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Cytotoxic T Lymphocyte Responses to Vaccinia Virus Antigens but not HIV-1 Subtype E Envelope Protein seen in HIV-1 Seronegative Thais

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SUMMARY The HIV-1 prime boost phase I/II vaccine trial using a recombinant canarypox vector, vCP1521, containing subtype E *env* (gp120), and subtype B *env* (gp41), *gag* and *protease* has started in Thailand. We have demonstrated that although 4 from 15 human immunodeficiency virus type 1 (HIV-1) seronegative individuals showed cytotoxic T lymphocyte (CTL) responses to vaccinia virus antigens, none of them showed specific CTL responses to subtype E Env after *In vitro* stimulation. This preliminary study suggests that specific CTL responses to subtype E envelope detected in HIV-1 seronegative individuals after vaccination should be considered as specific responses to the immunization.

A number of candidate HIV-1 vaccines are currently being tested in the USA and Europe. In Thailand, phase I/II trials of six different preventive or therapeutic candidate vaccines have been approved.[1] The ALVAC-HIV (vCP1521, Aventis-Pasteur, France) is a recombinant canarypox vector expressing subtype E Env (gp120), and subtype B Env (gp41), Gag, Protease. vCP1521 is being used in combination with oligomeric gp160 TH023/LAI-DID Env or gp120-CM235 + SF2 Env in ongoing trials in Thailand. Since HIV-1 subtype E is predominant in Thailand,[2] cytotoxic T lymphocyte (CTL) responses to subtype E Env protein should be examined in seronegative individuals to determine whether there is any non specific response to this protein after *in vitro* stimulation using the vaccinia recombinant vector, vP1536, carrying subtype E *env*. Previous vaccine trials with canarypox vectors have documented false positive CD8 CTL responses. These false positive responses were seen in some volunteers who received placebo and in some volunteers prior to receiving any vaccine.[3] We were concerned that the problem of false CTL responses may be exacerbated in Thai individuals who have much higher cytotoxic responses mediated by NK cells than do those of individuals in the USA.[4]

Global eradication of smallpox was declared by the World Health Organization (WHO) in 1980.[5] Vaccination against smallpox ceased to be carried out in all countries all over the world. Specific vaccinia virus CTL memory cells have been reported to persist for many years after vaccination.[6,7] However, other groups have not detected vaccinia virus CTL responses in normal volunteers following recombinant vaccinia revaccination.[8] By using the vP1536 recombinant vaccinia construct in the assay, we could investigate whether memory CTL responses to vaccinia virus antigens in these individuals still exist.

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MATERIAL AND METHODS

Subjects

Fifteen healthy individuals, 6 males and 9 females, aged 21-50 years old (mean age = 29.27 years) were included in this study. Blood samples from each individual was collected in tri potassium ethylenediaminetetraacetic acid (K_3 EDTA) blood collection tubes (Vacuette, Greiner, Stonehouse, Germany) following the protocol with written informed consent. Peripheral blood mononuclear cells (PBMC) were separated by standard ficoll-hypaque gradient centrifugation. All individuals were HIV-1 negative by serolgical tests, i.e. enzyme-linked immunosorbent assay (ELISA) and gel particle agglutination assay.

Recombinant vaccinia viruses

Recombinant vaccinia virus expressing HIV-1 subtype E Env, vP1536, was used for *in vitro* stimulation (IVS) and for autologous transformed B lymphoblastoid cell line (TBL) infection. The empty vaccinia virus, vP1170, was used as a control antigen. Both viruses were obtained from Virogenetics, Troy, New York, USA.

Preparation of HIV-specific CTL

Approximately 10 x 10^6 fresh PBMC were expanded into CTL effector cells through *in vitro* stimulation (IVS) with antigen expressing TBL. For each individual, autologous TBL were infected with vP1536 at 5 pfu/cell for 1.5 hours, washed and cultured overnight in RPMI 1640 with 10% fetal bovine serum. The cells were then washed, fixed with 1.5% paraformaldehyde for 15 minutes at room temperature, washed, incubated with 0.2 M glycine for 15 minutes at room temperature, washed and resuspended in PBS. The autologous fixed TBL were used as stimulators for each individual's PBMC by mixing them together at a ratio of 1 stimulator to 5 responders and cultured in 5% normal human serum-RPMI with 300 U/ml interleukin 7 (Genzyme, Boston, USA) at 2 x 10^6 cells/ml. Human recombinant interleukin 2 (Boehringer Mannheim, Indianapolis, USA) was then added to the culture at a concentration of 20 U/ml on day 7. The CTL assay was done on day 14.

Target cells

The autologous TBL of each individual were derived following PBMC infection with supernatant from B95-8 (Epstein Barr virus, EBV, producing cell line, ATCC, Rockville, MD, USA) and maintained in 10% fetal bovine serum RPMI 1640 with antibiotics.[9] TBL were used as target cells for the CTL assay after infection with vaccinia recombinants.

Cytotoxicity assays

Approximately 1 x 10^6 target cells from each individual were infected with vP1536, or vP1170 at 5 pfu/cell for 1.5 hours, washed, and then incubated with 100 μCi of ^{51}Cr. Effector cells were plated in triplicate and at 4 dilutions, starting from 3 x 10^5 cells/well in 96-well U bottom plate and then 5 x 10^3 cells/well of labeled targets were added to each well giving the effector to target (E:T) ratios of 60:1, 20:1, 7:1 and 2:1 in a final volume of 200 μl. To distinguish HIV-1 specific CTL from vaccinia-specific CTL, we used non-radiolabeled vP1170 infected TBL as cold targets (CT) at a ratio of 40:1 to the ^{51}Cr-labeled targets. This strategy has been developed for use in distinguishing HIV-1 specific CTL responses in volunteers receiving candidate HIV vaccines.[9-11] The plates were incubated at 37 °C for 5 hours and then the supernatants were harvested and counted (Top Count; Packard Instrument, Downers Grove, IL, USA). The maximum and spontaneous lysis of targets were determined by incubation of the targets with 10% SDS and culture medium in the absence of the effectors, respectively. The percent lysis at each E:T ratio was calculated with the following formula: [(mean test cpm - mean spontaneous release cpm)/(mean max release cpm – mean spontaneous release cpm)] x 100. We defined significant HIV or vaccinia specific lysis as >10% lysis at two E:T ratios and mean spontaneous lysis of less than 20%. Subtype E Env CTL responses were calculated by subtracting the % lysis of vP1170 + CT from vP1536 + CT. Since we did not perform any depletion to determine the phenotype of the effector cells, cytotoxic activity could be due to T cells or NK cells.

RESULTS

Using autologous fixed TBL infected with vaccinia recombinant as stimulator cells, we could investigate not only non-specific CTL responses to subtype E Env but also specific CTL responses to vaccinia in these seronegative individuals. The data from each individual is shown in Table 1. After subtraction the % lysis of vP1170 + CT from vP1536 + CT, a positive response would be present if the lysis was > 10% at two E:T ratios. The data in Table 1 shows that for each volunteer, there is no difference between the lysis seen against targets infected with vP1536 and vP1170 in the presence of cold tar-

Table 1 HIV-1 subtype E Env CTL activities in PBMC of HIV-1 seronegative individuals after *in vitro* stimulation (IVS)

Subject	% Lysis of target cells at different effector: target ratios								**Env specific lysis	
	vP1536*+CT				vP1170*+CT				(vP1536*+CT) - (vP1170*+CT)	
	60:1	20:1	7:1	2:1	60:1	20:1	7:1	2:1	60:1	20:1
M01	16	8	1	1	19	6	1	1	0	2
F02	10	4	2	1	7	2	2	0	3	2
M03	5	1	1	0	3	0	0	0	2	1
F04	5	1	1	0	4	2	0	0	1	0
M05	2	1	0	0	0	0	0	0	2	1
F06	5	2	1	1	3	0	0	0	2	2
F07	30	12	2	0	34	4	0	0	0	8
M08	6	4	3	0	7	3	1	1	0	1
F09	9	5	3	1	11	2	0	0	0	3
F10	4	0	0	0	2	2	0	0	2	0
M11	12	5	1	0	13	5	1	0	0	0
F12	31	11	4	0	32	10	4	1	0	1
F13	11	8	2	2	4	0	0	0	7	8
M14	3	2	0	0	4	0	0	0	0	2
F15	3	1	1	0	3	1	0	0	0	0

*^{51}Cr-labeled vaccinia infected transformed B lymphoblastoid cell line (TBL), CT = vP1170 infected TBL, M = male, F = female.
**Env specific lysis is calculated by subtracting the % lysis for vP1170*+ CT from the % lysis for vP1536*+ CT.

get inhibitor cells. The % lysis was not > 10% at two E:T ratios in any of the individuals. Thus, we did not detect any non specific CTL responses to subtype E Env in the individuals tested.

In general, effector cells specific for both vaccinia and EBV should be stimulated if vaccinia infected TBL are used as stimulator cells for IVS. In the absence of cold targets, 12 out of 15 subjects (M01-F12), demonstrated CTL responses to vaccinia infected B cells (Table 2). One method of comparing the % lysis of different subjects is to use a lytic unit value.[9] The mean lytic units in the volunteers who had CTL responses to vaccinia infected B cells (M01-F12) was 15, in those without a CTL response to vaccinia infected B cells (F13-F15) the lytic unit value was 3. Thus the magnitude of the CTL response was significantly higher in the group of subjects M01-F12 who had received the smallpox vaccine.

In order to distinguish between CTL activity to EBV antigens and vaccinia antigens we estimated the portion of the lysis that was due to EBV antigens and to vaccinia antigens by using cold target cells (either vaccinia infected or uninfected [UI] B cells). The formula for calculation of % lysis for vaccinia specific CTL responses is [(vP1170 no CT) - (vP1170 + CT)] - [(vP1170 no CT) - (vP1170 + UI)]. In our study, we found specific CTL responses to vaccinia in four (M01, M03, F04, F10) of the 12 individuals who had demonstrated responses to vaccinia infected TBL in the absence of CT (Table 3). These subjects had a history of smallpox vaccination during childhood.

DISCUSSION

The HIV-1 prime boost phase I/II vaccine trial has started in Thailand. Although the strategies of the vaccine are to induce both cell-mediated and humoral immunity against HIV,[12,13] specific CTL responses have shown a significant role in protection and control against HIV infection.[14] Thus, induction of a CTL response is crucial for the development of a preventive vaccine. This ALVAC-HIV vaccine includes HIV-1 subtype E *env* (gp120) in its construct.

Table 2 Vaccinia virus-specific CTL activities in PBMC of HIV-1 seronegative individuals after *in vitro* stimulation (IVS)

Subject	Age#	% Specific lysis of target cells at different effector: target ratios							
		vP1170*+UI				vP1170*no CT			
		60:1	20:1	7:1	2:1	60:1	20:1	7:1	2:1
M01$	23	32	24	14	6	51	41	29	15
F02	24	22	8	4	0	22	15	6	4
M03$	22	23	11	2	1	29	17	7	3
F04$	41	17	16	7	1	26	15	11	5
M05	24	16	8	4	0	19	11	5	2
F06	44	17	7	3	0	29	22	12	5
F07	30	16	3	0	0	39	28	10	2
M08	50	13	4	2	1	37	25	11	2
F09	22	12	2	0	2	27	20	12	2
F10$	25	16	13	6	3	32	24	16	3
M11	29	20	7	1	0	35	26	14	5
F12	35	31	18	7	2	63	48	41	24
F13	21	13	6	0	1	17	3	0	0
M14	21	8	4	0	0	11	7	4	1
F15	28	7	3	1	0	18	9	3	1

*^{51}Cr-labeled vaccinia infected transformed B lymphoblastoid cell line (TBL), CT = vP1170 infected TBL, UI = uninfected TBL, M = male, F = female, $ = positive CTL responses to vaccinia, # = Years, M01- F12 = smallpox vaccinated individuals, F13-F15 = unvaccinated individuals.

Table 3 Vaccinia virus-specific CTL activity in HIV-1 seronegative individuals

Subject	Age#	% Vaccinia specific lysis at different effector: target ratios			
		60:1	20:1	7:1	2:1
M01	23	13	18	13	5
M03	22	20	11	2	1
F04	41	13	14	7	1
F10	25	14	11	6	3

* % Vaccinia specific lysis were calculated by the formula:
% lysis = [(vP1170noCT) - (vP1170+CT)] – [(vP1170noCT) - (vP1170+UI)].
A positive vaccinia response is defined as >10% lysis at two E:T ratios,
M = male, F = female, # = years.

The detection of HIV-1 subtype E Env specific CTL responses in volunteers after vaccination would be a good indicator of this vaccine candidate eliciting a strong cellular immune response and thus for using this vaccine to prevent HIV-1 subtype E infection in Thailand. However, there should not be any non-specific CTL response to this glycoprotein prior to vaccination. We demonstrated here that there were no specific CTL responses to subtype E Env in all individuals tested. This preliminary result showed that any CTL responses to subtype E Env after vaccination should be the specific responses to this glycoprotein. Specific CTL responses to HIV-1 subtype B proteins which are included in the vaccine should be further examined in healthy individuals.

Twelve (M01-F12) individuals had been immunized against smallpox in early childhood while the other three (F13-F15) individuals are naïve to vaccinia vaccination. Four (33%) of the immunized individuals showed specific CTL responses to vaccinia whereas none were detected in unvaccinated individuals. The last smallpox vaccination in this country was around 1979. This means that vaccinia virus specific memory CTL exists for at least 20 years after vaccination, as no known exposure to the vaccinia virus had occurred. In general, poxvirus infections are acute infections without any involvement of latency or persistence.[15] Our data confirm other reports that the presence of antigen is not needed for the maintenance of memory CTL.[6,16,17] Selin *et al.*, reported that there were cross-reactivities in memory cytotoxic T lymphocytes in recognition of heterologous viruses.[18] These memory T cell populations could provide protective immunity depending on the virus sequence.[19] Whether the immunological cross reactivities between heterologous viruses play any roles on maintenance of memory CTL to vaccinia virus in human needs to be further investigated.

The studies described here first of all demonstrate that the CTL assay technology has been transferred successfully to Thailand, and that adequate quality control measures are in place. We also show the importance of including cold target cells in the assay, since 12/15 subjects would have demonstrated a positive response in the absence of cold targets. After addition of cold targets only four subjects still had demonstrable vaccinia specific lytic activity. Although we did not determine which cell type (CD4, CD8 or NK cells) was responsible for the lysis of vaccinia infected TBL, the addition of cold targets was sufficient to dampen this non-specific response. In future studies the phenotype of lymphocytes responsible for killing vaccinia infected target cells will be investigated. We anticipate that the majority of the cytotoxic lymphocyte activity against HIV gene product expressed in canarypox vectors will be mediated by CD8+ T lymphocytes, as shown in other HIV vaccine trials.[3,10] Our studies will provide valuable baseline reference information for ongoing and future HIV vaccine trials in Thailand and other countries in South East Asia.

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REFERENCES

1. AIDS Vaccine Research in Asia: Needs and Opportunities. Report from a UNAIDS/WHO/NIID meeting Tokyo, 28-30 October 1998. AIDS 1999;13: UNAIDS 1-13.
2. Amornkul PN, Tansuphasawadikul S, Limpakarnjanarat K, *et al.* Clinical disease associated with HIV-1 subtype B' and E infection among 2,104 patients in Thailand. AIDS 1999; 13: 1963-9.
3. Evans TG, Keefer MC, Weinhold KJ, *et al.* A Canarypox vector expressing multiple human immunodeficiency virus type 1 genes given alone or in combination with rgp120 elicits broad and durable CD8+ cytotoxic T lymphocyte responses in seronegative volunteers. J Infect Dis 1999; 180: 290-8.
4. de Souza MS, Karnasuta C, Markowitz LE, *et al.* A comparative study of the impact of HIV infection on natural killer cell number and function in Thais and North Americans. AIDS Res Hum Retroviruses 2000; 16: 1061-6.
5. Arita I. Standardization of smallpox vaccines and the eradication programme- a WHO perspective. Dev Biol Stand 1999; 100: 31-7.
6. Demkowicz WE Jr, Ennis FA. Vaccinia virus-specific CD8+ cytotoxic T lymphocytes in humans. J Virol 1993; 67: 1538-44.
7. Demkowicz WE Jr, Littaua RA, Wang J, Ennis FA. Human cytotoxic T-cell memory:long-lived responses to vaccinia virus. J Virol 1996; 70: 2627-31.
8. Graham S, Green CP, Mason PD, Borysiewicz LK. Human cytotoxic T cell responses to vaccinia virus vaccination. J Gen Virol 1991; 72: 1183-6.
9. Cox JH. HIV-1 specific cytotoxic T cell assays. In: Kim JH, Michael NL, eds. Methods in Molecular Medicine, HIV Protocols. New Jersey: Humana Press 1999; pp. 355-71.
10. Egan MA, Pavlat WA, Tartaglia J, *et al.* Induction of HIV-1 specific cytolytic responses in seronegative adults by a non-replicating, host range restricted canarypox vector (ALVAC) carrying the HIV-1 MN *env* gene. J Infect Dis 1995; 171: 1623-7.
11. Weinhold K, Tartaglia J, Paoletti E, *et al.* Specific activation strategies for amplification of low CTL signals. AIDS Res Hum Retroviruses 1992; 8: 1373.
12. Excler JL, Plotkin S. The prime-boost concept applied to HIV preventive vaccines. AIDS 1997; 11: S127-37.

13. Tartaglia J, Excler JL, Habib RE, *et al.* Canarypox virus-based vaccines: prime-boost strategies to induce cell-mediated and humoral immunity against HIV. AIDS Res Hum Retroviruses 1998; 14: 401-7.
14. Ada GL, McElrath MJ. HIV type-1 vaccine-induced cytotoxic T cell responses: Potential role in vaccine efficacy. AIDS Res Hum Retroviruses 1997; 13: 243-8.
15. Buller RM, Palumbo GJ. Poxvirus pathogenesis. Microbiol Rev 1991; 55: 80-122.
16. Mullbacher A, Flynn K. Aspects of cytotoxic T cell memory. Immunol Rev 1996; 150: 113-27.
17. Lau LL, Jamieson BD, Somasuandaram T, Ahmed R. Cytotoxic T-cell memory without antigen. Nature 1994; 369: 648-52.
18. Selin LK, Nahill SR, Welsh RM. Cross-reactivities in memory cytotoxic T lymphocyte recognition of heterologous viruses. J Exp Med 1994; 179: 1933-43.
19. Selin LK, Varga SM, Wong IC, Welsh RM. Protective heterologous antiviral immunity and enhanced immunopathogenesis mediated by memory T cell populations. J Exp Med 1998; 188: 1705-15.