9 November 2001
Cuernavaca

Dear Mr. President and Minister Pahad,

Below you will find a chronological accounting and documentation (with appropriate annotations) of what has transpired since October 19th.

VALIDATION OF HIV ELISA TESTING IN SOUTH AFRICA, ACT III

As with the previous two acts, this contains two interwoven plots, one, the science of the validation and the second, the human elements of the interaction between ourselves and the holdovers of apartheid represented in this case by the characters Schoub the Brazen, Williamson the Brave and Smith the Silent.

We begin normally enough with the following:

FIND ATTACHED THE CORRECT MINUTE OF THE WORKING GROUP MEETING - SEND YOUR COMMENTS TO ME ASAP
Date: Fri, 19 Oct 2001 15:53:05 +0200
From: "Ray Mabope" <RayMabope@health.gov.za>
To: <cwilliam@curie.uct.ac.za>, <asmith@health.gov.za>, "Lindiwe Makubalo" <MakubL@health.gov.za>,"A Ntsaluba" <NtsalA@health.gov.za>, "Ray Mabope" <RayMabope@health.gov.za>, "Nono Simelela" <SimelN@health.gov.za>, bialy@ibt.unam.mx>, <rasnick@mindspring.com>, <malegapuru.makgoba@mrc.ac.za>, <schoub@niv.ac.za>

MINUTE OF THE MEETING OF THE WORKING GROUP ON 'TESTING THE RELIABILITY OF HIV TESTING IN SOUTH AFRICA' OF THE PRESIDENTIAL ADVISORY PANEL ON AIDS HELD AT THE CROWNE PLAZA HOLIDAY INN SANDTON, JOHANNESBURG ON 18 OCTOBER 2001
1. Attendance: Dr. Ayanda Ntsaluba (Chair) ntsala@health.gov.za
Dr. Nono Simelela simeln@health.gov.za
Mr. Ray Mabope raymabope@health.gov.za
Prof. William Makgoba malegapuru.makgoba@mrc.ac.za
Prof. Barry Schoub schoub@niv.ac.za
Prof. Alan Smith asmith@health.gov.za
Dr. David Rasnick rasnick@mindspring.com
Dr. Harvey Bialy bialy@ibt.unam.mx or h.bialy@natureny.com
Prof. Sam Mhlongo ansie@wn.apc.org
Dr. Caroline Williamson cwilliam@curie.uct.ac.za

Dr. Lindi Makubalo makubl@health.gov.za

2. In his introductory remarks, Dr Ntsaluba reminded the Working Group that two outstanding tasks after the meeting of the Presidential Advisory Panel on AIDS which took place in July, 2000, were (1) the patterns of mortality and (2) the reliability of the tests. Whilst the work was on the patterns of mortality was ongoing under the leadership of StatsSA, this meeting was convened to take forward the work on the reliability of HIV testing. The meeting was informed that in 2001, the HIV Antenatal Survey would be expanded to include the following components: (1) the private sector; (2) behavioural surveys;and (3) incidence testing.

3. The main purpose of this meeting was to agree on the kind of tests to be done; to develop the protocol(s) for the tests; to assess the capacity to do the tests; to determine where the tests would be done; and to identify and commit resources for doing the tests.

4. Prof Barry Schoub made a presentation to the meeting on the first phase of the tests, which showed that there was 99.9% concordance between the HIV tests done in 5 sites in South Africa and the Centers for Disease Control and Prevention (CDC).

5. It was agreed that we should proceed towards the 2nd phase of the tests to answer the question: "When you do the tests, do they mean what we say it means". In this regard, it was agreed to discuss the pre-adsorption based protocol that was proposed by Dr Harvey Bialy.

6. The meeting adopted the protocol presented by Dr Harvey Bialy with the following changes: that the pre-adsorption antigens would be limited to diseases which are endemic and common in South Africa. These are TB, Malaria and Amoeba.Adenovirus should be included as well; and that this experiment will be done alongside the experiment proposed by Prof Barry Schoub, which is based on serial dilutions, which would achieve the same objectives.

7. It was agreed to use herpes and measles antigens as controls to monitor cross-reacting antigens and a reduction in the optical densities. [Prof Barry Schoub suggest that hepatitis A antibodies be considered in place of measles due to availability of highly sensitive Elisa tests and measles may not be as ubiquitously present throughout the population]

8. There was a substantial discussion on the interpretation of the results from the 2 experiments. It was agreed on how the results would be interpreted and everybody was committed to respect the outcomes of the two experiments and to work together in finding answers to further questions that may arise from the experiments.

9.. It was agreed that the revised protocol on pre-adsorption testing (Dr Harvey Bialy) and the new protocol on the serial dilution experiments (Prof Barry Schoub and Prof Alan Smith) must be circulated to the Working Group by Friday, 26 October 2001, and only one week would be allowed for comments from the Working Group.

10. It was agreed that the experiments would be done in South Africa at the laboratory facilities of the National Institute for Communicable Diseases (Prof Barry Schoub) and the Department of Virology of the University of Natal Medical School (Prof Alan Smith).

11. It was agreed that both Prof Barry Schoub and Prof Alan Smith would take the responsibility for coordinating the work on the experiments and compile a budget.

12. It was agreed that all the preparations for the experiments (protocols, antigens, reagents, consumables, personnel, etc.) must be done and completed by the end of January as Dr Roberto Stock of the Institute of Biotechnology of the Autonomous National University of Mexico, Cuernavaca, Mexico, one of the principal investigators, would be available to come to South Africa on 25 January 2001.

13. It was agreed that:

a. Dr Harvey Bialy would prepare the revised pre-adsorption protocol.

b. Prof Alan Smith and Prof Barry Schoub would prepare the serial dilution protocol.
c. Prof Terry Jackson would prepare the Entamoeba and Malaria antigens.
d. Dr Harvey Bialy would prepare the Adenovirus antigens.
e. Prof Mhlongo would prepare the 100 TB patients and approach his Ethics Committee.
f. Prof William Makgoba would approach Stellenbosch University for the TB antigen.
g. Prof Makgoba would present the work to the MRC Ethics Committee for approval.

14. The experiments would commence at the end of January 2002. The Working Group would meet at the end of February 2002 to look at the results and interrogate the data at that stage.

15. Dr David Rasnick and Prof Sam Mhlongo raised their concerns regarding the time it took to meet to agree on the 2nd phase experiments and inquired on the plan and timetable to do the other outstanding experiments, which are contained in the report of the Panel. Dr Ayanda Ntsaluba accepted the criticism for the late start. He assured the Working Group that there was no attempt to block or censor any experiments. He promised to give guidance on the process for the other experiments by e-mail to the Working Group with one calendar month.

16. It was agreed that Department of Health would consider whether or not it was necessary to issue a press statement and
if so, only one statement would be issued and that would be from the Department of Health.

END!

I reply to this as follows:

Subject: Re: FIND ATTACHED THE CORRECT MINUTE OF THE WORKING GROUP MEETING- SEND YOUR COMMENTS TO ME ASAP
Date: Tue, 23 Oct 2001 14:51:07 -0600
From: harvey bialy <h.bialy@natureny.com>
To: Ray Mabope <RayMabope@health.gov.za>
CC: cwilliam@curie.uct.ac.za, asmith@health.gov.za, Lindiwe Makubalo <MakubL@health.gov.za>, A Ntsaluba <NtsalA@health.gov.za>, Nono Simelela <SimelN@health.gov.za>, bialy@ibt.unam.mx,
rasnick@mindspring.com, malegapuru.makgoba@mrc.ac.za, schoub@niv.ac.za

Ray,

As you well know, this represents only one version of the minutes, and David, Sam and I do not agree that they represent the final word. The version of the minutes we prepared in your offices on Friday morning would seem to be equally appropriate to put on the group mailing, so that both sides can see where some disagreements still remain, and we can quickly iron them out. Thanks.

Harvey

Dave in the meantime sends this: (I include only the sections with his comments in italics)

Dear Ray,

Thanks for sending the summary of the Oct. 18 meeting. Below I add my comments and corrections.

on 10/19/01 6:53 AM, Ray Mabope at RayMabope@health.gov.za wrote:

> convened to take forward the work on the reliability of HIV testing. The
> meeting was informed that in 2001, the HIV Antenatal Survey would be expanded
> to include the following components: (1) the private sector; (2) behavioural
> surveys; and (3) incidence testing.

The survey's are for antibodies to HIV. They are not surveys of either HIV nor of AIDS. That must be specifically noted.

>
> 4. Prof Barry Schoub made a presentation to the meeting on the first phase of > the tests, which showed that there was 99.9% concordance between the HIV tests
> done in 5 sites in South Africa and the Centers for Disease Control and
> Prevention (CDC).

As far as I know, the members of Mbeki's AIDS Panel have not seen the specific protocols for nor detailed data from the collaboration between the MRC and the CDC.

> 6. The meeting adopted the protocol presented by Dr Harvey Bialy with the
> following changes: that the pre-adsorption antigens would be limited to
> diseases which are endemic and common in South Africa. These are TB, Malaria
> and Amoeba. Adenovirus should be included as well; and that this experiment
> will be done alongside the experiment proposed by Prof Barry Schoub, which is
> based on serial dilutions, which would achieve the same objectives.

I strongly recommend that all of the previously proposed antigens of the Bialy et al. study be included. The purpose of that study is to confirm the extent of non-specific antibody binding to a range of antigens.

> 8. There was a substantial discussion on the interpretation of the results
> from the 2 experiments. It was agreed on how the results would be interpreted
> and everybody was committed to respect the outcomes of the two experiments and
> to work together in finding answers to further questions that may arise from
> the experiments.

The proposed dilution experiment of Barry Schoub was indeed agreed to. However, it was not agreed by all members that Schoub's experiment would achieve the same objectives of Bialy et al. The discussion of the interpretation of the Schoub proposal was cut short and not concluded.

> 15. Dr David Rasnick and Prof Sam Mhlongo raised their concerns regarding the time it took to meet to agree on the 2nd phase experiments and inquired on the
> plan and timetable to do the other outstanding experiments, which are
> contained in the report of the Panel. Dr Ayanda Ntsaluba accepted the
> criticism for the late start. He assured the Working Group that there was no
> attempt to block or censor any experiments. He promised to give guidance on
> the process for the other experiments by e-mail to the Working Group with one
> calendar month.

Sam Mhlongo and Rasnick mentioned the remaining 9 experiments and studies specifically outlined in the interim report of Mbeki's AIDS panel.

So now I now modify my earlier email as follows:

Subject: Re: FIND ATTACHED THE CORRECT MINUTE OF THE WORKING GROUP MEETING- SEND YOUR COMMENTS TO ME ASAP
Date: Wed, 24 Oct 2001 15:50:41 -0600
From: harvey bialy <h.bialy@natureny.com>
To: Dave Rasnick <rasnick@mindspring.com>
CC: Ray Mabope <RayMabope@health.gov.za>, cwilliam@curie.uct.ac.za, asmith@health.gov.za, Lindiwe Makubalo <MakubL@health.gov.za>, A Ntsaluba <NtsalA@health.gov.za>, Nono Simelela <SimelN@health.gov.za>, bialy@ibt.unam.mx, malegapuru.makgoba@mrc.ac.za, schoub@niv.ac.za
References:
1

I am in complete agreement with Dave's email. Remember the question was reduced to disease conditions common in South Africa. HTLV I and II were claimed to be uncommon but as far as I can discover there is no large scale serological study published, and in all other studies on African populations the prevalence of positive tests is around 10%. Since HTLV I, II and III (HIV) are extremely similar, the inclusion of these two antigens as controls for HIV ELISAs is scientifically important. Finally, the addition of these two antigens only involves 8 ELISA plates and about 2 hours worth of pipetting. The other antigens omitted, i.e. those to common enteric infections contradicts our understanding to look at indigenous disease conditions that are generally considered AIDS related. The omission of the helminthic worm prep is similarly at variance, since schistosomiasis is not uncommon in South Africa. Similarly, M. leprae is becoming increasingly common, and the addition of this

antigen preparation is simple and would provide important, hitherto unavailable data. I would agree to leaving out pnemococcus if we have to omit anything.

With regard to the dilution controls. Again, I must reinforce Dave. We reached no mutual understanding of what such experiments would show. Importantly there was nothing that would allow the inclusion of the phrase "which would show the same thing" that appeared in the first version of the minutes. Since these are all the key points that Dave and I raised in our revision of Ray's minutes, I do not think it is necessary for Ray to circulate those as I had suggested earlier.

(Aside to Smith from me)

Subject: [Fwd: FIND ATTACHED THE CORRECT MINUTE OF THE WORKING GROUP MEETING- SEND YOUR COMMENTS TO ME ASAP]
Date: Wed, 24 Oct 2001 18:14:23 -0600
From: harvey bialy <h.bialy@natureny.com>
To: Alan Smith <ASmith@nu.ac.za>, Ray Mabope <RayMabope@health.gov.za>
Alan,
I have noticed that the email address for you in the group mailing is different from the above and does not function. I hope someone has been forwarding the exchange thus far, but in the event they have not here is a compendium of the discussion till now. (sent but not included here as it is only a repetition of the emails above)
Kind regards,
Harvey

Enter Williamson the Brave and Monochromic (who has not yet noticed that the emails to her professor Smythe are not being received as she uses the wrong email address):

Subject: HIV testing - working group
Date: Thu, 25 Oct 2001 11:48:17 +0200
From: "Williamson, C, Carolyn, Dr" <cwilliam@curie.uct.ac.za>
Organization: University of Cape Town
To: Dave Rasnick <rasnick@mindspring.com>, harvey bialy <h.bialy@natureny.com>
CC: Ray Mabope <RayMabope@health.gov.za>, cwilliam@curie.uct.ac.za, asmith@health.gov.za, Lindiwe Makubalo <MakubL@health.gov.za>, A Ntsaluba <NtsalA@health.gov.za>, Nono Simelela <SimelN@health.gov.za>, bialy@ibt.unam.mx, malegapuru.makgoba@mrc.ac.za, schoub@niv.ac.za

I believe the Chair of the meeting (Dr Ntsaluba) stated at the meeting that we would not enter into lengthy debates and that decisions made at the meeting would be carried forward. It was also

decided that the methodology, like any experiment, could be re-assessed once the experimental work had started and there were unforeseen complications.

Sincerely,

Carolyn Williamson

I next contribute the following:

Subject: Revised pre-adsorption protocol attached
Date: Thu, 25 Oct 2001 08:49:10 -0600
From: harvey bialy <h.bialy@natureny.com>
To: Ray Mabope <RayMabope@health.gov.za>
CC: cwilliam@curie.uct.ac.za, ASmith@nu.ac.za, Lindiwe Makubalo <MakubL@health.gov.za>, A Ntsaluba <NtsalA@health.gov.za>, Nono Simelela <SimelN@health.gov.za>, bialy@ibt.unam.mx, rasnick@mindspring.com, malegapuru.makgoba@mrc.ac.za, schoub@niv.ac.za, rstock@ibt.unam.mx
References:
1
Dear all,

This is for the table. In particular, since we have previously agreed to the essence of what you will read, and the only significant differences are in the number of antigens to be used, it is incumbent that counter-arguments to the inclusion of these antigens be substantive (i.e. not of the form: "this is what the chairman said"), especially as the entire study will consume less than 5% of the HIV test plates that were used in the recent MRC QC exercise and will provide considerably more practical information of immediate use in assessing the true HIV serological status of South Africans. The idea of doing a small set first and then a larger one depending on the outcome is an extremely ineffective way to accomplish the major goal of our deliberations since everything can be easily done at one time, and the total of the information that will be obtained by the same workers, in duplicate and at that time, will be many fold more valuable than a piecemeal data collection, as well as being considerably less logistically complex and obviously much more cost effective.

Here is the second version of our proposal.

Amended 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 Alagon (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)

The fundamental rationale of the protocol under discussion and precise formulation is to determine the accuracy of HIV ELISA tests currently in use when the sera that is being evaluated has first been pre-adsorbed to possibly confounding antigens.

Sample sizes and varieties:

150 HIV+ sera samples will be obtained after approval from the relevant SA ethics committee(s). One ELISA test will be chosen by the MRC side of the collaboration as representative of the apparent variety of tests now in use in SA, and it will be used to both determine the initial HIV + status of the sample set, and as the test to be evaluated in the adsorption studies..

The samples should be divided as follows:

10 samples off scale positive (OD greater than 4)
100 mid-range (OD 1.75-3.5)
40 lower limit (whatever value the test manufacturer suggests)

It is crucial that Dr. Stock be supplied with sufficient quantities of the sera samples **so that after the dilution that is recommended by the kit manufacturer, he will have at least 4 ml of each sample** in order to have sufficient quantities for all the tests that need to be performed.

The second set of sera samples will be supplied by Prof. Mhlongo, and consist of 100 TB patients whose HIV serological status has never been previously examined.

Antigens

The following is a minimal set of antigenic preparations that will be required to make a reasonably rigorous determination of HIV ELISA robustness. There was, and may continue to be considerable discussion about whether this in fact represents a minimal set. Let it be clear here why we think it does: There was general agreement at the meeting that we need to examine disease conditions common to SA that are generally considered to be AIDS-related. All the antigens listed below are from pathogens that fall into this category with the exception of M. leprae, BCG and HTLV I and II. The reasons for including the HTLV plates is that serological tests for these viruses have shown that they are extremely common in general African populations (prevalence app. 10%) and they are very closely related to HTLV III (HIV), there is no complete study of their prevalence in SA, the antigens are commercially available in kits produced by at least one of the same manufacturers that produces HIV tests and only a handful are needed for the study. Therefore there is no good scientific reason for leaving them out. 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.

All antigens will be initially quality controlled as follows:

(1) Protein concentrations determined by both teams in concert. Dr. Stock favors the 'BCA Protein Determination Kit' manufactured by Pierce.

(2) SDS polyacrylamide gels be run to ensure that we are working with complex protein mixtures. (If it becomes necessary, (i.e. after the studies are completed and either part of the collaboration wishes it) the samples can then be more rigorously authenticated through any of a number of possible means. If a suitable gel apparatus is not available in the laboratory chosen for the studies, Dr. Stock is prepared to bring a compact device from his laboratory that functions extremely well.

Antigens:

1. HTLV- I (commercially available)
2. HTLV- II (commercially available)
3. Mycobacterium t.b. (to be prepared according to a referenced protocol in SA)
4. Mycobacterium leprae (source to be determined)
5. BCG (commercially available)
6. Entamoeba histolytica (to be prepared in South Africa in concert with Dr. Stock who is an expert on this organism. Since the antigens are very unstable, this preparation needs to be made in situ and takes two days.
7. Adenovirus (to be supplied by Dr. Bialy)
8. Plasmodium falciparum (to be prepared according to a referenced protocol in SA)
9. Schistosomiasis (source to be determined)
10. Enteropathogenic E. coli (source to be determined)
11. Enteropathogenic Salmonella typhimurium (source to be determined)

All preparations need to be at approximately 1 mg total protein/ml and approximately 1 ml is required for all the proposed studies (see below).

It is necessary, considering the special conditions under which we are working, to insert a parenthetical set of remarks concerning the possibilities of either group deliberately attempting to fudge the outcome of the studies. Let us imagine that Drs. Stock, Alagon, myself and Mhlongo wished to dope the tests by using antigenic preparations that were 'spiked' with HIV antigens. In this case each and every initially positive HIV signal would be reduced by the same amount. Thus it is impossible for us to interfere with the outcome via this means. The only possible way we might accomplish this is by sleigh of hand at the moment the antigens are added to the NUNC plates. That is to say, Dr. Stock could (if he were adept at such maneuvers) slip a few hundred nanograms of HIV antigens into a few random wells on each pre-adsorption test plate. We contend this would be impossible for two reasons. (1) We insist that every laboratory manipulation that is carried out by Dr. Stock be under the direct supervision of several members of the MRC side of the collaboration. (2) Dr. Stock will be using a multi-channel pipetter that

will go only from the stock wells to the tests wells. As an aside to this: Dr. Stock strongly advises that he use his own multi-channel pipette device which he has personally calibrated. If not, then he wishes to be allowed to confirm to his own (and all observer's) satisfaction that the device he will use is accurate to his demanding specifications. From the other side, the only possibility of deliberating interfering with the outcome lies in the NUNC MAXISORP uncoated ELISA plates that will be used in the pre-adsorption phase of the study. For example, the plates could be pre-blocked with 300 ng/well of gelatin or BSA and therefore not adsorb any of the antigens that might be added. We therefore insist that the box of 100 plates that will be more than sufficient for all the studies be kept unopened, and that all the plates be in their original sealed packages.

HIV Test Plates Required

Each antigen to be tested as an HIV-ELISA sponge is pipetted into NUNC ELISA 96 well plates.

1. The 100 TB samples will only be tested against 4 antigens (M. Tb., M. leprae, BCG, plus the positive control – gelatin or BSA). This requires 2 HIV ELISA plates (x2) for each antigen for this part of the study. Thus for the 3 test antigens plus positive control, we need a total of 16 HIV ELISA plates

2. For the remaining 150 samples, the calculation is as follows: 4 plates are needed to accommodate 150 samples in duplicate, x (11 [if we use all the test antigens above] + 1 positive control) = 48 HIV ELISA plates.

Additional Materials

We will need to purchase 4 HTLV-I test plates and 4 HTLV-II tests in order to examine 150 samples in duplicate, and in addition to the 100 NUNC plates, and protein determination kits sufficient for 50 assays, Dr. Stock will also need 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 by technicians.

Protocol and timeframe

Day 1. Coating (a 24 hour process): dissolve or suspend the antigen stocks (and the positive control protein – gelatin or BSA) in 100 mM carbonate buffer, pH 9.5, at 2 micrograms/ml and pipette 100 microliters into all the necessary wells.

Day 2: Wash and dry all 'sponge' plates.

Day 3: Block all the unadsorbed sites with gelatin (BSA) to normalize the amount of protein in each well.

Days 4 – 9 (approximately): Dilute the sera samples as recommended by the kit manufacturer and pre-adsorb for 2 hours at ambient temperature against the bank of antigens plus the positive

control plates. Then immediately transfer the contents to HIV ELISA test plates and carry out the assay as is normally done. Dr. Stock can process 2-3 antigens/day (working 8 hours at the bench) and with the printout from the plate reader can process the raw data in his hotel overnight, so that there will be interpretable results the next morning. A reasonable estimate is 10 –12 days to complete the study. If Dr. Alagon accompanies Dr. Stock, processing time can be halved. Since Dr. Alagon is an internationally recognized expert in immunodiagnostics, we would highly recommend that he be invited to do so. (They are willing to share a hotel room, so it is only the additional cost of a ticket to accomplish this).

Both Drs. Stock and Alagon can come to South Africa on or about January 25, 2002. It is impossible for either of them to block out the time required before then.

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 non-specific reaction that makes the results of the test ambiguous with regard to the HIV serological status of the sample.

Almost simultaneously I send the following email to a select few:

Subject: some thoughts on the 'dilution' expt's of shoub and smith
Date: Thu, 25 Oct 2001 11:28:48 -0600
From: harvey bialy <h.bialy@natureny.com>
To: Dave Rasnick <rasnick@mindspring.com>, rstock@ibt.unam.mx, alagon@ibt.unam.mx, Ansie Olivier <ansie@wn.apc.org>, Ray Mabope <RayMabope@health.gov.za>

The expt as they want to perform it (and as I have it from Prof. Smythe via Ray in hardcopy):

Take samples dually reactive to HIV/Entamoeba and HIV/adenovirus. (According to Smith the amoebae test is good. I have no idea. The adeno is, I think, as good as an immunoassay can get.) and serially dilute the samples ('in dilution buffer' Prof. Smythe is careful to point out, lest we use sulfuric acid by mistake, though he does not specify which dilution buffer(s) he intends to use) until presumably reactivity to the 'non-HIV antigens' is lost at a dilution much lower than the HIV reactivity. What this is supposed to prove vis a vis HIV ELISA specificity under field conditions remains completely unclear to me, but even assuming it means what they would like to say it does, i.e. that the cross-reacting material can be removed and one is left with HIV-specific antibodies, it is not the correct control.

It seems to me the proper 'control' for our studies (via the dilution approach) would be to take all of the serum samples that became HIV negative after preadsorption to entamoebae and adeno and test those to see if indeed there is even evidence of dual infection. It is not at all certain that these samples will be dually reactive, particularly if the tests use purified proteins from these organisms which I presume they do. But again, suppose they are, trying to argue that the HIV test is still specific when it is performed at the dilution recommended by the manufacturer on a random serum sample holds about as much water as a punctured condom, even ignoring quantitative arguments based on the Gaussian distribution of antibody binding affinities in the sample

My suggestion in the final protocol would be to let them have all the rope they want and include both expts (gently suggest the second, leave the scientific critique of their meanings until the data is all in and it comes time to write the paper, but not allow them to insert any language in the protocol which would suggest that we think the two sets of studies show the same things.)

Roberto, Alejandro, Dave, Sam: Please your thoughts on this.
Ray: FYI only.

(I received affirmative responses from all)

Barry the Brazen now sends this:

Subject: Re: Draft protocol for Phase 2 studies for President's AIDS Panel
Date: Fri, 26 Oct 2001 14:00:50 GMT+0200
From: "Prof BD Schoub" <schoub@niv.ac.za>
To: Ntsala@health.gov.za, raymabope@health.gov.za, malegapuru.makgoba@mrc.ac.za, simeln@health.gov.za, makubl@health.gov.za, cwilliam@curie.uct.ac.za, asmith@health.gov.za, bialy@ibt.unam.mx, h.bialy@natureny.com, rasnick@mindspring.com, ansie@wn.apc.org

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DRAFT PROTOCOL PHASE TWO STUDIES OF PRESIDENTIAL AIDS PANEL

AIM: To investigate the effect on HIV serology of pre-adsorption of sera by antigenic material derived from infectious agents indigenous and common to South Africa.

STUDY DESIGN: The study will consist of two components:-

1. **The adsorption study:** Blank ELISA microtitre plates will be coated with

antigenic material from the following organisms:-

1. *Mycobacterium tuberculosis*
2. *Entamoeba histolytica*
3. *Plasmodium falciparum*
4. Adenovirus

Serum samples previously demonstrated to be HIV reactive will be added to the coated wells and incubated overnight to allow adsorption of antibodies. Samples will then be tested and OD reading before and after adsorption will be compared.

Pre- and post-adsorption serum samples will also be tested for highly prevalent antibodies, e.g. herpes simplex and hepatitis A to examine the effect of the adsorption process on these antibodies.

2. **The dilution study:** Serum samples dually reactive to HIV and *Entamoeba histolytica* or adenovirus will be serially diluted in dilution buffer and serological tests to determine dilution extinctions will be carried out.

METHODS:

A.

$ 100 random HIV positive serum samples from adults together with 100 HIV untested samples from adult tuberculosis patients (to be supplied by Professor S Mhlongo) will be collected. Approximately 4ml of serum (10ml blood sample) from each adult will be required.

$ Antigen preparations will be acquired from the following sources:-

1) *Mycobacterium tuberculosis* - to be supplied by Professor M W Makgoba from University of Stellenbosch TB facility.
2) *Entamoeba histolytica* and *Plasmodium falciparum* to be supplied by Professor Terry Jackson of the MRC.
3) Adenovirus capsid protein to be supplied by Dr Harvey Bialy.

$ Drs Roberto Stock and Alejandro Alagon (Institute of Biotechnology, autonomous national University of Mexico, Cuernavaca, Mexico) and Dr Harvey Bialy will come to South Africa towards the end of January (25^{th}) and supervise the adsorption experiments. Blank ELISA plates (NUNC) will be coated with the respective antigens mentioned above by dissolving or suspending the antigen preparations (2μg/ml) in 100mM carbonate buffer (pH 9.5) and pipetting 100μl into each well and leaving overnight at 4^{o}C. The plates will then be washed and gelatin or BSA (at 300ng/well) will be added. The 200 serum samples will then be added to each well for 2 hours at room temperature and then removed and tested for HIV antibodies using the same test as used before the adsorption procedure.

The serum sample pre- and post-adsorption would also be tested by standard routine diagnostic EIA tests for HSV (herpes simplex virus) and anti-hepatitis A antibodies.

B.

$ 50 serum samples dually reactive against HIV and *Entamoeba histolytica* and 50 serum samples dually reactive against HIV and adenovirus will be collected.

$ Serial 10-fold dilutions in diluting buffer will be made and each dilution tested for HIV and *Entamoeba histolytica* or adenovirus, respectively.

ETHICS: Both samples will be taken from adults after obtaining informed written consent. The study will be submitted for approval to the Ethics Committee of the Medical Research Council.

SITE OF STUDY:

Study A: The adsorption studies will be undertaken at the Department of Virology, University of Natal, as well as the HIV serology on the sera pre- and post-adsorption. The HSV and HAV serology on the pre- and post-adsorption sera will be performed at the NICD (National Institute for Communicable Diseases).

Study B: The dilution studies on sera dually reactive for HIV and Eh will be performed at the University of Natal and the HIV and adeno tests will be performed at the NICD.

BUDGET:

a) Department of Virology, University of Natal : to follow.

b) NICD: Costs of serology testing:-

A. **Adsorption study:**

200 pre- + 200 post-adsorption sera, tested for HSV and hepatitis A antibodies (IgG)

HSV	-	R34,40/test x 400	=	R 13 760
Hep A	-	R38,30/test x 400	=	R 15 320

B. **Dilution study:**

- Pre-screening of HIV+/adeno+ sera = 100 tests
- Dilution – 50 HIV/adeno+ samples
- 6 x 10-fold dilution = 300 tests

HIV	-	R24,90/test x 400	=	R 9 960
Adeno	-	R34,40/test x 400	=	R 13 760

Total: for single tests	R 52 710
Total: if done in duplicate	R105 420
Courier costs:	R 2 000

Now we begin to enter the realms of the surreal:

(William the Timid makes his one and only appearance until today at this point):

Subject: Re: Draft protocol for Phase 2 studies for President's AIDS Panel
Date: Fri, 26 Oct 2001 16:07:46 +0200
From: Prof W Makgoba <mwmakgoba@mrc.ac.za>
To: Prof BD Schoub <schoub@niv.ac.za>
CC: Ntsala@health.gov.za, raymabope@health.gov.za, malegapuru.makgoba@mrc.ac.za, simeln@health.gov.za, makubl@health.gov.za, cwilliam@curie.uct.ac.za, asmith@health.gov.za, bialy@ibt.unam.mx, h.bialy@natureny.com, rasnick@mindspring.com, ansie@wn.apc.org
References:
1

Dear Barry,
Thank you for this protocol. I have spoken to Prof Peter Cleaton-Jones about the ethical review process and he is ready and waiting. I have also discussed with Prof Terry Jackson of the MRC's Amoebiasis Unit to obtain the exact requirements from Alan so that he can start from now to prepare for the requirements.
I believe we are now ready to get on and do the studies as we have agreed upon.

yours sincerely,

Malegapuru)

However, Bialy the Bastard is not so passive and I write as follows:

Subject: Re: Draft protocol for Phase 2 studies for President's AIDS Panel
Date: Fri, 26 Oct 2001 08:33:44 -0600
From: harvey bialy <h.bialy@natureny.com>
To: Prof BD Schoub <schoub@niv.ac.za>
CC: Ntsala@health.gov.za, raymabope@health.gov.za, malegapuru.makgoba@mrc.ac.za, simeln@health.gov.za, makubl@health.gov.za, cwilliam@curie.uct.ac.za, asmith@health.gov.za, bialy@ibt.unam.mx, rasnick@mindspring.com, ansie@wn.apc.org
References:
1

Barry,

This proposal contains a number of deficiencies that need to be corrected before we can proceed to write the final protocol. I delineate some of the more serious problems here, and after they are cleared up we can move on.

You were charged with preparing a protocol for your part of the experiments, not to attempt a synthesized version.

You give an account of the pre-adsorption studies on both pages of your two page document in two completely different forms. One almost correct (and almost completely copied and pasted from the 'notes' we presented at our roundtable) the other completely incorrect and derived from who knows where. I have made comments in parentheses indicating what needs fixing. (a) incorrect "Serum samples previously demonstrated to be HIV reactive will be added to the coated wells and incubated overnight to allow adsorption of antibodies. Samples will then be tested and OD reading before and after adsorption will be compared" (This is NOT the experiment we discussed or proposed and should be omitted in toto. Read our protocol again so that you can understand the purpose of the BSA/gelatin positive control correctly. Please limit yourself to preparing a full protocol of the dilution experiments you wish to carry out. Including, the rationale, the exact kits you intend to use to test either amoebae and/or adeno (Please specify , one or the other or both.), and the dilution series you wish to use (ten fold dilutions are not good, 2 fold dilutions are much more common in serology). Further I would propose that in addition to the studies you will delineate in detail, we also add the following CRITICAL control. ALL samples that lose HIV reactivity after pre-adsorption to either amoebae and/or adeno should be examined by the dilution protocol using the same tests you wish to use for samples previously determined to be dually reactive. It is not at all certain that samples from the random sera collection that have large decreases in HIV signals after 2 hrs preadsorption to amoebae or adeno will even be reactive to the two pathogens on the tests you choose. (b) correct: (more or less): "Blank ELISA plates (NUNC) will be coated with the respective antigens mentioned above by dissolving or

suspending the antigen preparations (2?g/ml) (2 MICROGRAMS/ML. It is there in the document you copied most of this from) in 100mM carbonate buffer (pH 9.5) and pipetting 100 (microliters, ibid)?l into each well and leaving overnight at 4(superscript) oC. The plates will then be washed and gelatin or BSA (at 300ng/well) will be added (Not exactly, but the protocol we sent you has it right and it is not necessary for you to attempt to reproduce it. Once more, please limit yourself to preparing a proposal for the dilution experiments that you would feel comfortable submitting to an international granting agency and not a rubber stamp body.) The 200 serum samples will then be added to each well (Again, not exactly, only one sample will be added to each well, in total there will be several hundred) for 2 hours at room temperature and then removed and tested for HIV antibodies using the same test as used before the adsorption procedure."

With regard to the following: "The serum sample pre- and post-adsorption would also be tested by standard routine diagnostic EIA tests for HSV (herpes simplex virus) and anti-hepatitis A antibodies." : What exactly are these standard tests, and why should we use the random HIV+ sera set against them? Is there reason to think these patients will have herpes or hep A? It would seem that the correct control for these tests is to use sera that is known to be reactive to the two antigens in question and preadsorb them against the sponge bank we use for the HIV tests.

I cannot fathom your budget. Please indicate in a form similar to the one we used in our protocol how the material requirements break down so that purchasing agents can do the arithmetic.

The timetable logistics are not clear. Remember Dr. Stock needs to be present for every single laboratory manipulation, so an order needs to be set for all the work incorporating the day by day schedule we presented in our protocol. It is not acceptable that one part of the study be done in collaboration (preadsorption) and your study be done in isolation as it would appear from the document you sent.

It is not necessary for me to be in South Africa for the performance of these experiments. Drs. Stock, and Alagon are essential as is Prof. Mhlongo, I am not.

Finally, we come back to the question of the number of antigens. The set we sent you yesterday is the one we are presently discussing. I suggest you do two things. (1) prepare a revised protocol for you dilution studies that incorporates satisfactory responses to the issues raised above, and (2) if you have problems with the list of antigens we want to use, to address those in a separate communication antigen by antigen.

Thanks for sending this on time.

Harvey

Dave the Devoted sends his comments on Schoub et al:

Subject: Re: Draft protocol for Phase 2 studies for President's AIDS Panel
Date: Fri, 26 Oct 2001 09:40:32 -0700
From: Dave Rasnick <rasnick@mindspring.com>
To: Prof BD Schoub <schoub@niv.ac.za>, <Ntsala@health.gov.za>, <raymabope@health.gov.za>, <malegapuru.makgoba@mrc.ac.za>, <simeln@health.gov.za>, <makubl@health.gov.za>, <cwilliam@curie.uct.ac.za>, <asmith@health.gov.za>, <bialy@ibt.unam.mx>, <h.bialy@natureny.com>, <ansie@wn.apc.org>

I have read both protocols: Bialy et al; Schoub et al.

The Bialy et al. protocol is clear with appropriate specifics so that anyone skilled in the art of immuno-diagnostics should be able to carry out the experiments.

In addition, it is clear what the experiments of Bialy et al. are intended to do: "determine the accuracy of HIV ELISA tests currently in use when the sera that is being evaluated has first been pre-adsorbed to possibly confounding antigens."

"[A]ny signals that go from positive to negative will be taken to indicate a non-specific reaction that makes the results of the test ambiguous with regard to the HIV serological status of the sample."

The purpose of the Schoub et al. experiment, on the other hand, is not stated:

"The dilution study: Serum samples dually reactive to HIV and Entamoeba histolytica or adenovirus will be serially diluted in dilution buffer and serological tests to determine dilution extinctions will be carried out."

Okay, they will determine "dilution extinctions". What is the purpose of determining dilution extinctions? After the experiment is done, what will it tell us?

Dave

And I now send the following to a subset of the panel:

Subject: A question.
Date: Fri, 26 Oct 2001 10:42:33 -0600
From: harvey bialy <h.bialy@natureny.com>
To: Ray Mabope <RayMabope@health.gov.za>, Dave Rasnick <rasnick@mindspring.com>, Ansie Olivier <ansie@wn.apc.org>

Ray,
Please reply to this. After all is said and done through the emails, who will make the final decision on which protocol we will use? It is clear already that the other side does not wish to extend the list of antigens to include enough to make a real determination of the value of the hiv tests, as it is equally clear that they do not understand anything about the science of what we are doing. It remains unfathomable to me that they did not see the gigantic contradiction between the two forms of the preadsorption study they cited in their proposal and this is supposed to be the single most important health issue in the country! I can well imagine the amount of intellectual and other effort they devote to real diseases. And these are the people who are also supposed to train a new generation of world class South African scientists? Oy vey! In the next administration (when we have triumphed in the aids lucha and our President is fully empowered), I pledge all my efforts towards helping you establish a rational biotech policy. No charge. Only the first part of this requires an answer. Please pass this along to a select few.
Your friend,
Harvey

(I have not yet received any reply, despite several resends which I will not replicate here)

Now Williamson the Brave enters for the second time with the following:

Subject: Re: Draft protocol for Phase 2 studies for President's AIDS Panel
Date: Fri, 26 Oct 2001 21:04:13 +0200
From: Carolyn Williamson <cwilliam@curie.uct.ac.za>
To: Prof BD Schoub <schoub@niv.ac.za>, Ntsala@health.gov.za, raymabope@health. gov.za, malegapuru.makgoba@mrc.ac.za, simeln@health.gov.za, makubl@health.gov.za, asmith@health.gov.za, bialy@ibt.unam.mx, h.bialy@natureny.com, rasnick@mindspring.com, ansie@wn.apc.org
References:
1

Dear Professor Schoub,
Thank you for your protocol. Please find a couple of comments included in the text. I have used the tracking option in Word.
Thank you.

(Here it is)

DRAFT PROTOCOL PHASE TWO STUDIES OF PRESIDENTIAL AIDS PANEL

AIM: To investigate the effect on HIV serology of pre-adsorption of sera by antigenic material derived from infectious agents indigenous and common to South Africa.

STUDY DESIGN: The study will consist of two components:-

3. **The adsorption study:** Blank ELISA microtitre plates will be coated with antigenic material from the following organisms:-
   1. *Mycobacterium tuberculosis*
   2. *Entamoeba histolytica*
   3. *Plasmodium falciparum*
   4. Adenovirus

   Serum samples previously demonstrated to be HIV reactive will be added to the coated wells and incubated for 2 h to allow adsorption of antibodies. Samples will then be tested for HIV and OD reading before and after adsorption will be compared.

   Pre- and post-adsorption serum samples will also be tested for highly prevalent antibodies, e.g. herpes simplex and hepatitis A to examine the effect of the adsorption process on these antibodies. This will provide a control for non-specific adsorption of antibodies. .

4. **The dilution study:** Serum samples dually reactive to HIV and *Entamoeba histolytica* or adenovirus will be serially diluted in dilution buffer and serological tests to determine dilution extinctions will be carried out. The end point titres will be compared to determine if the antibodies are binding to different antigens.

METHODS:

A.

$ 100 random HIV positive serum samples from adults together with 100 HIV untested samples from adult tuberculosis patients (to be supplied by Professor S Mhlongo) will be collected. Approximately 4ml of serum (10ml blood sample) from each adult will be required.

$ Antigen preparations will be acquired from the following sources:-

4) *Mycobacterium tuberculosis* - to be supplied by Professor M W Makgoba from University of Stellenbosch TB facility.
5) *Entamoeba histolytica* and *Plasmodium falciparum* to be supplied by Professor Terry Jackson of the MRC.
6) Adenovirus capsid protein to be supplied by Dr Harvey Bialy.

$ s Roberto Stock (Institute of Biotechnology, autonomous national University of Mexico, Cuernavaca, Mexico) will come to South Africa towards the end of January (25th) and supervise the adsorption experiments. Blank ELISA plates (NUNC) will be coated with the respective antigens mentioned above by dissolving or suspending the antigen preparations (2μg/ml) in 100mM carbonate buffer (pH 9.5) and pipetting 200μl (this volume needs to match the sample volume) into each well and leaving overnight at 4°C. The plates will then be washed and 250 ul gelatin or BSA (at 300ng/well) will be added to block free sites on the ELISA plate. The gelatin or BSA will be removed, and the 200ul serum samples will then be added to each well for 2 hours at room temperature and then removed and tested for HIV antibodies using the same test as used before the adsorption procedure.

The serum sample pre- and post-adsorption would also be tested by standard routine diagnostic EIA tests for HSV (herpes simplex virus) and anti-hepatitis A antibodies.

OD reading before and after adsorption will be compared.

B.

$ 50 serum samples dually reactive against HIV and *Entamoeba histolytica* and 50 serum samples dually reactive against HIV and adenovirus will be collected.

$ Serial 10-fold dilutions in diluting buffer will be made and each dilution tested for HIV and *Entamoeba histolytica* or adenovirus, respectively.

$ End-point titres will be compared.

ETHICS: Both samples will be taken fom adults after obtaining informed written consent. The study will be submitted for approval to the Ethics Committee of the Medical Research Council.

SITE OF STUDY:

Study A: The adsorption studies will be undertaken at the Department of Virology, University of Natal, as well as the HIV serology on the sera pre- and post-adsorption. The

HSV and HAV serology on the pre- and post-adsorption sera will be performed at the NICD (National Institute for Communicable Diseases).

Study B: The dilution studies on sera dually reactive for HIV and Eh will be performed at the University of Natal and the HIV and adeno tests will be performed at the NICD.

BUDGET:

a) Department of Virology, University of Natal : to follow.

c) NICD: Costs of serology testing:-

A. **Adsorption study:**

200 pre- + 200 post-adsorption sera, tested for HSV and hepatitis A antibodies (IgG)

HSV -	R34,40/test x 400	=	R 13 760
Hep A -	R38,30/test x 400	=	R 15 320

C. **Dilution study:**

- Pre-screening of HIV+/adeno+ sera = 100 tests
- Dilution – 50 HIV/adeno+ samples
- 6 x 10-fold dilution = 300 tests

HIV -	R24,90/test x 400	=	R 9 960
Adeno -	R34,40/test x 400	=	R 13 760

Total: for single tests	R 52 710
Total: if done in duplicate	R105 420
Courier costs:	R 2 000

And then has the unmitigated gall to send the following to me:

Subject: Re: Revised pre-adsorption protocol attached
Date: Fri, 26 Oct 2001 22:14:21 +0200
From: Carolyn Williamson <cwilliam@curie.uct.ac.za>
To: harvey bialy <h.bialy@natureny.com>, Ray Mabope <RayMabope@health.gov.za>
CC: ASmith@nu.ac.za, Lindiwe Makubalo <MakubL@health.gov.za>, A Ntsaluba <NtsalA@health.gov.za>, Nono Simelela <SimelN@health.gov.za>, bialy@ibt.unam.mx, rasnick@mindspring.com, malegapuru.makgoba@mrc.ac.za, schoub@niv.ac.za, rstock@ibt.unam.mx
References:
1 , 2

Dear Dr Bialy,

Please see my comments on your amended protocol.

Sincerely,

Carolyn Williamson

(her comments, i.e. rewrite)

Amended 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 Alagon (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)

The fundamental rationale of the protocol is to determine if confounding antigens influence the accuracy of HIV ELISA tests currently in use.

Aim: The overall aim of this investigation is to determine if antibodies to diseases common to South Africans cross-react with antigens used in the HIV ELISA tests.

Study Design.

1. ELISA plates will be coated with proteins of common disease/infections in South Africa including *Mycobacterium tuberculosis, Entamoeba histolytica, Plasmodium falciparum,* Adenovirus.
2. HIV positive serum of known OD will be added to these plates and incubated for 2 h.
3. Serum will then be re-screened for HIV to determine if HIV antibody positive samples become HIV antibody negative.
4. To control for non-specific adsorption, a parallel tests will be perfomed on the same serum sample but assaying for another antibody such as hepatitis A virus or HSV.

. **Sample sizes and varieties:**

150 HIV+ sera samples (4 ml) will be obtained after approval from the MRC ethics committee. One ELISA test will be selected by the National Institute for Communicable Diseases as representative tests currently in use in SA. This test will be used throughout the study to determine the HIV status of all samples.

The samples should be stratified according to their the 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 TB patients of unknown HIV serological status.

Antigens

(not true that we selected AIDS related illnesses – we decided on common diseases) (screening for the prevalence of HTLV III antibodies is beyond the scopes of the aims of this study) All antigens will be quality controlled as follows:

(1) Protein concentrations will be determined by 'BCA Protein Determination Kit' (Pierce, ?USA).

(2) SDS polyacrylamide gels be run to ensure that we are working with complex protein mixtures.

Antigens:

3. Mycobacterium t.b. (to be prepared according to a referenced protocol in SA)

6. Entamoeba histolytica (to be prepared in Professor Jackson, MRC, Duban) 7. Adenovirus (to be supplied by Dr. Bialy)
8. Plasmodium falciparum (to be prepared according to a referenced protocol in SA)

)

Approximately 1 mg total protein is required for all the proposed studies (see below).

HIV Test Plates Required

Each antigen to be tested in NUNC ELISA 96 well plates. (the term sponge has no scientific definition and is open to different interpretations and should therefore be excluded). Serum will be incubated on plates containing antigens listed above. Put in calculated number of tests based on the agreed on antigens.

Additional Materials

Needs updating.

protein determination kits sufficient for 50 assays, Dr. Stock will also need 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.

Protocol

Day 1. Coating (a 24 hour process): dissolve or suspend the antigen stocks (and the positive control protein – gelatin or BSA) in 100 mM carbonate buffer, pH 9.5, at 2 micrograms/ml and pipette 100 microliters into all the necessary wells. Incubate overnight in humid container at 4 C.

Day 2: Wash plates and immediately block all unadsorbed sites with etc…below .
the unadsorbed sites with 250- 300 ul gelatin (or BSA) to prevent non-specific adsorption of serum proteins to the plate.

Remove blocking agent and store plates in sealed container at ?-20 C until use (I am not sure of the best storage conditions)

(approximately): Dilute the sera samples as recommended by the kit manufacturer and place in coated ELISA plates for 2 hours at ambient temperature. Immediately transfer the contents to HIV ELISA test plates and re-screen for HIV antibodies under standard conditions as recommended by manufacturer. Dr. Stock can process 2-3 antigens/day (working 8 hours at the bench) and with the printout from the plate reader can process the raw data in his hotel overnight, so that there will be interpretable results the next morning. A reasonable estimate is 10 –12 days to complete the study. If Dr. Alagon accompanies Dr. Stock, processing time can be halved. Since Dr. Alagon is an internationally recognized expert in immunodiagnostics, we would highly recommend that he be invited to do so. (They are willing to share a hotel room, so it is only the additional cost of a ticket to accomplish this). This section is not appropriate for a scientific protocol – move to addendum if necessary.

Data should be treated as normal confidential laboratory data, in numbered pages, signed by supervisor daily etc using normal accepted protocols to prevent fraud. This includes printouts etc. Data should not leave a laboratory.

If there are two people available one should to to Professors Smith laboratory in Durban and one to NICD.

NOTE: CONTROLS AS SPECIFIED BY PROFESSOR SCHOUB NEED TO BE INCLUDED. WITHOUT THESE CONTROLS THE RESULTS ARE COMPROMISED.
Interpretation

This statement is misleading and I would strongly disagree with this previous statement .

Differences in adsorptions will be interpreted in the context of the controls to assay for non-specific adsorption (see protocol from Professor Schoub). Signals that remain antibody positive will assume that the ELISA test is accurately diagnosing HIV infection. Where the signal goes from positive to negative, in the absence of evidence of non-specific adsorption, would indicate that antibodies to common disease in South Africa cross react with antigens used in the HIV ELISA tests.

Bialy the Bastard replies as follows:

Subject: Re: Revised pre-adsorption protocol attached
Date: Fri, 26 Oct 2001 15:48:01 -0600
From: harvey bialy <h.bialy@natureny.com>
To: Carolyn Williamson <cwilliam@curie.uct.ac.za>
CC: Ray Mabope <RayMabope@health.gov.za>, ASmith@nu.ac.za, Lindiwe Makubalo <MakubL@health.gov.za>, A Ntsaluba <NtsalA@health.gov.za>, Nono Simelela <SimelN@health.gov.za>, bialy@ibt.unam.mx, rasnick@mindspring.com, malegapuru.makgoba@mrc.ac.za, schoub@niv.ac.za, rstock@ibt.unam.mx

Carolyn,

We will deal with your extensive (and partially acceptable) rewriting of our proposal, and your modest brush strokes to the document of your senior professors in the near future. At this moment it is necessary to recall that Drs. Schoub and Smith were charged with doing the following (I quote from the 'official' minutes of the meeting):

"It was agreed that the revised protocol on pre-adsorption testing (Dr Harvey Bialy) and the new protocol on the serial dilution experiments (Prof Barry Schoub and Prof Alan Smith) must be circulated to the Working Group by Friday, 26October 2001, and only one week would be allowed for comments from the Working Group."

They specifically were not instructed to write a synthesized version.

Not to put too fine a point on it, but if I delete all the text of the Schoub et al proposal that has to do with the preadsorption studies, what remains is attached. Does this really pass muster?

Sincerely,

Harvey

(here is the attachment entitled protaidsmenos, Schoub's original was called protaids)

DRAFT PROTOCOL PHASE TWO STUDIES OF PRESIDENTIAL AIDS PANEL

5. **The dilution study:** Serum samples dually reactive to HIV and *Entamoeba histolytica* or adenovirus will be serially diluted in dilution buffer and serological tests to determine dilution extinctions will be carried out.

B.

$ 50 serum samples dually reactive against HIV and *Entamoeba histolytica* and 50 serum samples dually reactive against HIV and adenovirus will be collected.

$ Serial 10-fold dilutions in diluting buffer will be made and each dilution tested for HIV and *Entamoeba histolytica* or adenovirus, respectively.

ETHICS: Both samples will be taken from adults after obtaining informed written consent. The study will be submitted for approval to the Ethics Committee of the Medical Research Council.

SITE OF STUDY:

Study B: The dilution studies on sera dually reactive for HIV and Eh will be performed at the University of Natal and the HIV and adeno tests will be performed at the NICD.

BUDGET:

a) Department of Virology, University of Natal : to follow.

d) NICD: Costs of serology testing:-

D. **Dilution study:**

- Pre-screening of HIV+/adeno+ sera = 100 tests
- Dilution – 50 HIV/adeno+ samples
- 6 x 10-fold dilution = 300 tests

HIV	-	R24,90/test x 400	=	R 9 960
Adeno	-	R34,40/test x 400	=	R 13 760

Total: for single tests	R 52 710
Total: if done in duplicate	R105 420
Courier costs:	R 2 000

Dave replies to Williamson's two sendings more kindly but with equal force as follows:

Subject: Re: Draft protocol for Phase 2 studies for President's AIDS Panel
Date: Fri, 26 Oct 2001 16:57:56 -0700
From: Dave Rasnick <rasnick@mindspring.com>
To: Carolyn Williamson <cwilliam@curie.uct.ac.za>, Prof BD Schoub <schoub@niv.ac.za>, <Ntsala@health.gov.za>, <raymabope@health.gov.za>, <malegapuru.makgoba@mrc.ac.za>, <simeln@health.gov.za>, <makubl@health.gov.za>, <asmith@health.gov.za>, <bialy@ibt.unam.mx>, <h.bialy@natureny.com>, <ansie@wn.apc.org>

Dear Carolyn,

You added to Schoub's dilution experiment: "The end point titres will be compared to determine if the antibodies are binding to different antigens."

Different endpoint titers do not necessarily mean that the polyclonal antibodies are specifically binding to different antigens. It is also possible that all or part of observed binding is non-specific, with differential affinities for the two test antigens. It is the non-specific binding that is in question.

If non-specific binding is taking place, then the pre-adsorption experiment of Bialy et al. would likely show a previously positive HIV result going to negative.

The dilution experiment of Schoub et al. does not distinguish between specific and non-specific binding of polyclonal antibodies‹only differential affinity, some or all of which may be non-specific.

Dave

Subject: Re: Revised pre-adsorption protocol attached
Date: Fri, 26 Oct 2001 16:58:41 -0700
From: Dave Rasnick <rasnick@mindspring.com>
To: Carolyn Williamson <cwilliam@curie.uct.ac.za>, harvey bialy <h.bialy@natureny.com>,Ray Mabope <RayMabope@health.gov.za>
CC: <ASmith@nu.ac.za>, Lindiwe Makubalo <MakubL@health.gov.za>, A Ntsaluba <NtsalA@health.gov.za>, Nono Simelela <SimelN@health.gov.za>, <bialy@ibt.unam.mx>, <malegapuru.makgoba@mrc.ac.za>, <schoub@niv.ac.za>, rstock@ibt.unam.mx

Dear Carolyn,

The Bialy et al. experiment is not the Williamson et al. experiment. You are not at liberty to rewrite it.

You are supposed to critique it, ask questions and make suggestions.

If you wish a third expert titled the Williamson et al. experiment, then please send us your experimental protocol.

Dave

I enter once again:

Subject: A separate comment on your 'revised' protocol
Date: Sat, 27 Oct 2001 11:35:31 -0600
From: harvey bialy <h.bialy@natureny.com>
To: Carolyn Williamson <cwilliam@curie.uct.ac.za>, Ray Mabope <RayMabope@health.gov.za>, ASmith@nu.ac.za, Lindiwe Makubalo <MakubL@health.gov.za>, A Ntsaluba <NtsalA@health.gov.za>, Nono Simelela <SimelN@health.gov.za>, bialy@ibt.unam.mx, rasnick@mindspring.com, malegapuru.makgoba@mrc.ac.za, schoub@niv.ac.za, rstock@ibt.unam.mx, Ansie Olivier <ansie@wn.apc.org>, alagon@ibt.unam.mx

Carolyn et. al,

I think the two suggestions at the bottom of CW's exercise with "Word"'s edit function are excellent ones and should be made part of the finalized procedure we will follow. I put them here as a separate email because even though we will repeat them in the extensive reply (incorporating some of CW's input) forthcoming later today or tomorrow, I think it could get buried again, so here they be all by their lonesome.

1.As there are two sites and two sorts or studies that will be going on, the question of logistics is very important. Carolyn's idea that the team of Stock and Alagon be split between the two laboratories and that one participates in the pre-adsoprtion studies (Stock) and the other in the dilution ones (Alagon) is an ideal solution.

2. The Herpes and Hep A 'control' studies should be incorporated into the final pre-adsorption protocol. So please Barry et. al write a separate section for that protocol titled "Additional Controls" in which you detail the tests you wish to use, and the samples they will be tested against, as well as their rationale. We (myself, Roberto, Alejandro, Sam and Dave) continue to think that even if we use the tests against the HIV+ sera set, they need also to be used against at least as many known positives for each condition following exactly the same pre-adsorption procedures as with the HIV+ test for completeness sake. In any case, think well, all these additional tests will tell is whether they are as specific as they are

claimed to be when tested against a random, presumably HIV+ sera collection from South Africa. It is not at all clear what they will prove as a control for the pre-adsorption studies designed to determine the HIV ELISA's accuracy under field conditions, but we have no problem with including them.

Since these are such a simple matters, requiring no mathematical or biophysical training, the calculation of no affinity constants, no protein purifications, no semantic intrigues or semiotic debates over the meaning of meaning, etc, can we please agree to these two points now?

Thank you

Harvey

PS/Can we please use the above CC list as the common one for all future correspondence? It has the correct emails of everyone directly involved in this exercise. Thanks again.

Williamson the Unbelievable replies directly to this (i.e., she quotes it in her email but I do not reproduce it here) with the following:

Subject: c.v.s of Stock and Algon
Date: Sun, 28 Oct 2001 22:29:42 +0200
From: Carolyn Williamson <cwilliam@curie.uct.ac.za>
To: harvey bialy <h.bialy@natureny.com>, Ray Mabope <RayMabope@health.gov.za>, ASmith@nu.ac.za, Lindiwe Makubalo <MakubL@health.gov.za>, A Ntsaluba <NtsalA@health.gov.za>, Nono Simelela <SimelN@health.gov.za>, bialy@ibt.unam.mx, rasnick@mindspring.com, malegapuru.makgoba@mrc.ac.za, schoub@niv.ac.za, rstock@ibt.unam.mx, Ansie Olivier <ansie@wn.apc.org>, alagon@ibt.unam.mx

Dear Dr Bialy

Would it be possible to have c.v.s of Drs Stock and Alagon?

Sincerely,

Carolyn Williamson

And I, now a little ticked off, reply as follows:

Subject: Re: c.v.s of Stock and Algon

Date: Sun, 28 Oct 2001 14:41:54 -0600
From: harvey bialy <h.bialy@natureny.com>
To: Carolyn@natureny.com, Williamson@natureny.com, Dave Rasnick <rasnick@mindspring.com>,
roberto <rstock@ibt.unam.mx>, alagon@ibt.unam.mx, Ray Mabope <RayMabope@health.gov.za>

since they were copied on the email, it is likely they will provide you with copies. as the request does not come from the dept. of health i cannot do more. any particular reason?
could we have a copy of your own?
by the way: please prepare yourself to reply point by point to each antigen we have proposed. i have now stated, nicely & several times, that since we have taken the time to give reasons for their inclusion that you cannot simply eliminate them ad hoc. this is supposed to be a scientific discourse not an exercise in one upman or womanship. and as usual you have avoided any direct confrontation with the issues.

And in fact Stock and Alagon the Scientists do send their CVs, which I am attaching separately. But after a day or two her reply to the email above is typical of this spoiled brat child of undeserved privilege:

Subject: Re: c.v.s of Stock and Algon
Date: Mon, 29 Oct 2001 21:50:57 +0200
From: Carolyn Williamson <cwilliam@curie.uct.ac.za>
To: Ray Mabope <RayMabope@health.gov.za>, ASmith@nu.ac.za,
Lindiwe Makubalo <MakubL@health.gov.za>, A Ntsaluba <NtsalA@health.gov.za>,
Nono Simelela <SimelN@health.gov.za>, bialy@ibt.unam.mx, rasnick@mindspring.com,
malegapuru.makgoba@mrc.ac.za, schoub@niv.ac.za, rstock@ibt.unam.mx

Dear Dr Bialy,
With respect to you comment below on c.v.s - I think it is important that the process remains transparent and we understand the expertise of the people with whom we are working.
Sincerely,
Carolyn Williamson

We return to plot one and we send the final version of our protocol, incorporating those aspects of Williamson´s edit that are in fact useful.

Subject: Pre-adsorption protocol - version 3

Date: Sun, 28 Oct 2001 21:42:55 -0600
From: harvey bialy <h.bialy@natureny.com>
To: Ray Mabope <RayMabope@health.gov.za>, Ansie Olivier <ansie@wn.apc.org>, alagon@ibt.unam.mx
CC: Carolyn Williamson <cwilliam@curie.uct.ac.za>, ASmith@nu.ac.za, Lindiwe Makubalo <MakubL@health.gov.za>, A Ntsaluba <NtsalA@health.gov.za>, Nono Simelela <SimelN@health.gov.za>, bialy@ibt.unam.mx, rasnick@mindspring.com, malegapuru.makgoba@mrc.ac.za, schoub@niv.ac.za, rstock@ibt.unam.mx

Dear All,

Please find attached our newest version of the pre-adsorption protocol. Any comments, questions, amendments etc, should be communicated in separate emails detailing them point by point, and not please in the form of an edited rewrite. The only exception to this is the inclusion (if they finally decide to do so) of a section before "Interpretation" entitled "Additional Controls" in which Prof. Schoub et al. explain in detail what these controls are, how they will be performed, by whom, and detail the material and temporal requirements. As we have stated repeatedly, it is our well-considered scientific opinion that the experiment as we have proposed it already controls perfectly for 'non-specific' adsorption of antibodies which could result in an artifactual diminution of an HIV ELISA signal.

Sincerely,

Harvey Bialy
Roberto Stock
Alejandro Alagon
Sam Mhlongo

The latest working document from us:

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 3, 28 October 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 that 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.

(Note: The text above is not exactly what was sent in Version 3, as the email below, which I sent this morning, explains --- I have omitted the CC list and other heading details for ease of reading :

9 November, 2001

Dear All,

Please replace the first paragraph of our Preadsorption Protocol, which reads:

"The fundamental rationale of the protocol is to determine the specificity of the HIV ELISA tests currently in use in South Africa with the principal aim being to determine if disease agents common to South Africans produce antibodies capable of cross-reaction with antigens used in the HIV ELISA tests."

with

"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 that 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."

In our efforts to accommodate the 'suggestions' of Dr. Williamson we hybridized our original text inappropriately. Namely, the use of the phrase 'produce antibodies ...' is inaccurate for two reasons (1) B cells as we all know 'produce' antibodies, and (2) the studies as we have designed them do not directly address the question of what antigen elicited a cross-reactive antibody, only whether such cross-reactive antibodies are present in sera from South Africans. In order to properly experimentally ask the question of whether a given list of infectious agents is capable of eliciting HIV ELISA cross-reacting antibodies, it would be necessary to examine the sera of persons proved to be infected by one or more of the non-HIV agents being interrogated. This would necessitate, in addition to having an acceptable gold standard for an HIV infection, also having a gold standard for each other organism (a condition satisfied by most, but not all of the antigenic sources on our list), and in fact doing a completely different (and considerably more complicated) series of studies than the ones we have detailed. Therefore we think the simplified text above is preferable as it states accurately and clearly what we are intending to accomplish.

Harvey Bialy
Alejandro Alagon
Roberto Stock
Sam Mhlongo

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, as well as a positive control plate containing just the blocking protein to be used in the preparation of the pre-adsorption plates (gelatin). HIV positive 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.

Samples should 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 use of 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).

Antigens:

1- HTLV I (commercially available)
2- HTLV II (commercially available)
3- *Mycobacterium tuberculosis* (to be prepared according to a referenced protocol in SA)
4- *Mycobacterium leprae* (source to be determined)
5- BCG (commercially available)
6- *Entamoeba histolytica* (to be prepared by Prof. Jackson in direct or indirect collaboration with Dr. Stock).
7- Adenovirus (to be supplied by Dr. Bialy)
8- *Plasmodium falciparum* (to be prepared according to a referenced protocol in SA)
9- *Schistosoma mansoni* (source to be determined)
10- Enteropathogenic *E. coli* (source to be determined)
11- Enteropathogenic *Salmonella typhimurium* (source to be determined)

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 (non-specific) 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.

1- The 100 TB samples (in duplicate) will be incubated only against 4 antigens (M. tb, M. leprae, BCG and the control plate containing gelatin). This will require 2 HIV ELISA plates (x2) for each antigen for this part of the study. Thus for the 3 antigenic preparations plus the positive control, a total of 16 HIV ELISA plates will be required.
2- For the remaining 150 samples, the calculation is as follows: 4 plates are required to accommodate 150 samples in duplicate, multiplied by 12 if we use all the antigenic preparations listed above = 48 HIV ELISA plates.

Additional materials

We will need to purchase 4 HTLV I and 4 HTLV II test plates in order to examine 150 samples in duplicate, in addition to the 100 NUNC ELISA plates. 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.

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 100 µl of each preparation dispensed into each 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.

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 non-specific reaction that makes the results of the test ambiguous with regard to the HIV serological status of the sample.

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

It is necessary, considering the special conditions under which we are working, to guard against the possibilities of either group deliberately attempting to fudge the outcome of the studies. Let us imagine that Drs. Stock, Alagon, myself and Mhlongo wished to dope the tests by using antigenic preparations that were 'spiked' with HIV antigens. In this case each and every initially positive HIV signal would be reduced by the same amount. Thus it is impossible for us to interfere with the outcome via this means. The only possible way we might accomplish this is by sleigh of hand at the moment the antigens are added to the NUNC plates. That is to say, Dr. Stock could (if he were adept at such maneuvers) slip a few hundred nanograms of HIV antigens into a few random wells on each pre-adsorption test plate. We contend this would be impossible for two reasons. (1) We insist that every laboratory manipulation that is carried out by Dr. Stock be under the direct supervision of several members of the MRC side of the collaboration. (2) Dr. Stock will be using a micropipette that will go directly from the sample diluted in the HIV test kit dilution buffer to the preadsorption wells. From the other side, the only possibility of deliberately interfering with the outcome lies in the NUNC MAXISORP uncoated ELISA plates that will be used in the pre-adsorption phase of the study. For example, the plates could be pre-blocked with 300 ng/well of gelatin or BSA and therefore not adsorb any of the antigens that might be added. We therefore insist that the box of 100 plates that will be more than sufficient for all the studies be kept unopened, and that all the plates be in their original sealed packages.

Now more science with the following (as usual) unanswered series of emails from our side:

Subject: Comments on the comments of CW
Date: Sun, 28 Oct 2001 21:44:54 -0600
From: harvey bialy <h.bialy@natureny.com>
To: Carolyn Williamson <cwilliam@curie.uct.ac.za>, Ray Mabope <RayMabope@health.gov.za>,
ASmith@nu.ac.za, Lindiwe Makubalo <MakubL@health.gov.za>, A Ntsaluba <NtsalA@health.gov.za>, Nono Simelela <SimelN@health.gov.za>, bialy@ibt.unam.mx, rasnick@mindspring.com, malegapuru.makgoba@mrc.ac.za, schoub@niv.ac.za, rstock@ibt.unam.mx,
Ansie Olivier <ansie@wn.apc.org>, alagon@ibt.unam.mx

Carolyn,

The list of antigens in version 3 corresponds to our original submitted list. No reasons have been provided why they should not be included in these experiments. On the other hand, we have stated why they are necessary for a study of this scope, and

no antigenic preparation should be excluded without reason.

You rewrote the Interpretation section of our protocol as follows:

"Differences in adsorptions will be interpreted in the context of the controls to assay for non-specific adsorption (see protocol from Professor Schoub). Signals that remain antibody positive will assume that the ELISA test is accurately diagnosing HIV infection. Where the signal goes from positive to negative, in the absence of evidence of non-specific adsorption, would indicate that antibodies to common disease in South Africa cross react with antigens used in the HIV ELISA tests."

We have reinserted our original text as we think it stands scientific scrutiny. Yours does not for the following reasons:

1. There is no protocol from Prof. Schoub as yet, and so the word 'context' is contextless. Further, if you think about what is being proposed as a control you will first realize that it will triple the size of our proposed experiments without adding any data that bears on the purpose of our study. The controls you suggest are simply a repetition of the preadsorption experiments using another two serological test kits. This is clearly unnecessary and besides the validity of these other tests is not under scrutiny. This is so because the diseases they serve to diagnose are easily confirmed clinically (such as a herpes chancre or liver dysfunction).

2. According to what you have written, non-specific adsorption must be subtracted as a background for the HIV ELISA tests. However, the non-specific adsorption in question is that to the positive control plates which are coated with an inert protein that should not bind any antibodies. This is included to ensure that the HIV ELISA test is of any use whatsoever as antibodies detected by this assay cannot possibly be removed by incubation against a gelatin coated surface. If this were to be so, then antibodies could be adsorbed by the tubes used to collect the blood samples initially or prepare the serum. It is precisely, those non-specific antibodies which are removed by known antigens that serve to validate or otherwise the HIV ELISA. Nowhere do we assume that the antibodies present in the sera of HIV+ patients are in fact specific antibodies against any of the material we are using to coat the NUNC wells.

3. It is also necessary to remark that in the case of a herpes or hepatitis diagnosis based on a laboratory test the result of the test is not a death sentence. The case of the every-changing clinical spectra of diseases that are considered to be indirectly caused by HIV is quite the opposite.

Harvey Bialy
Roberto Stock
Alejandro Alagon
Sam Mhlongo

Subject: A final night's thought on the HepA/Herpes controls
Date: Sun, 28 Oct 2001 23:02:52 -0600
From: harvey bialy <h.bialy@natureny.com>
To: schoub@niv.ac.za
CC: Ray Mabope <RayMabope@health.gov.za>, ASmith@nu.ac.za, Lindiwe Makubalo <MakubL@health.gov.za>, A Ntsaluba <NtsalA@health.gov.za>, Nono Simelela <SimelN@health.gov.za>, bialy@ibt.unam.mx, rasnick@mindspring.com, malegapuru.makgoba@mrc.ac.za, rstock@ibt.unam.mx, Ansie Olivier <ansie@wn.apc.org>, alagon@ibt.unam.mx, Carolyn Williamson <cwilliam@curie.uct.ac.za>

Barry,

Earlier I had suggested that if we do the hepA/herpes tests on the 150 HIV+ sera pre and post-adsorption that we also perform the tests pre and post-adsorption on known positives for the two pathogens in question. As we pointed out in an earlier email of this evening, either protocol will lead to a massive amount of additional work, expense and time.

I now propose something much simpler and one to which I hope you will readily agree.

Let us perform the HepA/Herpes tests ONLY on those samples that go from HIV+ to HIV- on one or another of the pre-adsorption plates. There is no need to look at the samples that remain within the HIV+ boundaries. If we go this route then the amount of work/time becomes manageable and the results will be scientifically informative. What you say?

Harvey

(Thus far nada)

Now Stock and Alagon's comments on Shoub's Protocol. These are extremely rigorous and beautifully written, and will I hope appear almost verbatim in the paper we will eventually submit to a high profile journal like *Nature Biotechnology* (whose editor, Dr. Andrew Marshall is salivating at the possibility of running a paper entitled "How Valid is HIV ELISA Testing in South Africa?")

Subject: Comments on experiments
Date: Mon, 29 Oct 2001 20:38:30 -0600 (CST)
From: Roberto Stock <rstock@ibt.unam.mx>

To: schoub@niv.ac.za
CC: ASmith@nu.ac.za, cwilliam@curie.uct.ac.za, RayMabope@health.gov.za, malegapuru.makgoba@mrc.ac.za,
bialy@ibt.unam.mx, David Rasnick <rasnick@mindspring.com>, ansie@wn.apc.org, Alejandro Alagon <alagon@ibt.unam.mx>, mhlongo@uitweb.co.za

Dear all: Some comments on the preadsorption and serial dilution studies are attached. I hope they are of use in the interpretation of the data we will gather shortly.

Roberto Stock

Comments on the preadsorption and serial dilution experiments

The aim of the preadsorption study, as stated in the revised version of the protocol, is to assess the potential of infectious agents common to South Africans at eliciting antibody responses capable of cross-reactivity with HIV antigens used to determine the presence of unequivocally anti-HIV antibodies.

A few considerations on the information to be gathered by both studies, preadsorption and serial dilution, are necessary in order to clearly interpret the results in the context of the existing concern on the robustness of the HIV ELISA test as a diagnostic and epidemiological tool of value in SA.

We will begin with the preadsorption studies and their interpretation in those cases where preadsorption of sera results in significantly different readings from the positive control in the HIV ELISA test chosen.

The preadsorption experiment.

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

1- The first population may consist of antibodies truly elicited by *Entamoeba* (or adenovirus) infection which bind (at least some) HIV antigens used in the HIV ELISA test. Preadsorption lacking, such antibodies would yield a false positive 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.

2 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).
3 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 must 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 (in this case *Entamoeba* or adenovirus). The same is true if the decrease in signal does not 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.

2- The second population would be that of antibodies truly elicited by an HIV infection, a portion of which binds to *Entamoeba* (or adenovirus) 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.

a) 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).
b) 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 (viz *Entamoeba* or adenovirus antigens, in this case) 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.

The arguments presented thus lead us to an important conclusion regarding the preadsorption experiments: If the studies on preadsorption yield that a significant amount 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.

The serial dilution experiment.

The serial dilution study proposes that: "Serum samples dually reactive to HIV and *Entamoeba histolytica* or adenovirus will be serially diluted in dilution buffer and serological tests to determine dilution extinctions will be carried out. The end point titres will be compared to determine if the antibodies are binding to different antigens."

As mentioned earlier, the antibody populations under study are polyclonal. This means that there are a variety of epitopes on the antigens used in serodetection (HIV, *Entamoeba* and adenovirus antigens in this case) that are recognized by a variety of antibody molecules which differ in their specificity (that is, the epitope they recognize) and their affinity (their dissociation constant) for any given antigen. Polyclonal antibodies of the same specificity will present a spectrum of affinities, which generally follow a gaussian distribution.

The higher the affinity of an antibody population for a given epitope, the higher the dilution at which they will be useful as immunodiagnostic tools (that is, a lower concentration of antibody may be detected using a given antigen). This is why, ideally, monoclonal antibodies are used for immunodiagnosis; affinity for a given antigen/epitope is the same for all antibody molecules. Polyclonal antibodies present the said spectrum of affinities. Thus, an antibody population of low average affinity for a given antigen but at higher concentration will give an ELISA (for example) signal equivalent to that of another population of higher average affinity but lower concentration, provided that enough time is given to allow the antibody-antigen interaction to occur.

In a polyclonal population, the spectrum of affinities for a given epitope may well span orders of magnitude. This means that at any given antibody (or serum) dilution, low affinity antibodies will be detected with difficulty (if at all) whereas higher affinity ones will be easily detected. When a serial dilution of serum is performed, antibodies are sorted out by their relative affinities and by their relative concentrations for the antigens used in the detection.

The serial dilution experiment will inform us about the mean affinity of antibodies reactive to HIV, *Entamoeba* and adenovirus antigens in the sample population. Thus, three possible outcomes of the serial dilution experiment are possible:

1- The dilution series of the sera tested against HIV and *Entamoeba* (or adenovirus) antigens evidence a drop in signal that is faster for *Entamoeba* (or adenovirus) antigens than for HIV antigens.
2- The dilution series of the sera tested against HIV and *Entamoeba* (or adenovirus) antigens evidence a drop in signal that is slower for *Entamoeba* (or adenovirus) antigens than for HIV antigens.
3- The dilution series of the sera tested against HIV and *Entamoeba* (or adenovirus) antigens evidence a drop in signal that is the same for *Entamoeba* (or adenovirus) antigens than for HIV antigens.

Keeping in mind the considerations mentioned above, the only possible interpretations of the possible outcomes of the serial dilution experiment are the following:

1- The antibodies present in serum that bind HIV antigens have a higher mean affinity for HIV antigens than those present that bind *Entamoeba* (or adenovirus) antigens. Alternatively, the antibodies present in serum that bind HIV antigens are at a higher concentration than those present that bind *Entamoeba* (or adenovirus) antigens.
2- The antibodies present in serum that bind HIV antigens have a lower mean affinity for HIV antigens than those present that bind *Entamoeba* (or adenovirus) antigens. Alternatively, the antibodies present in serum that bind HIV antigens are at a lower concentration than those present that bind *Entamoeba* (or adenovirus) antigens.
3- The antibodies present in serum that bind HIV antigens have the same mean affinity for HIV antigens than those present that bind *Entamoeba* (or adenovirus) antigens. Alternatively, the antibodies present in serum that bind HIV antigens are at the same concentration than those present that bind *Entamoeba* (or adenovirus) antigens.

It is even theoretically possible that the same antibody molecules bind both antigenic preparations with similar or different mean affinities. Determining if this is the case would require a different set of experiments, all of them involving one type or another of competition for antigens, which are far from the scope of the study on the usefulness of the HIV ELISA test in the context of South African populations.

This concludes our analysis of the interpretability (meaning) of the two studies. If you have other interpretations, please make them explicit. Otherwise, we would expect to see some very close version of the text above in your final protocol, along with the experimental details of the precise tests you wish to perform, as well as the material and time requirements. Finally, we agree completely with Dr. Bialy's previous comment that you include as an essential control, an examination by whatever tests you finally choose, and whatever dilution protocol (we also think 2-fold dilutions are better than 10 fold) is decided upon, those samples which lose HIV+ reactivity after preadsoprtion to one or another of the test antigens.

We await your comments, and look forward to working with you early in the new year.

Sincerely,

Roberto Stock
Alejandro Alagon

(It is at this point that Stock sends the cvs to Williamson with the following curt note)

Subject: Re: c.v.s of Stock and Algon
Date: Mon, 29 Oct 2001 21:17:23 -0600 (CST)
From: Roberto Stock <rstock@ibt.unam.mx>
To: Carolyn Williamson <cwilliam@curie.uct.ac.za>
CC: harvey bialy <h.bialy@natureny.com>, Ray Mabope <RayMabope@health.gov.za>, ASmith@nu.ac.za, Lindiwe Makubalo <MakubL@health.gov.za>, A Ntsaluba <NtsalA@health.gov.za>, Nono Simelela <SimelN@health.gov.za>, bialy@ibt.unam.mx, rasnick@mindspring.com, malegapuru.makgoba@mrc.ac.za, schoub@niv.ac.za, Ansie Olivier <ansie@wn.apc.org>, alagon@ibt.unam.mx

Dear Dr. Williamson: The CVs of Drs. Alagón and Stock are attached as PDF files. Please note that the name (without accents) is Alagon.

Sincerely,

Roberto Stock

We chime in again, still in the spirit of what I had hopes would in fact become a real exchange of ideas.

Subject: An important semantic clarification
Date: Mon, 29 Oct 2001 21:19:38 -0600
From: harvey bialy <h.bialy@natureny.com>
To: Carolyn Williamson <cwilliam@curie.uct.ac.za>
CC: Ray Mabope <RayMabope@health.gov.za>, ASmith@nu.ac.za, Lindiwe Makubalo <MakubL@health.gov.za>, A Ntsaluba <NtsalA@health.gov.za>, Nono Simelela <SimelN@health.gov.za>, bialy@ibt.unam.mx, rasnick@mindspring.com, malegapuru.makgoba@mrc.ac.za, schoub@niv.ac.za, rstock@ibt.unam.mx, Ansie Olivier <ansie@wn.apc.org>, alagon@ibt.unam.mx

Dear All,

The terms specific and non-specific antibodies have been used a lot by all concerned with the finalization of mutually acceptable experimental protocols. Let us be clear that all antibodies are specific as well as non-specific. That is to say all antibodies have a range of binding constants to a variety of antigens, including monoclonal antibodies (although here the binding affinity for one particular epitopic set is orders of magnitude higher than the next member of the class). When we use the term 'specific' what we really mean is 'diagnostic'. That is to say, an antibody is considered specific if its binding to a particular antigen is diagnostic of an infection, disease, or other physiological condition in which that antigen participates.

Is there any disagreement with this definition of the term specific for the purposes of our study?

No reply will be taken to mean no disagreement.

Thank you.

Harvey Bialy
Roberto Stock
Alejandro Alagon
Sam Mhlongo

Dave replies, quickly, and in direct agreement:

Subject: Re: An important semantic clarification
Date: Mon, 29 Oct 2001 21:26:38 -0800
From: Dave Rasnick <rasnick@mindspring.com>
To: harvey bialy <h.bialy@natureny.com>, Carolyn Williamson <cwilliam@curie.uct.ac.za>
CC: Ray Mabope <RayMabope@health.gov.za>, <ASmith@nu.ac.za>, Lindiwe Makubalo <MakubL@health.gov.za>, A Ntsaluba <NtsalA@health.gov.za>, Nono Simelela <SimelN@health.gov.za>, <bialy@ibt.unam.mx>, <malegapuru.makgoba@mrc.ac.za>, <schoub@niv.ac.za>, <rstock@ibt.unam.mx>, Ansie Olivier <ansie@wn.apc.org>, <alagon@ibt.unam.mx>

Harvey,

That is a very good characterization of antibody-antigen binding specificity.

I would add that specificity also implies that the absence of binding between antibodies and antigen means that the putative cause (the antigen) is not responsible for the symptoms or the disease.

Dave

And now I write to Ray and a few others as follows:

Subject: Some morning thoughts on a morning that still does not place a Schoub in my Inbox
Date: Thu, 01 Nov 2001 07:48:34 -0600
From: harvey bialy <h.bialy@natureny.com>
To: Ray Mabope <RayMabope@health.gov.za>, Dave Rasnick <rasnick@mindspring.com>, "duesberg@uclink4.berkeley.edu" <duesberg@uclink4.berkeley.edu>, Robert Johnston <jaunts2@interlog.com>, roberto <rstock@ibt.unam.mx>, Ansie Olivier <ansie@wn.apc.org>, alagon@ibt.unam.mx

Ray mi amigo,

Your silence is a bit bewildering, but I know you are a very busy man, and reading and replying to the email barrage from myself and Dave could be a full time job. (By the way what has happened to the promises to Dave of the good, and aptly named, Dr. Bah?). Be all that as it might, it is now Thursday morning in Mexico and I despair of seeing the protocol from Schoub et al today or tomorrow. As you can tell by now, we (Roberto, Alejandro, Sam and myself) have done our part (and quite well I might add --- a number of independent, world-renowned scientists who have looked at the material we prepared agree. I would suggest that if finally the 'idiots', and I call them that with all due etymological respect, actually submit something they call a 'final dilution protocol', and/or they continue to dispute our own protocol, that both be submitted to a panel of three independent referees chosen by the editor of a high ISI ranking immunology journal that does not make a living from HIV papers, and they be asked to comment of the suitability of the two protocols. This is in the event that the Honorable Minister does not feel comfortable taking the final decisions by herself. Alternatively, I can recommend three guaranteed independent and more than qualified referees for her to send them to under conditions of confidentiality (I was not the presiding scientific editor of Nature Biotechnology for 13 years because I was known for bias in my selection of reviewers, nor my indiscretion in the handling of highly sensitive manuscripts often worth multiple millions of dollars to the companies whose researchers were submitting them).

Your friend,

Harvey

Meanwhile, Carolyn answers the 'semantic clarification' email thusly:

Subject: Re: An important semantic clarification
Date: Mon, 5 Nov 2001 13:59:55 +0200
From: "Williamson, C, Carolyn, Dr" <cwilliam@curie.uct.ac.za>
Organization: versity of Cape Town
To: Carolyn Williamson <cwilliam@curie.uct.ac.za>, harvey bialy <h.bialy@natureny.com>
CC: Ray Mabope <RayMabope@health.gov.za>, ASmith@nu.ac.za, Lindiwe Makubalo <MakubL@health.gov.za>, A Ntsaluba <NtsalA@health.gov.za>, Nono Simelela <SimelN@health.gov.za>, bialy@ibt.unam.mx, rasnick@mindspring.com, malegapuru.makgoba@mrc.ac.za, schoub@niv.ac.za, rstock@ibt.unam.mx, Ansie Olivier <ansie@wn.apc.org>, alagon@ibt.unam.mx

Dar Dr Bialy and all,

According to diagnositic definition (as you mentioned below):
specificity "describes the likelihood, expressed as a percentage, that the test results will be negative if HIV antibodies are not present. Specificity is determined by dividing the number of actual or true negative by all the non-infected samples. A higher specificity will mean fewer false-positive results"
(Martin and Simms, Feb. 2000, Vol 90, No 2, SAMJ).

To look at "non-specific" adsorption of antibodies - perhaps is confusing terminology.

As I mentioned at the meeting, certain bacterial proteins will bind immunoglobulins irrespective of the antibody specificity. The most well known example being Staphylococcus Protein A which binds the constant domain of immunoglobulin molecules.

In addition, certain chemicals/substances will inhibit the enzymes used for detection.

I do not mean that these specific compounds/proteins will be present, but that these scenarios are possible and that these factors

need to be controlled for in an experimental design which is founded on an undefined cocktail of proteins.

Sincerely,

Carolyn Williamson

Stock the Scientist tries to give her a little education:

Subject: Stoichiometry
Date Mon, 5 Nov 2001 21:27:25 -0600 (CST)
From: Roberto Stock <rstock@ibt.unam.mx>
To: william@curie.uct.ac.za
CC: schoub@niv.ac.za, ASmith@nu.ac.za, RayMabope@health.gov.za, malegapuru.makgoba@mrc.ac.za, David Rasnick <rasnick@mindspring.com>, ansie@wn.apc.org, mhlongo@uitweb.co.za, alagon@ibt.unam.mx, Lindiwe Makubalo <MakubL@health.gov.za>, A Ntsaluba <NtsalA@health.gov.za>, Nono Simelela <SimelN@health.gov.za>, HARVEY BIALY <h.bialy@natureny.com>

Dear Dr. Williamson: You are absolutely correct in pointing out the fact that certain antigenic preparations may have the capacity to bind a given amount on antibody present in the serum sample. This in itself would perforce invalidate the experiment if it were a large enough decrease. However, it is important to note some features of some of the antigenic preparations:

1- The HTLV-1 and HTLV-2 antigens will be from commercially available antigen-coated plates used for immunodiagnosis. Besides the fact that HTLV-1 and HTLV-2 have no reported Ig binding proteins, and that the plates are specifically designed to detect 'diagnostic' antibodies, they should presumably have very low to negligible 'non-specific' binding. If this were not so, they would be scarcely their value as immunodiagnostic tools.

3- In the case of adenovirus antigens, they also lack, to the best of my knowledge, antibody binding proteins of the type of the well known protein A from S. aureus (or any other).

3- The possible binding of immunoglobulins which you have shrewdly pointed out, in fact, should be the same for all serum samples for the given antigenic preparation, and irrespective of their initial positive OD value they should all exhibit a very similar decrease upon re-screening by the

HIV-ELISA test.

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, as suggested and exemplified in your email, and that it is small, 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, correspondifong 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 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).

It is also important to stress that a very similar decrease in OD should be apparent in all of the samples preadsorbed by comparison to the inert gelatin control, so the detection of this particular source of error, where it may apply, would be not only easy but also quantitated and, if deemed significant upon inspection, introduced as a correction in the calculation of the real decrease in OD by antigen binding by serum antibodies.

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. Although we have not been informed of the particular HIV ELISA we will be using in our study, we are using the information provided with the Abbot test as a guide.

We therefore think we may safely proceed with the preadsorption experiment, keeping in mind your valuable suggestion and with the confidence that if such immunoglobulin-binding proteins are indeed present in an antigenic mixture used there are but two possible outcomes: 1) It is a negligible source of error or, more unlikely, 2) will be easily detectable by the observed drop in OD of all serum samples preadsorbed against that particular antigen.

We hope that this will be of assistance in the rigorous interpretation of the data we will be collecting soon.

Sincerely,

R. Stock

Now Williamson becomes completely unbearable:

Subject: Interpretation
Date: Mon, 05 Nov 2001 22:53:24 +0200
From: Carolyn Williamson <cwilliam@curie.uct.ac.za>
To: schoub@niv.ac.za
CC: ASmith@nu.ac.za, RayMabope@health.gov.za, malegapuru.makgoba@mrc.ac.za, bialy@ibt.unam.mx, David Rasnick <rasnick@mindspring.com>, ansie@wn.apc.org, mhlongo@uitweb.co.za
References:

The interpretation of the experiments put forward by Drs Stock and Alagon do not agree with the outcome of the meeting. I cannot support their interpretation as:

At the meeting after lengthy discussions on interpretations it was agreed that reduction in OD would not be considered evidence of cross-reactivity. It was accepted that there could be adsorption of all immunoglobulins due to the experiment design i.e. use of a crude mixture of proteins to adsorb serum. It is known that certain bacterial proteins will bind

immunoglobulins irrespective of their specificity. This would result in reduction in OD. It is therefore essential for any experiment of this nature to measure for this. All results have to be interpreted in the context of controls (as outlined by Professor Schoub).

As you can tell, she either did not read Stock's explanation of the arithmetic involved or did not understand it because as is her style she repeats her nonsensical assertions and appeal to authority yet again. I reply to her as follows: (From here on I become quite insulting and nasty - assuming I was insufficiently abusive before. My justification is that there was a certain method to my madness in that I hoped to provoke Barry and Alan into at least some posture of scientific discourse. I know that if I received such comments from a fellow scientist, I would take not only umbrage but undertake some serious time in the library, as we used to call it, to properly defend myself. However, my language in the remaining emails is pure venom, and I can justify it only by saying that the insults to my intelligence, and that of my colleagues, by their continuing obtuseness, and the implied insults to the President by their continuing refusal to take this matter seriously, assuming they are intellectually capable, are far worse).

Subject: Re: Interpretation
Date: Mon, 05 Nov 2001 17:53:57 -0600
From: harvey bialy <h.bialy@natureny.com>
To: Carolyn Williamson <cwilliam@curie.uct.ac.za>
CC: schoub@niv.ac.za, ASmith@nu.ac.za, RayMabope@health.gov.za, malegapuru.makgoba@mrc.ac.za, bialy@ibt.unam.mx, David Rasnick <rasnick@mindspring.com>, ansie@wn.apc.org, mhlongo@uitweb.co.za, roberto <rstock@ibt.unam.mx>, alagon@ibt.unam.mx

Carolyn

Quite frankly this is stuff and nonsense. And you did not have either the courtesy or the courage to send this to Drs. Stock and Alagon directly. As you can see, I have forwarded this for their attention.

I then receive what at the moment is a puzzling email from Dave (it begins with my reply to the puzzlement).

Subject: Re: FW: Test protocols
Date: Tue, 06 Nov 2001 11:06:25 -0600
From: harvey bialy <h.bialy@natureny.com>
To: David Rasnick <rasnick@iqmail.net>

dave as you can see from the cc:s he got my email all wrong so i have no idea what he is doing or has done

send it to me asap
and call me
011 52 7 316 1294 if you can
this is serious if what i discern from your email is correct

David Rasnick wrote:

> Harvey,
>
> It appears to me that Barry Schoub is trying to call all the shots. It is my
> understanding that Bialy et al. would provide the details of their
> experiment and that Schoub et al. would do likewise for theirs. How is it
> that Schoub and Smith are now going to draw up the details for all
> experiments?
>
> What is your take on Shoub's control experiment using HAV and HSV? Is he
> trying remove non-specific binding from consideration even though measuring
> non-specific binding is the very purpose of the Bialy et al. experiment?
>
> I still don't understand the significance of the dilution experiment. Why is
> it necessary that the extinction for E. histolytica and presumably HIV be
> the same at dilution? I didn't really understand Schoub's sentence. Perhaps
> I misunderstood what he was driving at.
>
> Anyway, Alagon and Stock are the best ones to address Schoub's smokescreen.
>
> Dave
>
> ----------
> > From: "Liz Millington" <lizv@niv.ac.za>
> > Date: Tue, 6 Nov 2001 12:12:47 +0200
> > To: <ntsala@health.gov.za>, <simeln@health.gov.za>, <raymabope@health.gov.za>,
> > <malegapuru.makgoba@mrc.ac.za>, <asmith@health.gov.za>,
> > <rasnick@mindspring.com>, "wn.apc.org" <bialy@ibt.unam.mxansie>,
> > <cwilliam@curie.uct.ac.za>, <makubl@health.gov.za>, <alagon@ibt.unam.mx>,
> > <rstock@ibt.unam.mx>
> > Subject: Test protocols
> >
> > Dear Panellists,
> >
> > Attached herewith please find the outline for the test protocols as
> > discussed at the October 18th meeting.
> >
> > More detailed test methodologies will be complied by Professor Alan
> > Smith and myself and will be forwarded shortly.
> >
> > Prof Barry Schoub

I here insert the protocol that comes after all this time from Prof. Schoub (I received it from Dave, Schoub's secretary apparently did not notice that it bounced back to her. The email address of Sam was also incorrect and Dave sent him the material separately as well).

TEST PROTOCOLS : PRESIDENT'S AIDS PANEL

I would like to thank all the panellists as well as Drs Stock and Alagon, for their comments on the test protocols which were agreed upon at the meeting of the panel on October 18th. As there appears, from the comments, to be still some uncertainty regarding the rationale for the tests and the controls, I will again briefly reiterate them:-

A) **Adsorption experiment:**

1. Purpose: To determine whether infectious agents prevalent in South Africa could be responsible for the EIA-detectable HIV seropositivity due to cross-reactivity with HIV antigens.
2. Outline of methodology: Antigen preparations from 4 infectious agents, *Mycobacterium tuberculosis*, *Entamoeba histolytica*, *Plasmodium falciparum* and adenovirus, will be used as adsorbing "sponges". These antigens will be used to coat microplates onto which sera which are HIV seropositive by EIA will be added, incubated, and then subsequently retested to determine whether there are significant reductions in antibody titres.
3. Controls: To control for non-specific adsorption of immunoglobulin, EIA tests will be carried out on the pre- and post-adsorbed serum samples using 2 infectious agents prevalent in South Africa – hepatitis A (HAV) and herpes simplex (HSV).
4. Rationale for experiments: If EIA-detectable HIV seropositivity is due to other infectious agents prevalent in South Africa, the HIV titres should drop substantially or to undetectability due to adsorption by the putative cross-reacting antigens. If antibody titres for the control HAV and HSV antibodies similarly decline, then the interpretation of the experiments could be that adsorbing antigen preparations are non-specifically "sponging" out immunoglobulins including the anti-HIV antibodies.

B) Dilution experiments:

1. Purpose: ……..as for A) adsorption experiment.
2. Outline of methodology: Sera dually reactive for HIV and *Entamoeba histolytica* and adenovirus, respectively, will be serially diluted out. The highest dilution retaining a positive EIA result will be compared between HIV and the putatively cross-reacting *Entamoeba histolytica* or adenovirus.
3. Rationale of experiment: If the HIV positive EIA result is due to cross-reaction with either *Entamoeba histolytica* or adenovirus, the extinction of EIA positivity should be at the same dilution. This presumes that the manufacturers of the *Entamoeba histolytica* and the adenovirus EIA

diagnostic kits have designed their kits for maximal sensitivity, as they claim, and that these kits would, at the very least, be as sensitive to the agent they are designed to detect as to a putatively cross-reacting agent.

The details of the methodologies will be drawn by Professor Alan Smith and myself, and will be provided shortly.

I replied to Schoub as follows (there are two consecutive emails, one before I read the 'new' protocol and the second after).

Subject: Re: The latest Protocol from Schoub et al
Date: Tue, 06 Nov 2001 11:16:38 -0600
From: harvey bialy <h.bialy@natureny.com>
To: schoub@niv.ac.za
CC: Ray Mabope <RayMabope@health.gov.za>, ASmith@nu.ac.za, Lindiwe Makubalo <MakubL@health.gov.za>, A Ntsaluba <NtsalA@health.gov.za>, Nono Simelela <SimelN@health.gov.za>, bialy@ibt.unam.mx, rasnick@mindspring.com, malegapuru.makgoba@mrc.ac.za, rstock@ibt.unam.mx, Ansie Olivier <ansie@wn.apc.org>, alagon@ibt.unam.mx, "Williamson, C, Carolyn, Dr" <cwilliam@curie.uct.ac.za>

Barry,

You managed to get my email wrong on the last sending from you, so I have only an idea about it from Dave, who is, as I am, very concerned that you have appeared to have co-opted the ENTIRE protocol. This was explicitly (I don't know how many times this need be repeated to you) NOT what you were asked to do. YOU et al are to supply ONLY the protocol for your dilution experiments, and an insert into our own protocol for the preadsorption experiments, of what you deem a necessary control. However, I need point out to you (yet again) that the very purpose of our experiments is to remove non-diagnostic antibodies. We are not attempting to improve the tests as they are now used routinely to give life or death sentences to people. Both protocols will then be implemented independently. Stop trying to control the whole process. And stop stalling with promises of what Alan Smith will do.

Subject: Re: The NEW Protocol of Schoub et al
Date: Tue, 06 Nov 2001 13:04:32 -0600
From: harvey bialy <h.bialy@natureny.com>

To: schoub@niv.ac.za
CC: Ray Mabope <RayMabope@health.gov.za>, ASmith@nu.ac.za,
Lindiwe Makubalo <MakubL@health.gov.za>, A Ntsaluba <NtsalA@health.gov.za>,
Nono Simelela <SimelN@health.gov.za>, bialy@ibt.unam.mx, rasnick@mindspring.com,
malegapuru.makgoba@mrc.ac.za, Carolyn Williamson <cwilliam@curie.uct.ac.za>, rstock@ibt.unam.mx,
Ansie Olivier <ansie@wn.apc.org>, alagon@ibt.unam.mx

Barry,

I have now seen, thanks to Dave, your latest version. I will here only address the biggest flaws in your presentation (which contains many) Would you really consider submitting this to the US NSF or NIH, or the Mexican CONICIT? Be that as it might. You wrote.

. "If antibody titres for the control HAV and HSV antibodies similarly decline, then the interpretation of the experiments could be that adsorbing antigen preparations are non-specifically "sponging" out immunoglobulins including the anti-HIV antibodies."

This is completely wrong. If what you wrote were true then the interpretation of our studies is that the HIV ELISAs are bad only if every other test is. What can you possibly be thinking? The real interpretation is that there should be no change in the herpes or hep signals compared to the gelatin control pre and post-adsorption if the tests are as specific as they are claimed to be. But whatever the result, they say absolutely nothing about the HIV ELISA. Since the antigens provoking the antibodies are present in large amounts in these two cases, it is expected that that tests will be considerably better than HIV ELISAs. In the case of HIV, the antigenic stimulus is mild (so mild you need 30 cycles of pcr to see its presumed genomic source) and the corresponding antibody response much different. And now for the third time: If you really think otherwise INSERT the Hep/Herpes controls WITHIN the BODY of the latest version (#3) of our COMPLETE protocol providing the rationale, material and temporal requirements and ALL other details required in a standard proposal to undertake a small scientific investigation.

Now once again: The list of antigens. It is the most common of courtesies in normal scientific exchange to reply to questions raised by your presumptive collaborators. So, please point by point, why do you continue to systematically exclude the complete list of antigens and continue to refuse to give even an acknowledgment that you have been requested to give reasons for their exclusion (when we have taken the time to explain why we think they are necessary on several occasions) numerous times.

Finally: As has been pointed out in excruciating and completely rigorous detail

by Drs. Stock and Alagon, the rationale behind the preadsorption and dilutions is NOT the same. Are you incapable of understanding the comprehensive interpretation of the two studies that they sent you, or have you been too busy to read it?

Harvey

Williamson replies directly to this with the amazing:

Subject: Re: The NEW Protocol of Schoub et al
Date: Tue, 06 Nov 2001 21:46:15 +0200
From: Carolyn Williamson <cwilliam@curie.uct.ac.za>
To: harvey bialy <h.bialy@natureny.com>, schoub@niv.ac.za
CC: Ray Mabope <RayMabope@health.gov.za>, ASmith@nu.ac.za, Lindiwe Makubalo <MakubL@health.gov.za>, A Ntsaluba <NtsalA@health.gov.za>, Nono Simelela <SimelN@health.gov.za>, bialy@ibt.unam.mx, rasnick@mindspring.com, malegapuru.makgoba@mrc.ac.za, rstock@ibt.unam.mx, Ansie Olivier <ansie@wn.apc.org>, alagon@ibt.unam.mx

It has to be assumed that HSV and HAV diagnostic tests are accurate.

This comment (below) clearly explains the necessity of the controls and reaffirms that it is is essential that results are interpreted in the context of controls.

"If antibody titres for the control HAV and HSV antibodies similarly decline, then the interpretation of the experiments could be that adsorbing antigen preparations are non-specifically "sponging" out immunoglobulins including the anti-HIV antibodies."

Carolyn

And now I am completely furious and write the following:

Subject: Re: The NEW Protocol of Schoub et al
Date: Tue, 06 Nov 2001 14:07:56 -0600
From: harvey bialy <h.bialy@natureny.com>
To: Carolyn Williamson <cwilliam@curie.uct.ac.za>

CC: schoub@niv.ac.za, Ray Mabope <RayMabope@health.gov.za>, ASmith@nu.ac.za,
Lindiwe Makubalo <MakubL@health.gov.za>, A Ntsaluba <NtsalA@health.gov.za>,
Nono Simelela <SimelN@health.gov.za>, bialy@ibt.unam.mx, rasnick@mindspring.com,
malegapuru.makgoba@mrc.ac.za, rstock@ibt.unam.mx, Ansie Olivier <ansie@wn.apc.org>,
alagon@ibt.unam.mx

All you have done is repeat a completely incorrect statement (this is exactly by the way how HIV tests are validated, by repetition, and it goes without saying that if this were in fact a valid form of inference, we would not be engaged in what some of us take as a very serious exercise involving millions of human lives and not sophomoric pseudo-pedantry. Do you have a CV?
PS/ I see you have no objection to the term 'sponge' when it is used by one of your professors.

And I follow this with:

Subject: one more point of logic 101
Date: Tue, 06 Nov 2001 14:30:41 -0600
From: harvey bialy <h.bialy@natureny.com>
To: Carolyn Williamson <cwilliam@curie.uct.ac.za>
CC: schoub@niv.ac.za, Ray Mabope <RayMabope@health.gov.za>, ASmith@nu.ac.za,
Lindiwe Makubalo <MakubL@health.gov.za>, A Ntsaluba <NtsalA@health.gov.za>,
Nono Simelela <SimelN@health.gov.za>, bialy@ibt.unam.mx, rasnick@mindspring.com,
malegapuru.makgoba@mrc.ac.za, rstock@ibt.unam.mx, Ansie Olivier <ansie@wn.apc.org>,
alagon@ibt.unam.mx

The statement you so boldly present as clear and complete (even though it uses the term 'sponging') contains an internal fault (larger than the Rift Valley) that has nothing whatsoever to do with its 'scientific import'. Namely, it assumes to be true exactly the thing we are testing. Its premise is that 'real' hiv antibodies are in fact present, but are simply being masked!

Alejandro is more complete, not at all abusive and I think very helpful when today he sent the following:

Subject: Further Comments
Date: Fri, 9 Nov 2001 20:23:49 -0600 (CST)
From: Alejandro Alagon <alagon@ibt.unam.mx>
To: RayMabope@health.gov.za, schoub@niv.ac.za, ASmith@nu.ac.za, cwilliam@curie.uct.ac.za, rasnick@mindspring.com, ansie@wn.apc.org, rstock@ibt.unam.mx, h.bialy@natureny.com, MakubL@health.gov.za, NtsalA@health.gov.za, SimelN@health.gov.za, malegapuru.makgoba@mrc.ac.za

Dear All:

Please, enclosed find a new document entitled "Further comments on the pre-adsorption and dilution studies"

Dr. Alejandro Alagon Cano
Instituto de Biotecnologia - UNAM
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Mexico

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Further comments on the pre-adsorption and dilution studies

As already stated in the revised version of our protocol, the only purpose of this study is to determine the specificity of the HIV ELISA tests currently in use in South Africa by examining if antibodies that 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

Very few studies have been carried out to address the possibility of false positive results in the HIV-ELISA test caused by cross-reactive antibodies which can bind epitopes of the HIV antigens used in the test. Cross-reactivity should always be carefully examined in a biological context, since antibodies that are only slightly reactive (i.e., have low affinity) to the HIV antigens will significantly affect assay accuracy if they are present in samples at high concentration. Alternatively, although less likely in the present context, high affinity cross-reactive antibodies to the HIV antigens will interfere with assay specificity even if present at low concentration.

We want to stress the need of using as many as possible (our list includes 11) antigenic preparations in the pre-adsorption studies and not to limit them to preparations of only 4 infectious agents as suggested by Dr. Schoub. We have already pointed out that "the data

collected regarding one infectious agent allow no inferences regarding other infectious agents".

To control for non-specific adsorption of immunoglobulins we propose to also include pre-adsorption 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 (please, refer to the "Stoichiometry" document sent on Nov. 4th). 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.

Having Protein A pre-adsorption as the control for non-specific adsorption of immunoglobulins makes unnecessary the EIA tests for hepatitis A (HAV) and herpes simplex (HSV). On the other hand, being that HAV and HSV are prevalent in South Africa their antigens (provided in the commercial kits) could very well be included as two more antigenic preparations in the pre-adsorption studies.

We are pleased that Prof. T. F. Jackson will provide the necessary *Entamoeba histolytica* antigen preparation because of his undisputed expertise and experience with that protozoan.

Cross-reactivity between antigens means that they share at least one epitope. The shared epitope(s) can fall among two categories: (1) those which are identical, and (2) those which are similar but not identical. Antibodies that bind identical epitopes will have the same affinity for both epitopes while antibodies that bind similar epitopes will have different affinities for them. In the latter case, usually –but there are exceptions- the higher affinity is displayed against the epitope present in the antigen that elicited the antibody production. We expect that the cross-reactivity between HIV antigens and other infectious agents antigens, if present, will not be absolute, i.e., not all the epitopes will be shared, and the shared epitopes will fall, most likely, in the category of similar but not identical.

Therefore, we cannot agree with Dr. Shoub's statement for the Dilution Experiments that "If the HIV positive EIA result is due to cross-reaction with either *Entamoeba histolytica* or adenovirus, the extinction of EIA positivity should be at the same dilution". That could only happen if the HIV antigens were identical to *Entamoeba histolytica* or adenovirus antigens.

If the dual reactivity is due to cross-reaction by anti-*Entamoeba* or adenovirus antibodies, it is expected that the extinction of an EIA positive signal for HIV will occur at lower dilution than limiting dilutions in the *Entamoeba histolytica* or adenovirus tests. HIV extinction dilutions equal to or greater than those for amoebae or adenovirus imply that there is an authentic dual infection, and that EIAs for adenovirus and *Entamoeba histolytica* are many fold less sensitive than the HIV ELISA.

It would be a useful control, in the unlikely event the latter scenario occurs, to take such samples and run them against the entire library of preadsoprtion plates to determine if the HIV+ signal that remained at high dilution still remains positive. If it does, then we would have to agree that you most probably have a true HIV+ serological sample. (Whether this is due to the presence of an HIV genome is a separate issue that will involve us in the

molecular beacon or other PCR-based studies, which are beyond the scope of the present investigation).

The dilution experiments can be of great help to uncover cross-reactivity in the pre-adsorption experiments, particularly with the highly positive samples. Let us assume those samples are highly positive because cross-reacting antibodies (elicited against any other infectious agent) are present in very high concentration. Let us also assume that the pre-adsorption step for those high positive samples cannot significantly reduce the cross-reacting antibodies simply because the adsorbing antigens are present in limited amounts. Those samples could be treated as follows: first, in dilution experiments, find the dilution that still gives a high OD reading but is not saturating, then use that dilution in the pre-adsorption experiments.

Alejandro Alagon
Roberto Stock
Harvey Bialy
Sam Mhlongo

In conclusion, I can only hope that (a) you have had the stamina to read this far and that (b) having done so, you will be moved to get the preadsorption studies, which in the past were so readily agreed to, finally accomplished.

As I am sure you remember, William the Timid used my exact words (written originally on 20 August 2000) to present these studies (in a much less refined form) under his name in the Panel Report. And as the video of the breakout session documents, nobody had any problem with the simple and correct interpretation of what a loss of an HIV ELISA signal after a preadsorption would mean relative to the value of the tests.

So, after many months of agony, with every logical, and practically every logistical detail in place, I foolishly thought the hardest parts were behind us. Perhaps we really are all fools, except we are definitely not living in paradise.