Protocol for the pre-adsorption studies, as modified after discussions of the sub-group of the South African Presidential AIDS Advisory Panel held in Johannesburg on October 18, 2001, and prepared by Drs. Harvey Bialy, Roberto Stock and Alejandro Alagón (Institute of Biotechnology, Autonomous National University of Mexico, Cuernavaca, Mexico) and Prof. S. W. P. Mhlongo (Dept. of Family Medicine and Primary Care, Medical University of Southern Africa, Pretoria, SA). Version 7. 15 November 2001.

The fundamental rationale of the protocol is to determine the specificity of the HIV ELISA tests currently in use in South Africa by examining if antibodies which bind to antigenic preparations of infectious agents common to South Africa are also capable of binding antigens used in these tests efficiently enough to yield positive results.

Study design

ELISA plates will be coated with antigenic preparations (mostly protein) of common disease/infectious agents in South Africa including, but not limited to (see below) *Mycobacterium tuberculosis*, *Entamoeba histolytica*, *Plasmodium falciparum* and adenovirus, a positive control plate containing just the blocking protein (gelatin), as well as a negative control plate coated with Prot A will also be prepared. HIV+ serum samples of known OD will be added to these plates and incubated for 2 hours at room temperature. After incubation with the different antigens, the serum samples will be re-tested for anti-HIV antibodies using the same test that was initially used to determine the presence of HIV-reactive antibodies.

Sample sizes and varieties

150 HIV+ sera samples (it must be kept in mind that a minimal volume will be required depending on the total number of antigens to be used, we suggest a minimal volume of 4 ml of each sample at the dilution recommended by the HIV test). One ELISA test will be chosen by the National Institute for Communicable Diseases as representative of the tests now in use in SA, and samples will be collected after approval from the MRC ethics committee. The ELISA test selected will be used throughout the study to determine the HIV status of all samples. The studies (including the initial stratification of HIV+ samples) will be undertaken at the Department of Virology, University of Natal.

Samples will be stratified according to their ELISA OD as follows:

High OD (n=10)
Mid-range OD (n=100)
Low positive OD (n=40)

The second set of sera samples will be supplied by Prof. Mhlongo, and consist of 100 tuberculosis patients of unknown HIV serological status.

Antigens

All antigens will be quality controlled as follows: Protein concentrations will be determined using the "BCA Protein Assay Reagent Kit" (Pierce, Rockford, Illinois, USA).

SDS polyacrylamide electrophoresis gels will be run to ensure that the antigenic preparations consist of complex protein mixtures.

The following is a minimal set of antigenic preparations that will be required to make a reasonably rigorous determination of HIV ELISA robustness (see Appendix I).

HTLV I (commercially available)
HTLV II (commercially available)
Mycobacterium tuberculosis (to be prepared according to a referenced protocol in SA
Mycobacterium leprae (source to be determined)
Mycobacterium vaccae (available from the Durban Immunotherapy Trial Group)
BCG (commercially available)
Entamoeba histolytica (to be prepared by Prof. Jackson)
Adenovirus (to be supplied by Dr. Bialy)
Plasmodium falciparum (to be prepared according to a referenced protocol in SA)
Schistosoma mansoni (source to be determined)
Enteropathogenic *E. coli* (source to be determined)
Enteropathogenic *Salmonella typhimurium* (source to be determined)
HAV (commercially available)
HSV (commercially available)

Approximately 1 mg of each antigen, at a concentration of 0.5-1.0 mg/ml will be needed for the proposed studies. The concentration need be high as it will allow direct dilution in buffer for coating the preadsorption wells, without having to perform buffer exchange.

HIV Test plates required

Each antigenic preparation to be tested as an HIV ELISA antibody competitor will be immobilized in NUNC ELISA 96 well plates. Serum samples will be incubated on antigen-coated wells before re-testing for HIV antibody status.

The 100 TB samples (in duplicate) will be incubated only against 6 antigens (*M. tb, M. leprae, M. vaccae,* BCG and the control plates containing gelatin and Protein A). This will require 2 HIV ELISA plates (x2) for each antigen for this part of the study. Thus for the 4antigenic preparations plus the 2 control plates a total of 24 HIV ELISA plates will be required.

For the remaining 150 samples, the arithmetic is as follows: 4 plates are required to accommodate 150 samples in duplicate, multiplied by 16, if we use all the antigenic preparations listed above = 64 HIV ELISA plates.

Additional materials

We will need to purchase 4 HTLV I, 4 HTLV II, 4 HSV and 4 HAV test plates in order to examine 150 samples in duplicate, in addition to the 100 NUNC ELISA plates. A protein determination kit sufficient for 50 assays will also be needed, as well as a supply of sterile Eppendorf tips (in racks) in the following quantities: 5,000 blue tips (1 ml) and 10,000 yellow tips (250 microliters). The tips can be purchased in bags and then put in racks and sterilized.

Experimental Protocol and schedule

Day 0 -- QC of antigenic preparations as described above.

Day 1 – Coating (an overnight process): Stocks of antigenic preparations and gelatin are dissolved or suspended in 100 mM carbonate buffer pH 9.5, at 2 µg/ml, and, for each plate 100 µl of the appropriate preparation dispensed per well and incubated overnight at 4^{o}C in a humid chamber.

Day 2 – Coated wells will be washed 3 times in 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% (v/v) Tween 20. They will then be blocked by adding 200 µl/well of gelatin at 0.5% (w/v) in 50 mM Tris-HCl pH 8.0, 0.05% (v/v) Tween 20. Plates will be incubated for 2 hours at room temperature and washed 3 times in 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% (v/v) Tween 20, and stored at 4^{o}C until use.

Days 3 – 9 (approximately) Sera samples will be diluted in the dilution buffer supplied by the HIV ELISA test manufacturer and incubated in the antigen coated plates for 2 hours at room temperature (if the HIV ELISA requires 100 µl/test, then 150 µl of diluted sample will be preadsorbed to ensure transfer of an accurate volume to the HIV ELISA plate), after which the samples will be immediately transferred to the HIV ELISA plate and processed as is usually done.

Control

To control for non-specific adsorption of immunoglobulins we propose to also include preadsorption of serum samples using Protein A-coated plates, i.e., as if Protein A was another antigenic preparation. We do not foresee any significant effect of Protein A pre-adsorption. For a complete discussion of the stoichiometric reasons this is so, see Appendix II. This very strict control will preclude the possibility that a given antigenic preparation will decrease the absorbance value in the HIV test because of “non-specific” removal of immunoglobulins.

Interpretation

Any lowering of the positive control signal after pre-adsorption that remains within the positive values suggested by the test manufacturer will be taken to imply the presence of cross-reacting antibodies that are not sufficiently numerous to interfere with a correct HIV+ serological determination. However, any signals that go from positive to negative will be taken to indicate a cross- reaction that makes the results of the test ambiguous with regard to the HIV serological status of the sample. For a more complete discussion see Appendix III.

Appendix I

The antigens. There was, and may continue to be, considerable discussion about whether this in fact represents a minimal set. Let it be clear why we think it does: There was a general agreement at the meeting that we need to examine disease conditions common to SA. All the antigens listed fall into this category with the exception of *M. Leprae*, BCG and HTLV I and II. The reasons for the inclusion of HTLV plates is that serological tests for these viruses have shown that they are common in general African populations (prevalence app. 10%). Since these are closely related to HTLV III (now HIV), the antigen-coated plates are commercially available, only a handful are required, and there is no complete study of the prevalence of these viruses in SA, it is of interest to determine if antibodies reactive to HTLV I and II yield false positive results in the HIV test. We do not propose to carry out a survey to determine the prevalence of antibodies to HTLV I and II, we intend to determine if antibodies responsible for seropositivity to HIV (formerly known as HTLV III) are cross-reactive with those of HTLV I and II. M. leprae is important because leprosy is becoming an increasingly common condition in some parts of SA, and its inclusion would add important, hitherto unavailable data. A good commercial BCG preparation is essential to quality control the M. tb antigens, and to discover exactly how promiscuous the HIV test currently in use actually is. As it is inexpensive, easily obtained and scientifically informative, there is no reason to omit it from the study.

Appendix II

To further understand how this possibility would affect the results it is appropriate to do some simple stoichiometry:

Each preadsorption well will be coated with 200 ng of antigenic proteins, either complex mixtures (such as *Entamoeba*, *Mycobacteria*, etc.) or relatively simple mixtures (HTLV-1 & 2, adenovirus). Assuming that the hypothetical Ig-binding agent is a protein, say 10 kDa (actually, protein A is around 42 kDa), and that it makes up 100% of the antigen on the plate, then we must conclude that it will bind 3 micrograms of IgG (average MW of 150 kDa at a 1:1 stoichiometry).

Normal serum Ig (IgG + IgM) levels are around 10-20 g/l, or 10-20 micrograms/microliter. If the serum sample were diluted 1:10 and the volume used in preadsorption of 100 microliters then the 10 microliters of actual serum used would contain between 100-200 micrograms of imunoglobulin (actually, in the Abbot HIVAB test for HIV-1 & HIV-2 now in use in the

United States the dilution is much lower and 150 microliters of serum are used, corresponding to 1.5-3 mg antibody). A maximum of 3 micrograms IgG can be bound by 200 ng of 'non-specific' binding protein of 10,000 daltons. Thus, a decrease of maximum 1.7-3% could be reasonably expected to be due to this particular source of error of the experiment (if 10 microliters of serum were used). However, if the hypothetical Ig-binding protein were 1% of the complex antigenic mixture, the decrease in OD would be 0.017-0.03%. Furthermore, if the protein were the size of protein A, then the 'non-specific binding' would be considerably lower still.

If the original HIV ELISA OD readings are within these very small percentages of the cut-off, then this particular source of error may "move" the signal to a negative value. However, at least 110 of the total HIV+ samples will be far above the cut-off value than the worst theoretical possibility of error of this particular sort (the samples belonging to the medium and high signal groups which comprise the majority of the samples).

In fact such a source of error, as demonstrated, falls within the actual precision of the said Abbot HIVAB test. In the Specific Performance Characteristics of the test manual (Table I, p.4) the manufacturer reports an intra-assay variation of 3.7 to 25.8%. This was done using five replicate wells per sample and the figures represent the Coefficient of Variation (CV%) of the quotient of the Mean Sample Absorbance divided by the cut-off of the five determinations in the same assay.

Appendix III

Theoretically, we may expect two possible antibody populations to be responsible for the observed cross-reactivity:

The first population may consist of antibodies truly elicited by *Entamoeba* (or some other) infection which bind (at least some) HIV antigens used in the HIV ELISA test. Preadsorption lacking, such antibodies would yield a <u>false positive</u> result (of tremendous consequences to the quality of life of the patient). Thus, two different outcomes of the preadsortion of the (HIV+) sera may be expected when the samples are re-screened in the HIV ELISA test.

The signal falls below the cut-off values of the HIV test (this could be reasonably expected of samples which give low or mid-range positive values in the HIV ELISA).

The signal decreases but remains above the cut-off (this could be reasonably expected of samples which give very high or off-scale positive values in the HIV ELISA).

If upon preadsorption a decrease in signal results in values below the cut-off of the HIV ELISA test, it <u>must</u> be concluded that the false positive result was due to antibodies not sufficiently specific (that is, diagnostic) of HIV infection recognizing (at least some) antigens used in the HIV ELISA test. These HIV antigens are therefore not appropriate for use to screen a population in which a significant amount of people are exposed to agents capable of generating these particular cross-reactive antibodies. The same is true if the decrease in signal does <u>not</u> diminish below the cut-off. Both outcomes of the experiment would then

reveal flaws in the specificity of the HIV ELISA test, as specificity is defined as the percent of true seronegatives correctly determined to be so under the (serological) conditions prevalent in the population under study, when compared with a gold standard, something that for the HIV ELISA test has never been published.

The second population would be that of antibodies truly elicited by an HIV infection, a portion of which binds to *Entamoeba* (or other test) antigens with sufficient affinity to be removed efficiently by preadsorption against those antigens. If these antibodies are abundant enough, preadsorption will result in a significant decrease in signal in the HIV ELISA test. Thus, two different outcomes upon preadsortion of the (HIV+) sera may be expected when the samples are re-screened in the HIV ELISA test.

The signal falls below the cut-off values of the HIV test (this could be reasonably expected of samples which give low or mid-range positive values in the HIV ELISA).

The signal decreases but it remains above the cut-off (this could be reasonably expected of samples which give very high or off-scale positive values in the HIV ELISA).If upon preadsorption the decrease in signal results in values below the cut-off of the HIV ELISA test, this would also reflect flaws in the specificity of the test, as antigens not specifically diagnostic of HIV infection have been used as tools for detection of antibodies that should be generated solely by a response to HIV. If the signal remains above the cut-off, then the patient was correctly diagnosed as seropositive, demonstrating the sensitivity of the test, as sensitivity is the percentage of true seropositives correctly diagnosed to be so under the (serological) conditions prevalent in the population under study. The sensitivity of the test would be attributable to the fact that not all HIV antigens used by the HIV ELISA test are recognized by antibodies that may be the product of responses to different agents and that the test is sufficiently sensitive to detect that HIV-specific subpopulation of antibodies.

It follows from the previous arguments that each antigenic preparation to infectious agents of high prevalence, or at least common, in SA may yield information on the specificity and sensitivity of the HIV ELISA test. More importantly, the data collected regarding one infectious agent allow no inferences regarding other infectious agents, as it is possible that, for example, *Entamoeba* infection commonly elicits cross-reactive antibody responses whereas another pathogen does not. This is the fundamental justification for the choice of a variety of antigens in the preadsorption studies.

For a test to be used to screen massive amounts of samples, and whose positive results so affect the quality of life (and indeed, amount of life) of the patient, it is of the utmost importance that its reliability be ascertained in every population tested. Furthermore, there is no gold standard to prove or disprove the specificity of the serological test since clinical diagnostic criteria are variable and subject to the physician's discretion (particularly in Africa), and virus/antigen detection is not available. The HIV ELISA test stands alone in that its outcome severely alters the life of the patient and that there is no way to prove or disprove the diagnosis of large number of samples by any means, clinical or biochemical, other than serology.

These considerations lead to an important conclusion regarding the preadsorption experiments: If the studies on preadsorption show that a significant number of samples are of dubious HIV serological status, the HIV ELISA test lacks enough robustness to be used as a diagnostic tool by itself. A false positive result is equivalent to a death sentence applied to an innocent. The preadsorption study will reveal what are the risks of using the HIV ELISA test alone as a tool for the epidemiology of HIV infection on one hand, and as a tool for individual diagnosis of infection on the other.