autologous B-cell lines as targets for PBMC after in-vitro stimulation with recombinant vaccinia-HIV-1 vectors. No specific lytic activity above background vaccinia-infected targets was seen, in contrast to the readily obtained cytolytic activity from virtually all symptom-free HIV-1-positive donors assayed in this system.⁸ In addition, 2018BP’s CD4 lymphocyte subset numbers and percentages held steady over the 2 years of putative infection.

Lymphoproliferation assay results were less clear cut. HIV-1 antigen-specific lymphoproliferation is usually poor among even the healthiest long-term or newly infected symptom-free HIV-1-positive patients, but it can be enhanced by vaccination.⁹–¹² On repeated visits, 2018BP’s PBMC were stimulated with various HIV-1 and non-HIV-1 recall antigens. The PBMC showed good reactivity to several HIV-1 antigens on repeated testing, including epitopes and strains not included in the vaccine. However, all of these HIV-1 reactivities have been observed among a minority of normal, uninfected controls tested in our laboratory (albeit rarely in cells from a single person). PBMC from 2018BP initially resisted challenge with HIV-1MN on three consecutive occasions (a phenotype we observe in PBMC from many symptom-free infected donors, but only among about 10% of uninfected controls) but subsequently supported infection over the next three occasions. Interestingly, this switch in susceptibility to in-vitro infection coincided with the development of increased lymphoproliferative reactivity to HIV-1 antigens (figure).

Finally, since the HIV-1 infection status of this volunteer remained unresolved, further serum from samples previously tested for HIV-1 RNA were sent to an independent licensed commercial laboratory for repeat RNA RT-PCR testing by the Roche Amplicor Monitor assay system. All samples were negative for HIV-1 RNA (<200 copies/mL), including the cluster that had previously been reported as positive and the original positive plasma sample from day 832 that had triggered the extensive investigation.

Discussion

This report of a seronegative patient is an unusual but informative example of the difficulties in diagnosing HIV-1 infection among high-risk participants in HIV-1 vaccine trials. Despite repeated and unequivocal negative serology from screening EIA and confirmatory western blot (except for a few vaccine-related envelope-reactive bands), the general assumption was that a single positive plasma or serum viraemia as measured by RT-PCR was sufficiently specific and sensitive to establish a diagnosis of infection. Furthermore, a hypothesis was put forward that a vaccine-
induced immune response, present during critical stages of infection, might have led to an abortive infection with abrogation of seroreactivity—arguably a very desirable outcome.

This notion triggered a retrospective assessment of serum RNA, which showed a cluster of positive results over a limited period, convincing many investigators of the validity of the original positive result. The subsequent investigation extended for 6 months and revealed intriguing suggestions of HIV-1-specific cellular immune responses and initial in-vitro resistance to HIV-1 superinfection but no cytotoxic T-cell activity, no EIA reactivity, no decrease in CD4 percentage or number, no culturable virus, no proviral DNA in whole PBMC, and, finally, no serum or plasma RNA on repeat testing in certified laboratories. This volunteer’s PBMC initially resisted in-vitro challenge with exogenous HIV-1 capsid at a time when negligible HIV-1-specific lymphoproliferative responses were seen in parallel cultures, whereas samples from later times showed susceptibility to infection and good lymphoproliferation to several HIV-1 antigens. These data suggest that HIV-1-specific lymphoproliferation will not be a useful surrogate marker for protective immunity.

Further points regarding the approach to exposed HIV-1-vaccine-trial participants detailed in this case report should be considered. A very conservative estimate of the cost of the analysis presented here is $5000. The advertised cost of a single quantitative RNA RT-PCR assay is almost $200. The positive RNA RT-PCR results were obtained in the context of repeatedly negative results of licensed diagnostic screening and confirmatory assays, whereas RNA RT-PCR has not yet been approved in the USA for either phase I or phase II vaccine trials. These tests were ordered, carried out, and interpreted by a group of recognised clinical and scientific experts with perhaps the world’s greatest combined experience in caring for participants in HIV-1 vaccine trials.

This case is informative because the volunteer remained seronegative despite continued high-risk behaviour. Continued seronegative status was taken by some as evidence of probable protective immunity, possibly induced by 2018BP’s previous vaccination. We cannot know how the original false-positive results would have been interpreted if the serology had backed up the RNA RT-PCR results. The greater challenge, therefore, is to determine the status among vaccine-trial participants who are seropositive after vaccination with products containing antigens from several HIV-1 genes. The third set of RNA PCR tests would have been highly unlikely to be ordered if volunteer 2018BP had been seropositive, and in these circumstances even the described investigation might not have convinced all that 2018BP was uninfected. The volunteer was never told about the PCR results until these results were confirmed as negative. Had the volunteer been given a diagnosis of HIV-1 infection based on the earlier PCR results it is unclear whether such information would have had significant sequelae.

For the foreseeable future, monitoring of large numbers of phase II or phase III vaccinees by RNA RT-PCR assays as currently proposed will not be feasible. By some estimates, an HIV-1 vaccine will have to cost less than $5 to be practicable in many developing countries, and even developed countries will not be able to afford thousands of dollars per volunteer and months of delay to establish infection status on repeated occasions. Only if new vaccine candidates are developed in parallel with sensitive and specific diagnostic antibody tests to differentiate between infection and vaccination will efficacy trials be possible. The latest candidate vaccines have been designed to omit a segment of gp41 containing epitopes that react with nearly 100% of all clade B HIV-1-positive serum samples. Use of this limited gp41 epitope in screening EIAs should provide sufficient specificity and sensitivity for use in the setting of HIV-1 vaccine trials. However, current efforts to generate more complex vaccines including oligomeric envelope products may lead to inclusion of this region in vaccines. Inclusion of this immunodominant gp41 epitope could compromise our ability to track the epidemic among populations where large vaccine trials occur.

Contributors
David Schwartz coordinated all aspects of the study including laboratory tests (except plasma viral RNA measurements, which were not done at Johns Hopkins) and wrote the paper with assistance from all co-authors. Renan Castillo, Oliver Laeyendecker, and Silvio Arango-Jaramillo did the laboratory tests at Johns Hopkins and assisted with data analysis. Mary-Jane Reynolds provided clinical care and collected clinical data.

Acknowledgements
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References