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30. Homogenates (2.5%) of unfertilized *Lytechinus pictus* eggs (Marinus, Inc., Long Beach, CA) were prepared (*15*) in a buffer consisting of 250 mM potassium gluconate, 250 mM *N*-methylglucamine, 20 mM Hepes (pH 7.2), 1 mM $MgCl_2$, 0.5 mM ATP, creatine kinase (2 U/ml), 4 mM phosphocreatine, oligomycin (1 μg/ml), 1 mM sodium azide, and 2 μM fura-2 pentapotassium salt. The concentration of free Ca^{2+} in 1-ml portions was measured in a spectrofluorometer with 340-nm excitation, 510-nm emission, and 5-nm bandpasses; as appropriate, ratios of the 340 nm to 380 nm fluorescence were checked to ensure that intensity changes monitored at 340 nm accurately reflected changes in the concentration of Ca^{2+}. Basal concentrations of Ca^{2+} were typically 100 nM. All measurements were conducted at 24° ± 0.5°C; similar results were observed in experiments at 17°C. Test agents were added in volumes not exceeding 10 μL with vigorous mixing. Calcium release was calibrated by sequential additions of known amounts of $CaCl_2$ to comparable portions without allowing time for resequestration between additions. The total available sequestered Ca^{2+} was measured by release with ionomycin (5 μM) and was nearly constant in similar portions of homogenate (averaging 1.3 μM). IP_3 was from Calbiochem, ryanodine was from Calbiochem or a gift of Merck, Sharp & Dohme, and cADPR was prepared from mammalian brain homogenates (*13*). Addition of vehicle (DMSO or water for ryanodine, ethanol for ionomycin, and water for other agents) had no effect on the fura-2 signal in homogenates. All figures are representative of experiments repeated at least three times.
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Technical Comment

Conserved Sequence and Structural Elements in the HIV-1 Principal Neutralizing Determinant: Further Clarifications

It has come to our attention that some researchers are attempting to use nucleotide sequences encoding the HIV-1 principal neutralization determinant (PND) (*1*) and related information submitted by us to GenBank and to the Human Retroviruses and AIDS Database to measure variability among HIV-1 viruses infecting a single individual (intra-individual variation). Such analyses presume the generation of, through molecular cloning and nucleotide sequencing, an accurate representation of the genes encoding different viruses present in the individual at the time the sample is taken. For several reasons, the nucleotide sequence data supporting the conclusions of our analysis could be misleading if they are used to reach conclusions about intra-individual HIV-1 variation. These reasons are as follows:

1) It is possible that a subset of the infectious virus genomes present in the infected PBMCs were preferentially amplified during culturing.

2) The oligonucleotide primers used for PCR amplification in our study may have hybridized more efficiently to the envelope gene of a subset of viral genomes.

3) The list of amino acid sequences shown in our figure 1 includes only amino acid sequences that differed in each PCR product and amino acid sequences occurring more than once from each PCR product (sibling sequences) that were not included or identified in the list of sibling sequences and sibling relationships submitted to the Human Retroviruses and AIDS Database.

4) Because our study involved working with samples from a large number of infected individuals, many viral samples were cultured and the DNA was extracted and PCR amplified in parallel. It is possible that contamination occurred between such samples from different individuals or from exogenous sources such as the HIV-1 IIIB isolate that was being used for other projects in several laboratories involved in our study. For example, there are nine sequences (numbers 166, 169, 170–175, and 225 in our figure 1) that are identical or similar to IIIB-like sequences. In addition, there are instances in which sibling sequences differed significantly (for example, sequences 115, 174, and 201; 151 and 225; and 175, 224, and 229). These sequences are therefore either from individuals that are infected with significantly different HIV-1 viruses or there was contamination of these virus or DNA preparations. In view of these uncertainties, samples that gave rise to IIIB like sequences or sibling sets with significant sequence heterogeneity are being re-examined. We therefore do not recommend the use of the LaRosa sequence set for analyzing intra-individual variation until the conclusion of this re-examination.

The focus and conclusions of our study were the identification of conserved elements (both sequences and predicted secondary structures) in the PND to aid in the design of immunogens able to elicit antibodies that neutralize a large percentage of HIV-1 virus isolates. There are at least three ways to compile the amino acid sequences we obtained. The first includes all duplicate sequences obtained from PCR products from different individuals and does not include duplicate sequences obtained from the same PCR product, The second is that in which all sequences are included, and the third is that in which no duplicate sequences are included. We chose the first method so that we would not overestimate the occurrence of conserved PND sequences by including the large number of identical sibling sequences we had obtained. Regardless of the method used to compile the sequences, and therefore regardless of whether any possible contamination occurred, the conclusions of our study are unchanged; the consensus PND sequence is unchanged; a single amino acid occurred at PND positions 11, 14, 16, 19, 20, 21, 22, 23, and 25 in 80% or more of the sequences, and the frequency of occurrence of each of the sequences listed in our table 1 was not significantly altered.

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