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ORIGINAL ARTICLE

Designer antigens for elicitation of broadly neutralizing antibodies against HIV

Tuckweng Kok[1,2], Adriana Gaeguta[1], John Finnie[1,2], Paul R Gorry[3], Melissa Churchill[3] and Peng Li[1]

Broadly neutralizing antibodies (bNAbs) are a consistent protective immune correlate in human immunodeficiency virus (HIV) patients as well as in passive immunotherapy studies. The inability to elicit bNAbs is the core reason underlining the repeated failures in traditional HIV vaccine research. Rare monoclonal bNAbs against HIV, however, have been produced. The significance of producing and studying more monoclonal bNAbs against HIV is underlined by its capability of defining critical epitopes for antigen designs aimed at the development of a serum-neutralizing HIV vaccine. In this regard, traditional antigen preparations have failed. There is a need to clearly advocate the concept, and systematic study, of more sophisticated 'designer antigens' (DAGs), which carry epitopes that can lead to the elicitation of bNAbs. Using an extremely efficient cell-to-cell HIV infection model for the preparation of HIV prefusion intermediates, we have investigated a novel and systematic approach to produce (not screen for) potential bNAbs against HIV. We have established the concept and the experimental system for producing formaldehyde-fixed HIV DAGs that carry temperature-arrested prefusion intermediates. These prefusion intermediates are structures on the cell surface after viral attachment and receptor engagement but before fully functional viral entry. Using defined HIV prefusion DAGs, we have produced monoclonal antibodies (mAbs) specific to novel epitopes on HIV prefusion intermediates. These mAbs do not react with the static/native surface HIV or cellular antigens, but react with the DAGs. This is a paradigm shift from the current mainstream approach of screening elite patients' bNAbs.
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Elicitation of broadly neutralizing antibodies (bNAbs) against the constantly mutating HIV is at the heart of meeting the global health challenge of human immunodeficiency virus (HIV) vaccine development and treatment. In recent years, we have been repeatedly reminded of the fundamental lesson that a successful prophylactic HIV vaccine must elicit bNAbs. Our inability to do so is the core reason underlining the repeated failures in HIV vaccine development.[1–4] Moreover, animal and clinical studies suggest that passive infusion of monoclonal antibodies (mAbs) that neutralize HIV isolates *in vitro* can suppress virus replication in macaques and humans.[5,6] Generation of HIV bNAbs will also help the development of passive immunotherapy against drug-resistant HIV.[7–9]

HIV is a rapidly mutating virus with vast sequence variants/quasi species, glycan shielding and conformational masking of surface glycoproteins, for example, gp120 that binds to CD4.[10] This may explain why bNAbs are rarely found in sera of acutely infected patients. In addition, NAbs elicited during early infection are usually strain specific.[11] Traditional HIV antigen preparations and viral antigen expression regimes have not been able to induce bNAbs in vaccine trials.[12–14]

Large-scale screening of HIV patients' sera has yielded some rare bNAbs with unusual characteristics, for example, with long complementarity-determining region (CDR) H3,[15–18] which were produced by extremely rare patients' B cells. The bNAbs were subsequently cloned by genetic engineering and humanized. However, this technically demanding and labour-intensive approach essentially selects the rare genetic background of elite patients.[19]

One of the reasons for the rarity of HIV bNAbs may lie in the fact that their 'broadly neutralizing antigen determinants' are not easily recognized by the host immune system during infection. Following HIV attachment to the host cellular receptor (CD4), productive virus entry is mediated by co-receptor (CCR5/CXCR4) engagement followed by fusion of the viral and cellular membranes. This fusion process begins with the formation of viral prefusion intermediates with extended coiled coil and other prehairpin structures carrying conserved antigen determinants that are exposed/induced/shaped by components of both viral envelope proteins and cellular CD4 and CCR5/CXCR4. Some of the best-characterized HIV bNAbs (b12, 2F5, 2G12 and 4E10) and other bNAbs (Fab), such as X5 and 17b, apparently target conserved epitopes that are exposed, induced or shaped by receptor and co-receptor binding.[20–22] This type of B cell epitope may well be the Achilles' heel of HIV.

In the natural course of HIV infection at body temperature (37 °C), these conserved and potentially broadly neutralizing epitopes on the HIV prefusion intermediates are progressively induced and transiently exposed, thus the host immune system does not have sufficient time to

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recognize them and to produce specific antibodies. The virus–cell interaction process is a continuously changing target and membrane fusion is a temperature-dependent process. There is a practical possibility of making 'designer antigens' (DAGs) under prefusion temperature arrest states (TAS), which will carry those potentially conserved, broadly neutralizing B-cell epitopes in fixed forms to mimic the transient state of HIV prefusion intermediates and consistently induce bNAbs against the virus. This report describes the cellular model for the production of novel DAGs at selected TAS using a high multiplicity of infection cell-to-cell transmission model,[23] (reviewed by Sattentau[24] and Sattentau[25]) selection and characterization of mouse monoclonal DAG-specific antibodies for potentially broadly neutralizing activity against HIV.

RESULTS

Cellular model for temperature-arrested HIV infection—I: efficient viral receptor binding

Using indicator cells and a dye-based fusion assay, Mkrtchyan and colleagues[26] showed that a ternary HIV prefusion intermediate complex, composed of viral envelope proteins gp120/gp41, CD4 and CCR5/CXCR4, can be arrested at 23 °C. By using T cells and a B clade HIV in a cell-to-cell HIV infection model that has been successfully used in our laboratory for more 20 years, we first show that the viral receptor binding is very efficient.

Figure 1 shows the immunofluorescence (IF) staining of cell-to-cell HIV infection temperature arrested for 3 h at 23 °C (HuT78: H3B = 10:1, d–f are higher magnification of panel a–c). The CD4 molecules are identified by red staining (arrows in d). HIV antigens are identified by green staining (stained with a HIV patient's serum, arrows in e). When both green and red fluorophores were excited, it is apparent that the vast majority of HuT78 cells were dual-stained for both HIV and CD4, indicating HuT78 cells have grabbed the virus from virus donor H3B cells, that is, virus-receptor engagement is accomplished efficiently (arrows in f). Note that under temperature-arrested prefusion intermediate state, all transferred HIV is studded only on the surface of HuT78 cells and there is no cell fusion despite 3 h incubation after receptor engagement (also see Figure 2).

Cellular model for temperature-arrested HIV infection—II: no functional viral entry

Using the above cell-to-cell HIV infection model, when H3B virus donor and HuT78 virus acceptor cells were co-incubated at selected TAS, we showed that the fusion-mediated HIV infection was unable to progress further.

H3B virus-donor and HuT78 virus-acceptor cells were co-incubated in a 1:4 mix respectively at 23 °C for 15 min. (cell processing time, essentially no low temperature arrest) or incubated for 3 h (temperature arrested). The co-culture mix was then further incubated at 37 °C for 0–120 min. Functional viral entry and replication was judged by estimation of episomal DNA with analysis for early and late HIV reverse transcription products by real time PCR using primer pairs specific for HIV minus strand strong-stop DNA (ss-DNA) and HIV DNA after first (1ST-DNA) and second (2ST-DNA) strand-transfer[27] The levels of early ss-DNA (a), 1ST-DNA (b) and late 2ST-DNA (c) were expressed as copy numbers per 2×10^6 mixed cells (Figure 2)

The data in Figure 2 show that: (1) HIV does not fully enter virus-acceptor cells after 3 h at low temperature arrest (similar levels of viral DNA in black and grey columns, with or without 3 h co-incubation at 23 °C, before temperature shift to 37 °C); and (2) co-incubation at 23 °C prepares the virus-acceptor cells for HIV entry, as progression of viral reverse transcription was much faster (black columns) than without a prolonged incubation at 23 °C (grey columns), when the temperature was subsequently increased from 23 to 37 °C. The target is a stable cellular state where viral entry events have been initiated but arrested at prefusion intermediate stage.

In summary, the above cellular model that we used delivered efficient viral receptor/co-receptor binding but stops short of viral entry. However, cellular receptor /co-receptor engagement and the formation of viral prefusion intermediates have changed the conformational structure at the cell–virus interface. The TAS has prepared viral acceptor cells for HIV entry. As a result, the onset of viral reverse transcription is expected to be much faster when the co-culture incubation temperature is subsequently shifted to 37 °C than without a prolonged incubation step at a temperature below 25 °C. This and recent studies by Henderson and Hope[28] suggest that, before the fatal exposure of gp41 N-terminal fusion peptide, stable HIV prefusion

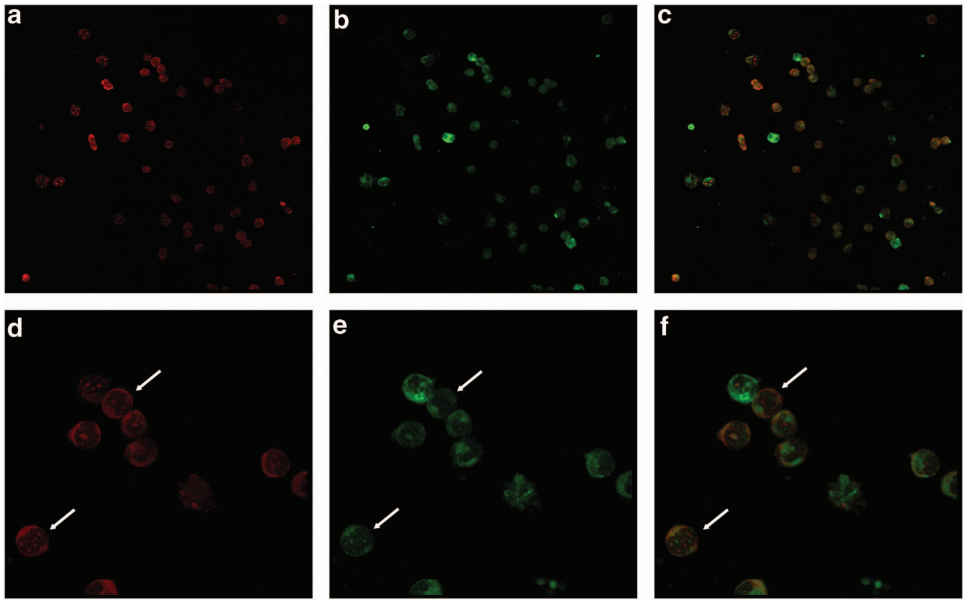

Figure 1 Cell-to-cell HIV infection temperature-arrested at 23 °C.

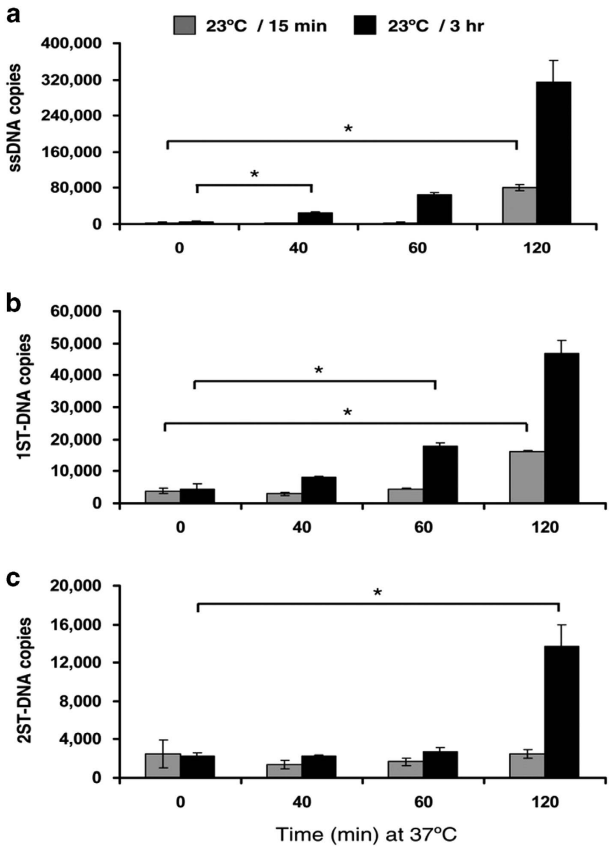

Figure 2 Temperature-arrested HIV infection followed by incubation at 37 °C. Functional viral entry was monitored by reverse transcription, as judged by estimation of HIV episomal DNA copies. Error bars represent ranges of duplicate infections. *Statistical significance ($P<0.05$, Student's *t*-test) between time point samples denoted at the ends of each horizontal line. Grey columns: Cell processing time. Black columns: co-culture mix temperature arrested for 3 h. (A. Davis and P. Li (unpublished))

intermediates can be induced and arrested *in vitro* by using live HIV infection models.

Antisera derived from T cell-tropic HIV prefusion DAGs neutralize both T cell-tropic and macrophage-tropic HIV

In a preliminary experiment, DAGs prepared at TAS at 16, 19 or 23 °C (viz. DAG16, DAG19 or DAG23) were used to immunize guinea pigs to produce polyclonal sera. The antisera against DAGs were heat inactivated at 56 °C for 30 min to minimize complement activity. The antisera were absorbed against 3.5×10^6 freshly prepared viable HuT78 cells once, then with 3.5×10^6 formaldehyde (final 0.05%) fixed HuT78 cells separately and then absorbed with 3.5×10^6 formaldehyde fixed (final 0.05%) H3B cells twice. These immune sera were referred to as 'absorbed sera'. The absorbed sera are expected to contain antibodies against the viral DAG prefusion intermediates, but minimal or no antibodies against static/native HIV antigens and cellular antigens that are not involved in viral fusion. One half volume of each of the absorbed immune sera was then further absorbed against the corresponding DAG16, DAG19 or DAG23 (*ca.* $2–3\times10^4$ cell equivalent). These were referred to as 'depleted sera'.

The depleted sera (depleted of those antibodies against the viral prefusion intermediates) and the absorbed sera were compared for their ability to neutralize the virus in a HIV single cycle infectivity assay. The results in Figure 3 showed that after extensive absorption to

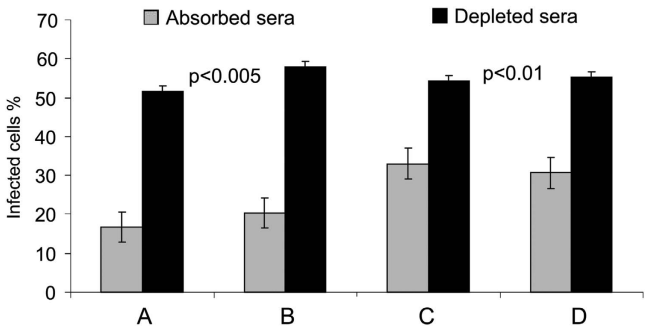

Figure 3 Absorbed (grey columns) and depleted guinea pig sera (black columns) at 1:10 dilution were tested for their ability to neutralize the infectivity of T-cell tropic HIV-HXB2 (a, b) and macrophage tropic HIV-Bal (c, d) on TZM-b1 cells. Columns with error bars represent the average if HIV-infected cell numbers from triplicate 48th post infection.

remove antibodies directed against static HIV and cellular antigens that were not involved in the fusion process, the T-cell tropic HIV-HXB2 prefusion DAG-derived antisera contained neutralizing antibodies which reduced T-cell tropic HIV-HXB2 infectivity by 65–68% and, importantly, reduced macrophage-tropic HIV-Bal infectivity by 39–46%.

HIV prefusion DAG-specific mAbs

We were encouraged by the guinea pig antisera results described above. To further study the properties of DAG-specific antibodies, we have produced mouse mAbs using DAGs that were prepared at TAS of 19 °C (DAG19).

Figures 4a and b show the IF staining of different cells by a HIV-specific mAb (8B3), which detects a static/native antigen of HIV and that of a HIV prefusion DAG-specific mAb (5E1), which detects an antigen only present on the viral prefusion intermediates. The results clearly show that mAb 5E1 does not react to mature or budding HIV particles on H3B cells—rather, it specifically captures a previously undescribed novel epitope that is unique to HIV prefusion intermediates. As expected, cells that were fixed prior to mixing did not show receptor engagement, (IF negative with 5E1). We also obtained convincing trifluorophore IF staining patterns of DAG cells and H3B cells by mAb 8B3 or by mAb 5E1 (see below).

Further confirmation of DAG specificity with HIV patient's serum and mAbs using confocal trifluorophore IF

The novel DAG specificity was further confirmed with confocal IF staining using HIV patient's serum, mouse mAb 5E1 and 8B3 and rat anti-human CD4. Figure 5a shows 5E1-specific staining (green) against DAG19 but not H3B cells—likely directed against novel, prefusion DAGs. The patient's serum showed specific fluorescence (blue) with DAG19 and H3B cells, which were virus positive. As expected, staining with rat anti-CD4 showed specific fluorescence (red) with the HuT78 cells ($CD4_+$) but not against H3B cells, which are CD4 negative. Colocalized imaging showed blue, red and green fluorescence against the DAG19 cell but only reaction of patient's serum with HIV (blue) against H3B cells.

Figure 5b shows that specific fluorescence staining was observed in DAG19 and H3B cells when reacted with 8B3 mAb—likely directed to static/native HIV antigens. The HIV patient's serum showed specific fluorescence against DAG19 and H3B cells, both of which contain the virus. Similarly, anti-CD4 showed specific fluorescence (red) against HuT78 (CD4 pos) but not H3B cells (CD4 neg). Colocalized imaging showed blue, red and green fluorescence against DAG19 cells but only

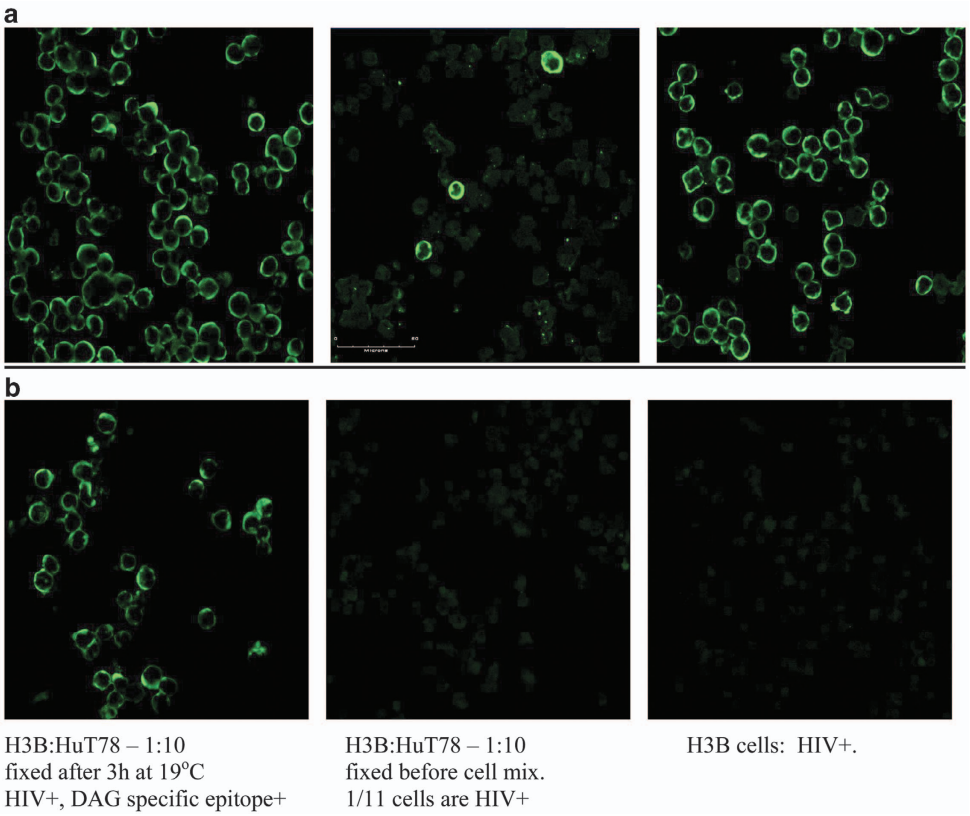

Figure 4 Immunofluorescence staining with mAb 8B3 (HIV native/static antigen) and a pre-fusion DAG19-specific mAb 5E1 against DAG19, H3B and Hut78. (**a**) IF: mAb 8B3 (static/native HIV specific). (**b**) IF: mAb 5E1 (novel DAG specific).

blue (patient's serum) and green fluorescence (8B3) against the virus in H3B cells. Table 1 summarizes the results of the IF staining (Figures 5a and b) of DAG19 and H3B cells.

The results of the IF staining and colocalized imaging with the four antibodies (5E1, 8B3, patient's HIV serum and anti-CD4) against DAG19 and H3B cells provide further confirmation of the novel DAG specificity.

DISCUSSION

The work described in this paper has shown the successful production of HIV DAGs using a synchronous one-step, cell-to-cell transmission model in which the viral donor H3B cells (B-clade HIV) were co-cultured with viral acceptor HuT78 T-cells. At selected temperature-arrest states, transiently formed and exposed epitopes—as a result of the interaction between HIV gp120, CD4 and co-receptor CXCR4—were fixed with 0.05% formaldehyde prior to viral fusion. Using indicator cells and fusion assays, Mkrtchyan *et al.*[26] showed that HIV prefusion intermediate complexes, composed of viral envelope proteins gp120/gp41 and CD4 and CCR5/CXCR4, could be arrested at 23 °C. Poon *et al.*[29] have also showed that formaldehyde-stabilized, heat-inactivated clade B virions could produce bNAb responses against heterologous HIV from clades A and C.

Our results suggest that the HIV prefusion intermediates can be induced and arrested *in vitro* by using the high multiplicity of infection model that has been successfully used in our laboratory to study HIV replication for more than 20 years.[23] We have shown that the viral fusion process can be 'frozen' and viral prefusion intermediates can be arrested at various temperatures below 25 °C. This TAS does not allow fusion-mediated HIV infection to progress further despite prolonged co-incubation of virus donor and acceptor cells. The transiently formed epitopes were fixed as the DAGs—which were subsequently used to produce mouse mAb. The specific 5E1 mAb showed reaction with the DAG 19 cells but not against the static/native viral or cellular antigens on H3B or HuT78 cells, unlike the 8B3 mAb (see Figures 5a and b).

This unorthodox approach of using fixed, temperature-arrested HIV prefusion intermediates as novel 'designer antigens' can be used for the induction of potential bNAbs against HIV. bNAbs are a crucial protective immune correlate in controlling HIV but traditional approaches have so far failed to induce them (see review by McCoy and Weiss[30]). The human body ' …is indeed capable of producing potent, broadly neutralizing antibodies;',[31] and epitopes following CD4 binding have been shown as promising targets.[6,22,32,33] In the natural course of HIV infection at body temperature (37 °C), these epitopes are only transiently exposed, thus the host immune system does not have sufficient time to recognize them and to produce specific antibodies. On the contrary, the DAGs reported here, in fixed form, can be used to induce potential 'designer HIV bNAbs'—once present in adequate concentrations, the antibodies would be able to react with the epitopes transiently expressed (induced) on the HIV prefusion intermediates and may halt the fusion process—thus inhibit HIV infection. The HIV DAGs can be used for quantity immune sera production in large farm animals. The resulting immune sera, as biological antiviral agents, potentially would contribute to current efforts in formulating non-abrasive anti-HIV topical microbicides.

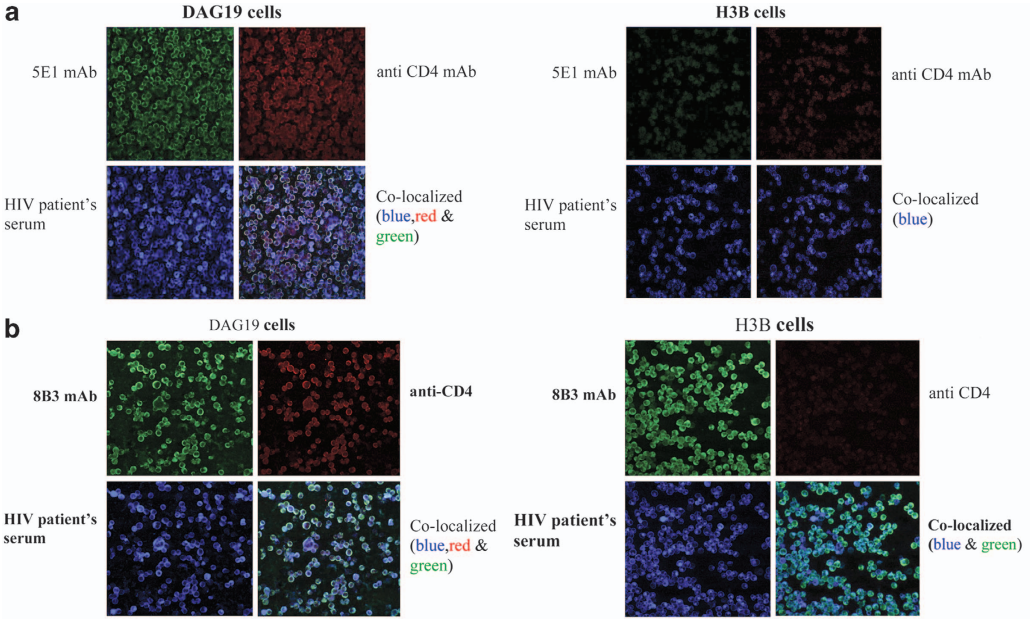

Figure 5 (**a**) Confocal trifluorophore IF staining of DAG19 and H3B cells with 5E1 mAb, patient's HIV serum and anti-human CD4. (**b**) Confocal trifluorophore IF staining of DAG19 and H3B cells with 8B3 mAb, patient's HIV serum and anti-human CD4.

Table 1 Confocal immunofluorescence staining of DAG19 and H3B cells with 5E1 mAb, 8B3 mAb, patient's HIV serum and anti-human CD4 with three fluorophores

Primary Ab	*Secondary Ab (conjugate)*	*DAG19*[a]	*H3B*[b]
Patient's anti-HIV	Goat anti-human IgG (blue)[c]	Pos	Pos
Rat Anti-human CD4	Goat anti-rat (red)[d]	Pos	Neg
5E1 mAb anti-novel epitope	Goat anti-mouse immunoglobulins (green)[e]	Pos	Neg
8B3 mAb anti-HIV[f]	Goat anti-mouse immunoglobulins (green)	Pos	Pos

Abbreviations: Ag, antigen; DAG, designer antigen; HIV, human immunodeficiency virus; mAb, monoclonal antibody; Neg, negative; Pos, positive. [a]HIV pos, human CD4 pos and novel epitope pos. [b]HIV pos, human CD4 neg and novel epitope neg. [c]Alexa Fluor 350 [d]Alexa Fluor 594. [e]Fluorescein isothiocyanate (www.lifetechnologies.com). [f]Static/native antigen

METHODS

Production of DAGs

HIV donor H3B cells (CD4 negative) were co-cultured with HuT78 viral acceptor cells ($CD4^+$) at a ratio of 1:10, respectively. H3B cells on average contain two HIV proviral DNA copies per cell and a culture of 5×10^7 cells produces a virus titre of 5×10^5 $TCID_{50}\,ml^{-1}$.[23] The co-culture mix of H3B and HuT78 cells ($1–3\times10^6\,ml^{-1}$ in phosphate-buffered saline (PBS)) was incubated for 3 h at selected temperatures 4, 10, 15, 19 or 23 °C (temperature-arrested state, TAS). An equal volume of 0.1% formaldehyde (Ultrapure, methanol-free) pre-equilibrated at the selected temperature was then added to the culture, mixed gently and incubated at the same temperature for 1 h. The DAG cells (viz. DAG4, DAG10, DAG15, DAG19 or DAG23) were then washed twice with PBS and stored in 0.05% formaldehyde (15.6 mM) in PBS containing 0.05% merthiolate as preservative. To test for possible viable virus in the DAGs, an equal volume of the co-culture mix with HuT78 cells was incubated at 37 °C for 1 week. There was no cytopathology or presence of giant cells—suggesting the absence of viable HIV. A similar mix of the DAG cells that were fixed in 0.005% formaldehyde (1.56 mM), including a separate batch that was fixed in 0.05% formaldehyde, was inoculated into TZM-bl indicator cells (NIH AIDS Research and Reference Reagent Program, www.aidsreagent.org). There were no positive indicator cells observed in the HIV single cycle infectivity assay (see below), which confirmed the absence of viable virus.

Production of mouse mAbs

Six to 8-week-old female BALB/c mice were immunized with 5×10^6 cells with an equal volume of complete Freund's adjuvant in each mouse via the subcutaneous route. The second dose was given via the intraperitoneal route with 10^6 cells in an equal volume of Freund's incomplete adjuvant 5 to 6 weeks post first immunization. The final dose was given with 5×10^5 cells in PBS via the intravenous route 4 to 5 weeks post second dose. The spleens were then extracted from the immunized mice 4 days later according to the SA Pathology Animal Ethics Committee (AEC) guidelines (www.sapathology.sa.gov.au). The immune spleen cells were fused with Sp2/O-Ag14 myeloma cells (www.cell-lines-service.de or www.sigmaaldrich.com/australia.html) in the ratio of 5:1 respectively, using polyethylene glycol MW1500, according to standard protocols.[34]

Selection and characterization of DAG specific antibody

Cultures that showed the presence of hybridoma colonies were tested by the IF test for DAG-specific antibodies. The culture supernatant fluids were reacted overnight at 4 °C against DAG, H3B and HuT78 cells, which were fixed with 0.05% formaldehyde onto silane-coated glass slides. The unreacted antibodies were washed three times with PBS and then reacted with fluorescein isothiocyanate-goat anti-mouse immunoglobulin A, G and M (www.abcam.com). The latter were incubated at 37 °C for 30 min. and then washed three times with PBS to remove unreacted fluorescein isothiocyanate conjugate. Fluorescent mounting fluid (Dako, www.dako.com/au) was then added to the stained cells and viewed with an Olympus BX51 microscope equipped with LED fluorescence excitation. Hybridoma fluids that stained the DAG but not H3B or HuT78 cells were selected for further passage and subcloning by limiting dilution. Anti-human CD4 mAb (rat) was purchased from www.lifetechnologies.com. Characterization of DAG-specific mAb was described in Results.

Production of DAG antibodies in guinea pigs

DAGs *ca.* $2\text{-}3\times10^6$ cells (prepared at 16, 19 and 23 °C) were mixed with an equal volume of complete Freunds adjuvant and immunized subcutaneously in 12-week-old guinea pigs (www.sahmri.com). The second, third and fourth inoculations ($5–10\times10^5$ DAG cells with Freunds incomplete adjuvant) were given subcutaneously at 3 to 4 week intervals. Sera from the immunized guinea pigs were obtained by cardiac puncture one week after the final inoculation, according to the AEC guidelines (as above).

HIV single cycle infectivity assay

Tissue culture 48-well microplate with 2×10^4 TZM-bl cells was infected with HIV-HXB2 cell free virus stock (H3B cell culture supernatant—prepared as described in Kok *et al.*[35]). The virus was treated with an equal volume of 1/5 dilution of normal guinea pig serum, or one of the 'absorbed sera', or one of the 'depleted sera' for 1 h at 4 °C prior to infection. The nominal multiplicity of infection used was 0.002. At 24 and 48 h post infection, the TZM-bl cells were fixed with 1% formaldehyde in PBS and stained with X-gal according to established laboratory protocols. HIV-infected cells (blue) were counted after 90 min staining.[36,37]

ACKNOWLEDGEMENTS

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