Immunology 1995 **86** 85–96

Th1 cells specific for HIV-1 gag p24 are less efficient than Th0 cells in supporting HIV replication, and inhibit virus replication in Th0 cells

A. VYAKARNAM, P. M. MATEAR, S. J. MARTIN & M. WAGSTAFF *Department of Immunology, University College London Medical School, London, UK*

SUMMARY

This report provides three lines of evidence to suggest that T-helper type 1 (Th1) and type 0 (Th0) cells could play an opposing role in acquired immune deficiency syndrome (AIDS). Using a panel of Th1 and Th0 clones specific for human immunodeficiency virus-1 (HIV-1) gag p24, derived from seronegative volunteers immunized with gag p24 : Ty virus-like particles, a Th1 clone specific for tuberculin (PPD), and a Th0 clone derived by random activation from the same volunteer, we have demonstrated the following differences in the capacity of these clones to regulate the *in vitro* replication of HIV. (1) Th1 clones were less efficient than Th0 clones in supporting HIV replication, both in their resting state (by 10–1000-fold) and after antigen activation (by five to 100-fold). Furthermore, the infectious titre of HIV recovered from the Th0 population was more than 1000-fold higher than virus from the Th1 population, and the number of HIV-infected Th0 cells was five to 16 times higher than the number of infected Th1 cells. (2) Antigen- or mitogen-activated Th1, but not Th0 clones, inhibited HIV in bystander CEM-4 cells. Th1 cells also inhibited HIV in autologous and allogeneic Th0 cells. The level of inhibition in these experiments ranged from 50% to 100% and was three to 10-fold higher and more sustained in the presence of p24-specific clones compared to the PPD-specific Th1 clone. The capacity of Th1 cells to inhibit HIV in neighbouring cells was also reflected in the reduced replication of HIV in the clones immediately after antigen activation compared to unstimulated cells. Kinetic studies of virus production, cytokine release and proliferation showed that inhibition of HIV was associated with peak cytokine release and preceded proliferation. (3) The Th1 clones had higher cytolytic potential than the Th0 clones. Therefore, the HIV inhibitory activity of Th1 cells could be partly due to cell to cell killing. These data demonstrate the opposing effects of Th1 and Th0 cells on the *in vitro* replication of HIV, and suggest that Th1 cells might be important in immunity whereas Th0/Th2 cells might lay a role in promoting disease.

INTRODUCTION

Clonal analysis of murine and human T cells suggests that $CD4^+$ T cells can be classified into subsets on the basis of the cytokines they secrete. At least three subsets have been identified in the murine system: $CD4^+$ T cells that produce high levels of interferon-γ (IFN-γ) and interleukin-2 (IL-2) but no IL-4 fall in the T-helper cell type 1 (Th1) category $CD4^+$ T cells that produce IL-4 but no IL-2 or IFN-γ fall in the Th2 category; and $CD4^+$ T cells that produce all three cytokines are believed to be precursors of the Th1/Th2 subsets and are referred to as Th0 cells.[1–3] Unlike the murine system, human Th1 and Th2 cells have partially overlapping cytokine profiles. Most human Th1 clones also produce low levels of IL-4 and, conversely, human Th2 cells produce low levels of IL-2 and IFN-γ.[4–9] Despite this overlap, it is clear that many antigens can induce a polarized Th1/Th2 response in humans.[10] For example, many bacterial antigens preferentially induce Th1 cells,[4,5,7,8] whereas many allergens and antigens derived from some parasites induce Th2 cells.[6–8] Both murine and human Th1/Th2 cells, despite differences, share common properties. Th1 cells in both systems induce delayed-type hypersensitivity and are relatively inefficient compared to Th2 cells in inducing antibody production, although Th1 cells can induce IgG2a synthesis. On the other hand, Th2 cells provide superior help for antibody production and induce IgG1 and IgE production.[1,11–13]

Recent studies in mice and humans show two striking features of Th1 and Th2 cells. First, *in vitro* experiments show that these subsets can interconvert with considerable overlap in cytokine profiles, suggesting that Th1/Th2 cells probably represent $CD4^+$ T cells at different stages of differentiation.[1,11–14] In general, cytokines produced by one population

Received 22 February 1995; revised 4 May 1995; accepted 4 May 1995.

Correspondence: Dr A. Vyakarnam, Department of Immunology, UCL Medical School, Arthur Stanley House, 40–50 Tottenham Street, London W1P 9PG, UK.

down-regulate or inhibit cytokine production of the second subset. For example, IL-4 and IFN-γ are mutually inhibitory[11,15] and IL-10 may be a particularly potent inhibitor of Th1 cells.[16,17] Furthermore, human $CD4^+$ T cells can be induced to differentiate into Th1 and Th2 cells by the exogenous addition of IFN-γ and IL-4, respectively.[11–14]

The second important feature of Th1/Th2 cells is that they have been shown to play opposing roles in many diseases, partly reflecting the mutually inhibitory nature of the cytokines produced by each subset. This has been clearly illustrated in several murine model systems. In many diseases, Th1 cells have been shown to be important in immunity, with Th2 cells promoting disease (leishmaniasis, *Bordetella pertusis* and candidiasis),[15,18–20] although there is at least one example of Th2 cells playing a protective role (*Microfilariae*).[21] These observations have also been extended to murine leukaemia virus (LP-BM5) infection in mice, which has been shown to induce murine acquired immune deficiency syndrome (MAIDS). In this system, too, Th2 cells were associated with progression to disease and Th1 cells with a disease-free state.[22] These findings, taken together with the recent observations of Clerici & Shearer[23] and Clerici *et al.*[24] who have demonstrated, from a study of over 100 human immunodeficiency virus (HIV) patients, an association between Th1 cytokine patterns (high IL-2, low IL-4) and healthy HIV seropositives, and conversely Th2 cytokine patterns (high IL-4, low IL-2) and HIV patients with disease, suggest that Th1 cells may be associated with immunity and Th2 cells with progression to disease in acquired immune deficiency syndrome (AIDS).

In this paper we provide evidence from *in vitro* studies of human Th1 and Th0 clones specific for the HIV-1 core protein gag p24, as well as clones specific for tuberculin (purified protein derivative; PPD) and those derived by random activation with phytohaemagglutinin (PHA), to suggest that Th1 and Th0 cells may indeed play an opposing role in HIV infection. $CD4^+$ Th1 and Th0 clones specific for HIV-1 gag p24 were generated from the peripheral blood mononuclear cells (PBMC) of seronegative volunteers immunized, as part of a Phase 1 clinical trial, with fusion proteins containing the HIV-1_{IIIB} sequence and the yeast TYA retrotransposon gene product p1, which is capable of assembling into virus-like particles (p24:Ty-VLP).[25,26] The details of immunization and the results of this Phase 1 clinical trial have been reported elsewhere[26] and show that immunization with p24:Ty-VLP does lead to the induction of both a T-cell proliferative and an antibody response to recombinant p24 protein. The successful generation of Th1 and Th0 clones specific for p24 from five individuals who received this immunization, apart from demonstrating for the first time that a Th1/Th0 response can be induced to a HIV protein, has enabled us to study the association between these subsets and HIV with homogeneous populations of cells. Using a panel of these clones, we provide data to suggest three mechanisms by which Th1/Th0 cells could play an opposing role in AIDS. (1) Th1 clones are intrinsically less efficient than Th0 clones in supporting HIV replication. (2) The activation of Th1 clones reduces HIV replication in the clones themselves and in neighbouring cells. (3) Th1 clones have higher cytolytic potential than Th0 clones and may therefore be important in killing HIV-infected cells.

MATERIALS AND METHODS

Media

T-cell cloning and all assays of T-cell clones were carried out in RPMI-1640 + 10% pooled human AB serum (HS). Washing medium consisted of Hanks' balanced salt solution (HBSS) + 2% HS. Epstein–Barr virus (EBV)-transformed B-cell lines (B-LCL) were maintained in RPMI-1640 + 10% fetal calf serum (FCS).

Stimuli

Recombinant soluble HIV-1_{BRU} p24 from the baculovirus expression system (American Biotechnologies Inc., Cambridge, MA) was obtained from the UK Medical Research Council's (MRC) AIDS Directed Programme (ADP) repository. Recombinant human IL-2 and HIV-1_{SF2} gag p24 20-mer peptides, which overlapped by 10 amino acids covering amino acids 133–353 of the p24 sequence, were also obtained from the ADP repository (NIBSC, Potters Bar, UK).

T-cell cloning (isolation of Th1/Th0 clones specific for gag p24)

Donors. PBMC from five donors recruited into the Phase 1 clinical trial of HIV-1 gag p24:Ty-VLP, conducted by Professor J. Weber (St Mary's Hospital, London, UK) and British Biotechnology Ltd UK (Abingdon, UK), were used. The details of this clinical trial and the results of the cell-mediated and antibody responses observed in the volunteers before and after immunization have been reported elsewhere.[26] The volunteers (named V13, V14, V5, V9 and V12 in the trial) received 500 μg p24:Ty-VLP in aluminium hydroxide subcutaneously at 0, 4 and 12 weeks, as detailed elsewhere.[26] Cells for cloning were obtained from PBMC frozen down in liquid nitrogen 6 weeks after the first immunization and 2 weeks after the first boost (see previous publication for isolation and testing of PBMC).[26]

Cloning and characterization of clones. Frozen PBMC of the above study were rapidly thawed at 37°, washed three times and cultured in 24-well plates at 2×10^6/ml with recombinant p24 (5 μg/ml) for 7 days. The cultures wre expanded in 20 U/ml of recombinant IL-2 for 10 days, before the cells were plates out in 96-well round-bottomed plates at 0·3 cells/well in the presence of autologous irradiated EBV-transformed B-cell lines (B-LCL as antigen-presenting cells; APC), recombinant p24 (2 μg/ml) and 20 U/ml IL-2. Cultures were fed every 3–4 days with IL-2 alone, and every 12–14 days with antigen and APC exactly as above, and wells positive for growth expanded using a similar feeding protocol to that used for cloning. Expanded clones were characterized first for their ability to recognize p24 by proliferation. Cloning efficiency for line 13 was 13%, for line 14–6%, for line 12–22%, for line 9–16%, and for line 5–4%. The clones were phenotyped using a panel of antibodies specific for T cells and natural killer (NK) cells and analysed by flow cytometry. All clones were $CD4^+$ and $CD3^+$ and $CD8^-$ and $CD16^-$ (data not shown).

Isolation of clones specific fof PPD and by random activation with PHA

These clones were derived from a single seronegative volunteer in an identical manner to that described above, using autologous B-LCL as APC and either tuberculin (PPD) from Statens Seruminstitut (Copenhagen, Denmark) at 5 μg/ml to

generate PPD-specific clones, or PHA-L (Sigma, Poole, UK) at 1 μg/ml to derive randomly activated clones. One PPD-specific clone, MS-PPD-12-C1, and one randomly activated clone, MS-cl-PHA-1, were included in this study.

Cytotoxicity assays

2×10^6 B-LCL target cells were labelled with 100 μCi Cr in 100 μl for 1 hr at 37°. Cells were washed three times and 1×10^4 cells/well cultured with the clones (effectors) in triplicate in the presence and absence of antigen for 12 hr at a final volume of 200 μl. Cr released into 100 μl culture supernatant was measured (c.p.m.) and the mean percentage specific lysis of triplicate wells calculated as c.p.m. test wells–c.p.m. control/total c.p.m.–c.p.m. control $\times$ 100. The control was the spontaneous release of Cr in the absence of effectors, and the total c.p.m. was determined by measuring the total number of target cells cultured per well.

Proliferation assays

T-cell clones (5×10^3–2×10^4 cells/well) were cultured in triplicate in 10%HS medium in the presence and absence of various stimuli. The cells were pulsed with [^{3}H]thymidine (1 μCi/well) for the last 6 hr of culture and harvested onto glass fibre disks with an automatic cell harvester. Filters were processed through a Beta Plate (Pharmacia, Uppsala, Sweden) and the incorporated thymidine calculated as c.p.m.

Cytokine assays

T-cell clones were cultured at 5×10^5/ml in 24-well plates in the presence and absence of stimuli. Cell-free supernatants were harvested and snap-frozen at −70°. Supernatants were tested by enzyme-linked immunosorbent assay (ELISA) for the following cytokines using commercially available paired antibodies (protocols as recommended by the manufacturers): IL-4 (AMS Biotechnology Ltd, Whitney, UK; sensitivity 20 pg/ml using recombinant IL-4 from AMS as standard); IFN-γ [AMS Biotechnology Ltd; sensitivity < 1 IU/ml using reference preparation from National Institute of Biological Standards and Control (NIBSC), Potters Bar, UK as standard]; IL-2 (Medginex, Milton Keynes, UK; sensitivity 30 pg/ml using reference preparation from NIBSC as standard).

Virus stocks, infection and HIV replication

The procedures for the generation of virus stocks, the titration of virus stocks and the acute infection of cells were exactly as described elsewhere.[27] The HIV-1 isolate NY5/LAV-1 was used in these studies and was maintained in H9 cells. The tissue culture infectious dose (TCID) of stocks was determined by end-point titration on C8166 cells using the appearance of syncytia as an indication of HIV replication.[27] Acute infection of cells was achieved by culturing 5×10^6 cells in a volume of 0·5–1 ml with 1–5 $TCID_{50}$ of HIV for 2–4 hr at 37°. Infected cells were washed five times before use. HIV replication was monitored by three assays. The level of p24 antigen in cell-free culture supernatants was determined by a commercial p24 antigen ELISA kit (Coulter Electronics, Luton, UK). The protocol for this assay was exactly as described by the manufacturers. Second, the TCID of virus in cell-free supernatants was determined by end-point titration on C8166 cells, as described above. The third assay for HIV replication included determining the proportion of cells productively infected by staining cells see with Rhodamine-conjugated anti-p24 antibody (Coulter Electronics, Luton, UK) and enumerating the stained cells by flow cytometry.

Virus inhibition experiments

Virus replication was monitored in the clones, and the capacity of the clones to inhibit HIV in neighbouring target cells was also tested.

Target cells. These consisted of either the CEM cell line or Th0 clones. Target cells were infected with HIV as described above, washed and cultured for 24 hr, then washed again and cultured with Th1 or Th0 clones under different experimental conditions. All assays were set-up in triplicate in 96-well plates in a final volume of 200 μl in 10% HS medium. Target cells cultured in the absence of clones served as controls. Virus replication was monitored by measuring the levels of p24 antigen in the culture supernatant, and the mean p24 antigen in triplicate cultures determined. The level of inhibition was calculated as follows: percentage inhibition = (p24 level in control supernatant − p24 level in test supernatant/p24 level in control supernatant) $\times$ 100.

In experiments where HIV replication was followed in the clones themselves, the cells were first infected with HIV exactly as described above and cultured in triplicate in 96-well plates in a final volume of 200 μl under different conditions. Cells cultured in the absence of any stimuli served as controls. p24 antigen levels were monitored and the level of inhibition in test cultures relative to controls was established as above.

RESULTS

Characteristics of Th1 and Th0 clones

Table 1 shows the specificity and cytokine profile of the clones used in this study. Specificity was determined by [^{3}H]thymidine uptake in a standard 3-day proliferation assay. The p24 clones were tested against a series of 20-mer overlapping peptides covering the entire sequence of p24. Each clone recognized only one peptide in this series. Clones 13–43, 13–20 and 13–41 recognized peptide 8, which covered amino acids (aa) 71–90 of the HIV-1 gag p24 sequence; clones 14–10.7 and 14–10 recognized peptide number 3 of the same series, which covered aa 21–40 of the p24 sequence; and clones 5–3, 9–1 and 12–6 recognized peptides 3, 1 and 14, respectively, of the same series (the aa sequence recognized by each of the clones is indicated in Table 1). All these clones proliferated to recombinant p24 (Fig. 1). The specificity of the PPD- and the PHA-specific clones was established on their basis to proliferate to PPD and PHA, respectively. A typical time–course of the proliferative response to cognate antigen (recombinant p24 or PPD) is shown in Fig. 1. All the clones showed peak proliferation at 3/4 days.

Blocking experiments with antibodies to MHC class I and class II showed that the proliferative response to p24 could be blocked by anti-class II but not anti-class I antibodies (data not shown). The MHC class II alleles important in antigen presentation were established, for some of the p24 clones, using a panel of class II matched and mismatched allogeneic

Table 1. Characteristics of Th1/Th0 clones

Clone	Specificity	Peptide no.	MHC restriction	IFN-γ (IU/ml)	IL-2 (IU/ml)	IL-4 (pg.ml)	Clone type
13–20	p24 (aa 71–90)	8	DR7	3886	55·0	0	Th1
13–41	(as above)	8	DR7	2187	26·0	0	Th1
13–43	(as above)	8	DR7	1002	24·4	0	Th1
14–10	p24 (aa 21–40)	3	DR4	240	3·2	1307	Th0
14–10.7	p24 (aa 21–40)	3	DR4	114	5·2	1276	Th0
5–3	p24 (aa 21–40)	3	DR2	676	8·8	899	Th0
9–1	p24 (aa 1–20)	1	DR1	878	30·0	8·8	Th1
12–6	p24 (aa 131–150)	14	ND	380	6·6	1890	Th0
MS-PPD-12-C1	PPD	—	ND	1012	38·0	13·6	Th1
MS-c1-PHA-1	PHA	—	ND	534	8·2	599	Th0

Figures represent cytokine levels produced after antigen stimulation. 5×10^5 cloned cells were cultured with 2×10^4 autologous B-LCL (APC) and 2 μg/ml cognate peptide or antigen. Cytokines were measured in 24 hr cell-free supernatants. Cells cultured with APC in the absence of antigen did not contain IL-2 or IFN-γ and < 5 pg/ml IL-4 (data not shown).

ND, not done.

B-LCL. The 13-series clones recognized peptide 8 in the context of HLA-DR7, the 14-series clones recognized peptide 3 in the context of HLA-DR4, and the clones 5–3 and 9–1 recognized cognate peptide in the context of DR2 and DR1, respectively.

Cytokines were induced by culturing cells with the relevant peptide (Table 1) or recombinant p24 (Fig. 2). All the clones produced IFN-γ and IL-2, although clones 13–20, 13–43, 13–41, 9–1 and MS-PPD-12-C1 produced higher levels of both cytokines than the others. The major difference between the clones was in the production of IL-4. Clones 13–20, 13–41 and 13–43 failed to produce IL-4, whereas clones 9–1 and MS-PPD-12-C1 produced low levels of this cytokine. In contrast, clones 14–10, 14–10.7, 12–6, 5–3 and MS-cl-PHA-1 produced high levels of IL-4. A typical time–course of the cytokine response to cognate antigen (recombinant p24 or PPD) is shown in Fig. 2. None of the clones produced cytokines constitutively. Peak cytokine release in response to antigen stimulation was 24–48 hr, after which time cytokine levels fell sharply. For the V13 and V14 series of p24 clones, the MHC restriction of cytokine induction was also confirmed by using class II matched and mismatched B-LCL, and was identical to the proliferative response (data not shown).

On the basis of cytokine production, the clones which produced low (or undetectable) levels of IL-4 and high levels of IL-2 and IFN-γ fell in the Th1 category, whereas the clones which produced high levels of IL-4 and lower levels of IFN-γ and IL-2 than the Th1 clones fell in the Th0 category.

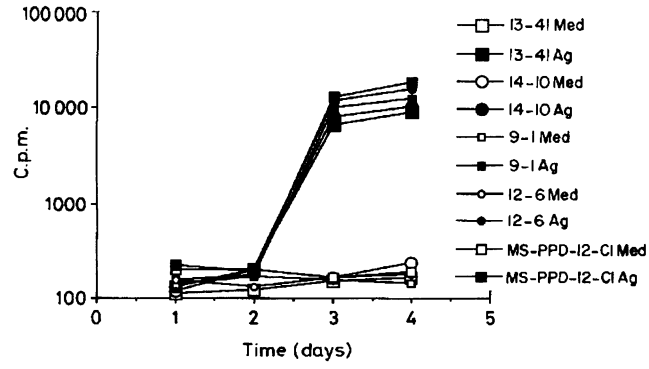

Figure 1. 2×10^4 cells (squares = Th1 clones; circles = Th0 clones) were cultured/well along with irradiated autologous B-LCL (APC) at 5×10^3/well and 2 μg/ml recombinant p24 (for the p24-specific clones) or 2 μg/ml PPD for MS-PPD-12-C1 (closed symbols). Cells cultured without stimuli served as controls (open symbols). Cultures were set up in triplicate. Data show uptake of [^{3}H]thymidine at 1–4 days. SD of replicates was less than 5%.

HIV replicates less efficiently in Th1 compared to Th0 cells

HIV-infected Th1 clones 13–20, 13–41, MS-PPD-12-C1 and the Th0 clones 14–10 and 5–3 were cultured in the presence of IL-2 at limiting cell concentrations (2×10^5, 4×10^4, 8×10^3/well). Cultures were fed every 3/4 days with fresh medium ± IL-2, and the p24 antigen concentration followed over 11 days (Fig. 3a, b, c). These experiments clearly showed the p24

Table 2. HIV replication in Th1/Th0 clones

Clone	% infected cells (range)	$Log_{10}TCID_{50}$ (range)
Th0	42–67	1·99–3·34
Th1	4·1–8·9	0·18–0·39

A series of HIV-infected (MOI = 2) Th1 and Th0 clones (gag p24-specific) were cultured in 24-well plates at 1×10^6/ml with 2×10^4 irradiated autologous APC/well and 20 μg/ml of the specific p24 peptide. Five days post-infection culture supernatants were harvested for p24 antigen measurement. The $TCID_{50}$ of each supernatant was also established by end-point titration on C8166 cells. The cells from each culture were recovered and stained with a rhodamine-conjugated anti-p24 antibody and the numbers of stained cells determined by flow cytometry.

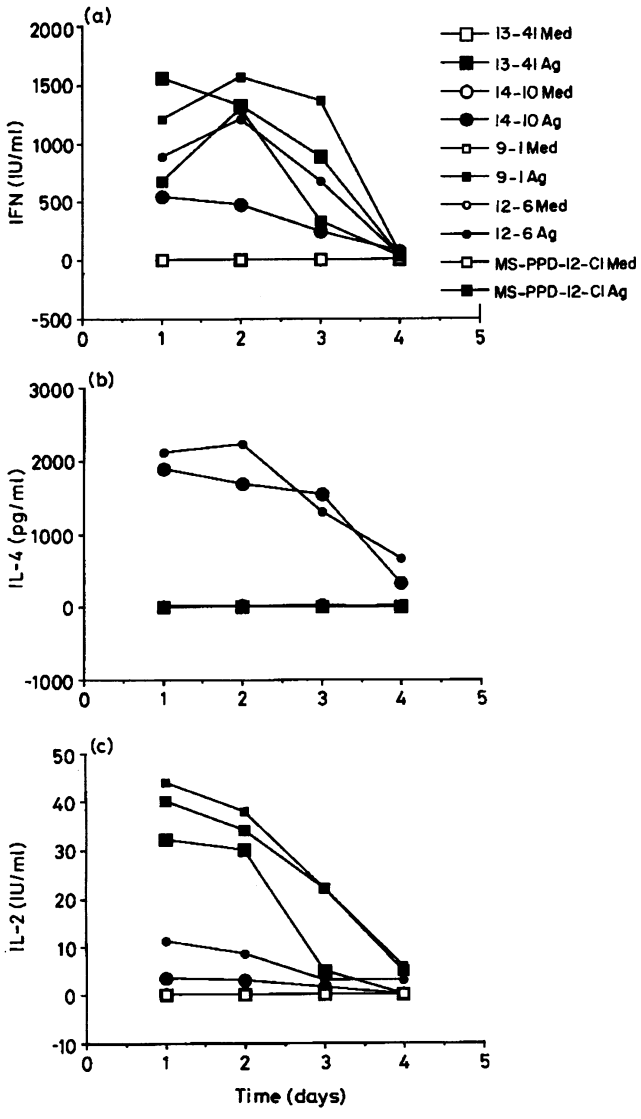

Figure 2. Cytokine assays were set up in parallel to Fig. 1. Th1 clones (squares) 13–41, 9–1 and MS-PPD-12-C1, and Th0 clones (circles) 14–10 and 12–6, were cultured at 5×10^5/ml with 2×10^4 autologous irradiated B-LCL in the absence of antigen (Med; open symbols) or in the presence of 2 μg/ml cognate antigen (Ag; recombinant p24 or PPD; closed symbols). Supernatants were harvested at each of the time-points and the level of IFN-γ (a), IL-4 (b) and IL-2 (c) in each culture measured.

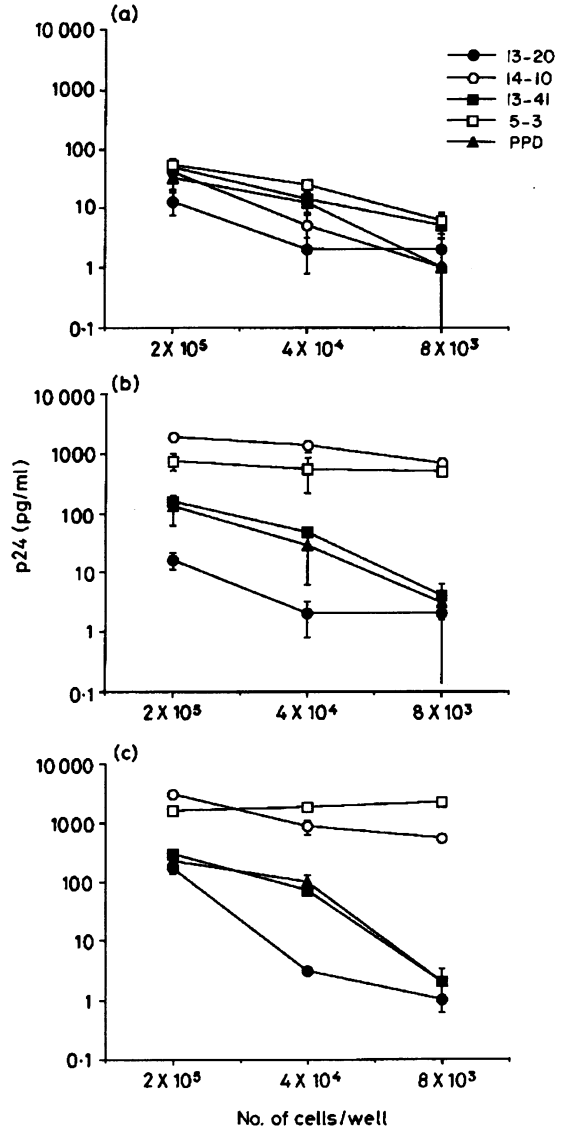

Figure 3. The Th1 clones (closed symbols) 13–20, 13–41 and MS-PPD-12-C1 (marked PPD), and the Th0 clones (open symbols) 14–10 and 5–3, were infected with NY5-LAV-1 (MOI = 5), washed five times and plated out at the indicated concentrations in 96-well plates. Cells were cultured in triplicate in 20 IU/ml IL-2 and fed every 3/4 days with fresh IL-2 at the same concentration. p24 antigen levels were measured at (a) 2, (b) 5 and (c) 8 days post-infection. Data show the mean of triplicate cultures from two separate experiments.

antigen concentrations in the three Th1 clones to be lower than the two Th0 clones, despite clonal differences between the Th1 clones; clone 13–20 was the least efficient of the Th1 clones in supporting HIV. The difference between the Th1 and Th0 clones was particularly striking at the lowest cell concentration, where high levels of p24 were recovered from the Th0 clones with little evidence of virus production in the Th1 clones, suggesting that for a given multiplicity of infection (MOI), the proportion of the HIV-infected Th0 cells may be higher than the numbers of HIV-infected Th1 cells. This was confirmed to be the case.

The proportion of virus-infected cells was determined by flow cytometry after staining with an anti-p24 antibody. Table 2 shows the numbers of infected cells to be five to 16 times higher in the two Th0 clones compared to the three Th1 clones. Furthermore, culture supernatants titrated onto C8166 cells showed the infectious titre of the virus recovered from Th0 cells to be upto 1800-fold higher than Th1 cells (Table 2).

Activation of Th1 cells has dual effects on HIV: it causes an initial reduction in HIV replication in the clones followed by conditions that support virus replication

A representative experiment of HIV replication in activated

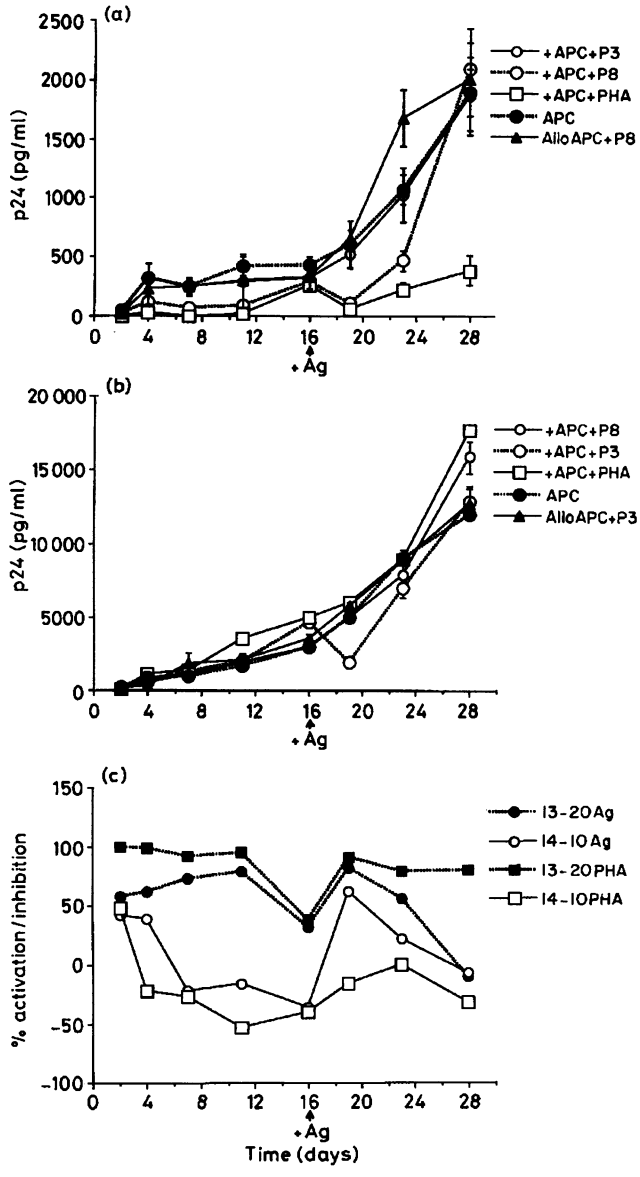

Figure 4. Clone 13–20 (a) and 14–10 (b) were infected with NY5-LAV-1 (MOI = 1), washed five times and plated out at 2×10^5 cells/well in 96-well plates. Cells were cultured in triplicate in 20 IU/ml IL-2 with 5×10^3 autologous irradiated B-LCL/well (APC); APC + cognate peptide (APC + P8 for 13–20 and + P3 for 14–10); APC + irrelevant peptide (APC + P3 for 13–20 and + P8 for 14–10); APC + PHA at 2 μg/ml; MHC mismatched APC and cognate peptide (AlloAPC + P8 or + P3). Peptide concentration was 20 μg/ml. Cultures were fed every 3/4 days with fresh IL-2 at the same concentration and on day 16 with antigen/APC as above. Data in (a) and (b) show the mean of triplicate cultures from a representative experiment; (c) shows the same data represented as percentage inhibition of HIV.

Th1/Th0 clones is shown in Fig. 4. The Th1 clone 13–20 and the Th0 clone 14–10 were infected and cultured with autologous B-LCL and IL-2, in the presence and absence of relevant or irrelevant p24 peptide or PHA. Cultures were fed every 3/4 days with IL-2, and on day 16 cultures were fed once more with antigen/APC. Figure 4a shows that the activation of Th1 cells with the relevant peptide resulted in significant inhibition of HIV (60–80% inhibition; Fig. 4c) compared to the unstimulated control population (dotted line). The inhibitory effect resulting from antigen activation fell gradually with time and could be restored after the second round of activation on day 16. However, with time antigen-activated cells supported HIV replication more efficiently than unstimulated cells, as shown by virus levels in the peptide 8-stimulated population being higher at the 28-day time-point compared to the unstimulated population. A similar trend was also observed after activation with PHA, although the overall level of inhibition was higher (100% inhibition was observed at the early time-points; Fig. 4c) and sustained over the 28-day culture period. The specificity of antigen-induced inhibition was demonstrated by the failure of the irrelevant peptide on autologous B-LCL or the cognate peptide on MHC-mismatched B-LCL to alter virus replication compared to the control unstimulated population. These experiments demonstrate the dual effect of activation on HIV replication — leading first to HIV inhibition and with time to enhanced virus production.

Although, virus replication was also inhibited in the Th0 clone after antigen activation (Fig. 4b), the level of inhibition (40–50%) was less than the Th1 clone (Fig. 4c). Moreover, the inhibitory effect was not sustained, as antigen- or mitogen-activated Th0 cells went on rapidly to support HIV replication more efficiently than the control unstimulated population (Fig. 4b, c).

Further experiments (data not shown) demonstrated the level of inhibition in the Th1 population to be dose dependent and was not only lower but rapidly lost in cells cultured with suboptimal concentrations of peptide. Similar effects were also noted with PHA, although the overall level of inhibition was higher in cells activated with PHA rather than antigen. Parallel experiments with the Th0 clone 14–10 showed that the dose of peptide or PHA did not alter the failure of this clone to inhibit HIV efficiently (data not shown).

Finally, the experiments shown in Fig. 4 demonstrate that activation with antigen did not alter the differences in HIV replication between Th1 and Th0 clones, as p24 levels were consistently higher in the Th0 compared to the Th1 clone.

Comparison of HIV replication in Th1/Th0 clones, specific and non-specific for HIV (gag p24)

The above experiments were extended to more p24-specific clones (clones 9–1 and 12–6) and a comparison was made of HIV replication in p24-specific clones and non-p24 specific clones (MS-cl-PHA-1 and MS-PPD-12-C1). All four clones were infected with HIV and cultured with IL-2 and autologous APC in the presence or in the absence of the relevant p24 peptide or PHA. Cultures were fed with fresh IL-2 medium every 3/4 days and on day 13 received a further round of antigen/APC. Virus production on day 24 in unstimulated cells (dotted line in Fig. 6) was substantially lower in the two Th1 clones 9–1 and MS-PPD-12-C1 (600–5000 pg/ml p24) compared to the two Th0 clones 12–6 and MS-cl-PHA-1 (10–16 000 pg/ml p24). Consistent with observations in clone 13–20 (Fig. 4a), virus production was inhibited after the first and second rounds of antigen activation in the p24-specific Th1 clone 9–1, and the effect of activating cells with PHA resulted in more pronounced and sustained inhibition (Fig. 5a). This was also evident, albeit to a lesser extent, in the p24-specific Th0

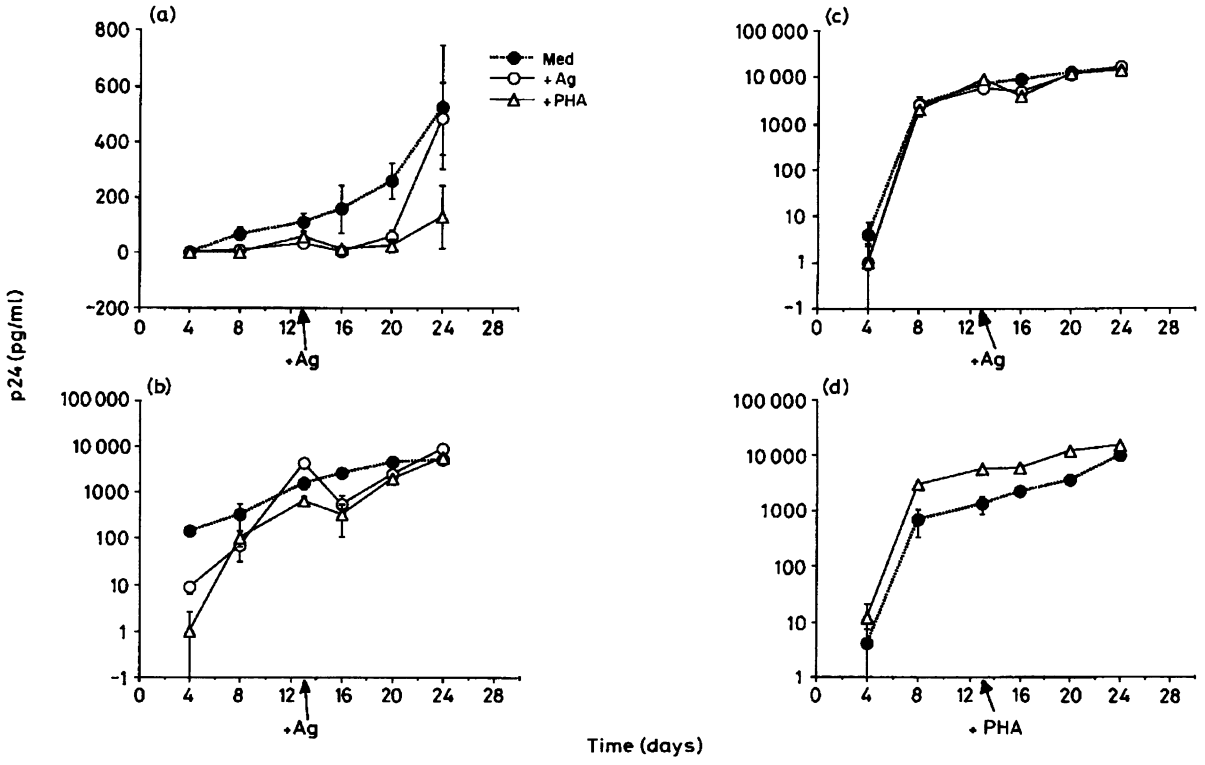

Figure 5. Clones 9–1 (a), MS-PPD-12-C1 (b), 12–6 (c) and Ms-cl-PHA-1 (d) were infected with HIV (MOI = 3), washed five times and cultured at 2×10^5/well in triplicate in 20 IU/ml IL-2 with 5×10^3 autologous irradiated B-LCL (APC; Med, dotted line); APC + antigen at 20 μg/ml (Ag) or APC + PHA at 2 μg/ml (PHA). Cultures were fed every 3/4 days with fresh IL-2 at the same concentration, and on day 13 with a further round of antigen or mitogen/APC. Data show the mean of triplicate cultures.

clone 12–6 (Fig. 5c) (also consistent with the observations in clone 14–10). However, although HIV replication was inhibited after antigen activation in the PPD-specific clone, the level of inhibition was three- to 10-fold lower than that seen in the p24-specific Th1 clones and was not sustained, as antigen-activated cells went on rapidly to support virus replication more efficiently than the control unstimulated population (Fig. 5b). Interestingly, the phenomenon of reduced virus replication in response to activation was not observed in the Th0 clone MS-cl-PHA-1 (Fig. 5d); in this clone HIV was activated immediately after activation.

Activation of Th1 cells leads to inhibition of HIV in neighbouring cells

Effect of CEM cells. Clones 13–20 and 14–10 were cultured with autologous irradiated B-LCL (APC) in the presence or absence of cognate peptide. Twelve hours later HIV-infected CEM cells were added to all cultures at an effector : target (E : T) ratio of 10 : 1. Cultures were maintained over 11 days by replacing culture supernatant with fresh 20 IU/ml IL-2 medium. Data in Fig. 6a show substantial HIV replication in target CEM cells cultured in the absence of clones (dotted line). At the first time-point of 2 days, virus replication was significantly inhibited in the presence of antigen-activated clone 13–20 (10-fold inhibition), and to a lesser degree (two- to three-fold inhibition) by 14–10 cells activated with antigen. However, it was only in the presence of antigen-activated clone 13–20 that virus inhibition was sustained over 10 days. Unstimulated clone 13–20 cells failed to reduce HIV replication in CEM cells, demonstrating that the capacity of 13–20 cells to inhibit HIV in neighbouring cells was most marked after antigen activation.

Effect of Th0 cells. Similar experiments to those described above were carried out to establish if antigen-activated Th1 cells could inhibit HIV in MHC mismatched Th0 cells. Two p24-specific clones, 13–41 and 9–1, the PPD-specific clone MS-PPD-12-C1 and the p24-specific Th0 clone 12–6 were cultured in the presence of cognate peptide and autologous APC. Twelve hours later 14–10 cells (targets), which had been previously infected with HIV and activated with PHA for 12 hr, were added at an E : T ratio of 2 : 1. Cultures were maintained in IL-2 medium and were fed every 3/4 days with fresh IL-2. At the 13-day time-point, cultures were fed with a further round of cognate antigen and APC. Data in Fig. 6b show substantial virus replication in the target cells cultured on their own (dotted line). HIV was significantly inhibited in the presence of the Th1 clones: maximum inhibition was observed in the presence of clone 9–1 (~40-fold inhibition on day 4) compared to the Th1 clones 13–41 and MS-PPD-12-C1 (~eightfold inhibition). On the other hand, the Th0 clone 12–6 failed to inhibit HIV. However, the inhibitory effect of the activated Th1 clones was gradually lost over the first 13 days of culture and could only be partly recovered after the second round of antigen activation. Furthermore stimulated Th1 cells failed to inhibit HIV in the Th0 clone (data not shown).

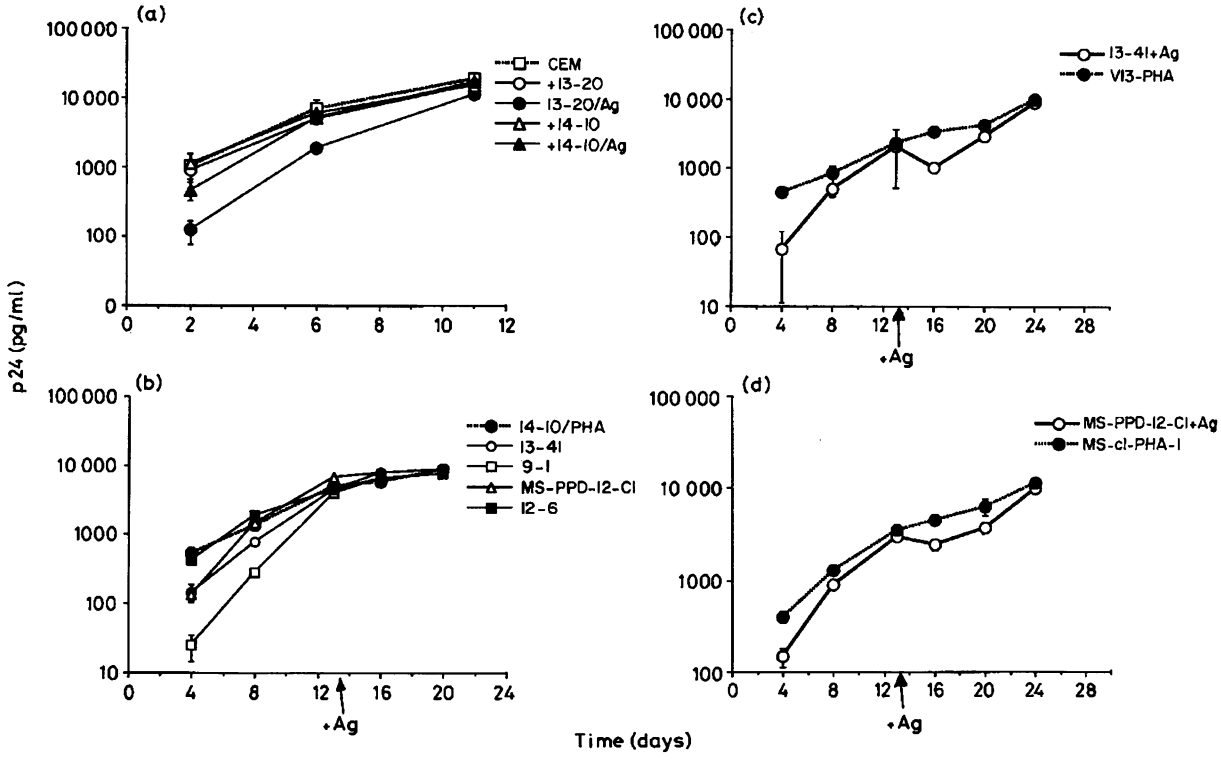

Figure 6. (a) 10^5 13–20 or 14–10 cells were first cultured with 5×10^3 autologous APC (marked 13–20 or 14–10) or APC and 20 μg/ml cognate peptide (marked 13–20 + Ag or 14–10 + Ag) in triplicate in 96-well plates in a final volume of 100 μl. Twelve hours later 1×10^4 HIV infected CEM targets (MOI = 1) were added per well and the final volume adjusted to 200 μl. Targets cultured in the absence of effectors served as controls (dotted lines). Cultures were fed every 3/4 days with 20 IU/ml. Data shows mean p24 of triplicate cultures. (b) 10^5 cloned cells (open symbols = Th1 clones; closed symbols = Th0 clone) were first cultured with 5×10^3 autologous APC and 20 μg/ml antigen in triplicate in 96-well plates in a final volume of 100 μl. Twelve hours later 1×10^5 HIV-infected 14–10 targets (MOI = 1) previously activated with 2 μg/ml PHA for 12 hr were added per well and the final volume adjusted to 200 μl. Targets cultured in the absence of effectors served as control (dotted line). Cultures were fed every 3/4 days with 20 IU/ml and on day 13 received a further round of APC and cognate peptide. Data shows mean p24 of triplicate cultures. (c, d) 10^5 Th1 clones 13–41 or MS-PPD-12-C1 cells were first cultured with 5×10^3 autologous APC in the presence of 20 μg/ml antigen in triplicate in 96-well plates in a final volume of 100 μl. Twelve hours later 1×10^5 HIV-infected (MOI = 1) targets previously activated with 2 μg/ml PHA for 12 hr were added per well and the final volume adjusted to 200 μl. Clone 13–41 received the V13-PHA Th0 line (c) and MS-PPD-12-C1 received clone MS-cl-PHA-1 (d). Targets cultured in the absence of effectors served as control (dotted line). Cultures were fed every 3/4 days with 20 IU/ml and on day 13 received a further round of APC and cognate peptide or PPD. Data shows mean p24 of triplicate cultures.

Further experiments were performed to establish if Th1 cells inhibited HIV in autologous Th0 cells. The Th1 clones 13–41 and MS-PPD-12-C1 were tested against autologous Th0 cells in an experimental system set up exactly as described above. MS-PPD-12-C1 was tested against the Th0 clone MS-cl-PHA-1, whereas clone 13–41 was tested against a $CD4^+$ Th0 line derived from autologous PBMC by repeated stimulation with PHA/autologous B-LCL and IL-2 for 14 days. These experiments reveal substantial inhibition of HIV in 13–41 cultures (~eightfold inhibition), which was gradually lost with time and could be partly recovered by a second round of antigen activation (Fig. 6c). Consistent with the above observations, the capacity of the Th1 clones to inhibit HIV was linked to the cells being stimulated, and was most marked immediately after the first round of antigen activation. The capacity of the PPD-specific clone to inhibit HIV in Th0 cells also followed the same pattern, although the level of inhibition was lower (~threefold inhibition) than that achieved with 13–41 cells (Fig. 6d).

Th1 cells have higher cytolytic potential than Th0 cells

In order to establish if differences in the ability of Th1 and Th0 cells to inhibit HIV was linked to differences in cytolytic potential, we followed the capacity of each clone to kill antigen-coated autologous B-LCL in standard Cr-release assays. The three Th1 clones (13–20, 13–41 and 13–43) and the two Th0 clones (14–10, 14–10.7) were cultured with Cr-labelled autologous B-LCL targets at different E:T ratios. Each clone was cultured with and without the relevant peptide to follow the effects of antigen activation on cytolysis. Figure 7a shows that the three Th1 clones killed targets cultured with peptide 8. Maximum killing under these conditions was 25–30% and titrated to low E:T ratios. The specificity of killing was

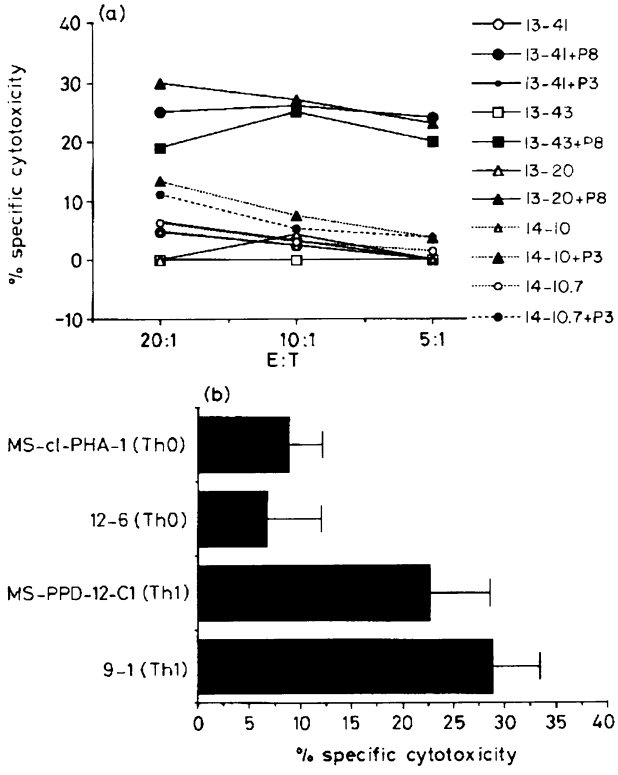

Figure 7. (a) Cytotoxicity assays were set up as described in the Materials and Methods. 1×10^4 B-LCL cells were cultured in 96-well plates with different concentrations of clones to give a final E : T ratio of 20 : 1, 10 : 1 and 5 : 1. Cultures were set up in triplicate in the presence of 2 μg ml cognate peptide (closed symbols) or in the absence of peptide (open symbols). Clone 13–41 was also tested with the irrelevant peptide (13–41 + P3). Mean percentage specific release of Cr in triplicate cultures at 12 hr is shown. Unbroken lines represent Th1 clones and broken lines Th0 clones. (b) Cytotoxicity assays were set up with clones 9-1, 12-6, MS-PPD-12-C1 and MS-cl-PHA-1. 1×10^4 autologous B-LCL cells were cultured in 96-well plates with 2×10^5 clones to give a final E : T ratio of 2 : 1. Cultures were set up in triplicate in the presence of 20 μg ml specific antigen. Figures represent mean percentage specific Cr release at 12 hr. Spontaneous Cr release was < 15%.

demonstrated by the failure of the clones to kill in the absence of antigen or in the presence of an irrelevant peptide (13–41 tested with peptide 3), confirming that the capacity to kill, like the ability to inhibit HIV in neighbouring cells, is linked to the Th1 clones being activated with cognate antigen. In contrast to the Th1 clones, both Th0 clones killed peptide-coated targets weakly — maximum killing by clones 14–10 and 14–10.7 was 10% and was only apparent at the highest E : T ratio (Fig. 7a). Further experiments of cytolytic activity of additional Th1/Th0 clones showed that the Th1 clones 9–1 and MS-PPD-12-C1 killed autologous targets to the same extent as the 13-series of clones, whereas the Th0 clones 12–6 and MS-cl-PHA-1 killed inefficiently (Fig. 7b).

DISCUSSION

The results of experiments described in this paper suggest three mechanisms by which Th1 cells might be important in immunity, and conversely why Th0 cells may be important in promoting disease in response to HIV infection. Firstly, we have demonstrated that Th1 cells do not support HIV replication as efficiently as Th0 cells, an observation further confirmed by measuring the virus load in Th1/Th0 cells by a semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) assay.[28] Secondly, HIV-specific Th1 but not Th0 cells inhibited HIV replication in neighbouring cells. Thirdly, we have shown that Th1 cells have higher cytolytic potential than Th0 cells and therefore could be important in the elimination of HIV-infected cells. These observations were consistent in many experiments. Furthermore, the differential interaction of these subsets with HIV, although associated with the cytokine profile of the clones, is unlikely to reflect clonal differences in the kinetics of proliferation or cytokine production, as we have shown that both the Th1 and the Th0 clones have similar profiles regarding proliferation and kinetics of cytokine release (Figs 1 and 2). Indeed, recent observations of HIV replication in a panel of Th1/Th0/Th2 clones revealed the most marked differences in HIV replication to be between non-IL-4-producing (low HIV replication) and IL-4-producing clones (high HIV replication), with clones producing low levels of IL-4 showing an intermediate capacity to support HIV.[29]

Four main characteristics of these phenomena are described in this paper. (1) Firstly, it is clear that Th1 cells are intrinsically less efficient than Th0 cells in supporting HIV. This is supported by the observation that HIV levels were lower in Th1 compared to Th0 cells whether or not the cells were activated. (2) Secondly, the specificity of the cells could be important in governing the HIV inhibitory activity of Th1 cells. Th1 cells specific for HIV were more efficient than a PPD-specific clone in inhibiting HIV. (3) Thirdly, the ability of Th1 clones to inhibit HIV in bystander cells was linked to the activation of cells with mitogen (PHA) or through the T-cell receptor complex (HIV was inhibited when cells were activated with the correct peptide presented in the context of the correct MHC). (4) Fourthly, the activation of Th1 cells could have dual effects on virus production, leading initially to the inhibition of HIV in neighbouring cells and in the clones themselves. However, this inhibitory effect is gradually lost, leading to conditions which supported HIV growth. Some of the mechanisms for these observations are discussed below, but they are not donor dependent as Th1 clones from several individuals were consistently poor in supporting HIV compared to Th0 clones. Furthermore, a Th1 clone (PPD-specific) and a Th0 clone (isolated by random activation) derived from the same individual differed in their ability to support HIV. Our observations complement and extend the observations of Maggi *et al.*[30] who showed that HIV replication was lower in a range of PPD and tetanus toxoid-specific Th1 compared to Th0 clones derived from several individuals. They also confirm our first observations of differential HIV replication in Th1 and Th0 clones specific for p24[31] with an expanded series of clones.

Two mechanisms may account for the HIV inhibitory function of antigen-activated Th1 cells. One possibility is that Th1 cells produce a soluble factor capable of inhibiting HIV. Indeed, the capacity of antigen-activated Th1 cells to inhibit HIV in MHC mismatched bystander cells that cannot directly activate the effectors is consistent with the idea that Th1 cells

inhibit HIV through a soluble factor. This is also supported by our observation that maximum inhibition was concomitant with peak cytokine release (Fig. 2). There are three examples of HIV replication being inhibited by soluble factors: IL-10,[32] transforming growth factor-β (TGF-β)[33] and IL-13[34] are three such factors, and Walker & Levy have shown that HIV replication can be inhibited by a soluble factor(s) secreted by $CD8^+$ T cells.[35] Establishing the HIV regulatory role of the factors produced by these Th1 and Th0 clones is a subject of study in our laboratory[36] and recent experiments show that cell-free supernatants of Th1 but not the Th0 clones can inhibit HIV, although the level of inhibition is not as high as that observed with the cells themselves (A. Vyakarnam, unpublished observations).

The second mechanism for HIV inhibition by Th1 cells could be linked to their ability to kill. We have shown that antigen-stimulated Th1 clones kill antigen-coated targets more efficiently than Th0 clones. Indeed, it can be argued that the direct recognition of HIV antigens by the HIV-specific Th1 clones through the T-cell receptor complex might result in more efficient cell–cell killing than targets being killed by bystander lysis in the presence of non-HIV-specific Th1 cells. This possibility is currently being tested and might explain why the gag p24-specific Th1 clones were more efficient than the PPD-specific Th1 clone at inhibiting HIV in neighbouring cells and in themselves after antigen or mitogen stimulation.

The seemingly paradoxical observations of Th1 cell activation resulting in HIV inhibition and in HIV replication remains to be resolved. We are currently investigating two possibilities. Firstly, we have data to suggest that HIV might be differentially cytopathic for Th1 and Th0 cells, with Th1 cells more prone to undergoing cell death than Th0 cells.[29] Secondly, Th1 cell activation results in the production of multiple cytokines [e.g. IL-2 and IFN-γ (as shown in Table 1), and tumour necrosis factor (TNF), lymphotoxin and IL-10 (data not shown)]. Some of these cytokines have been shown in separate studies to enhance HIV replication,[27,37–39] and others could have dual effects on virus production, e.g. IFN-γ, TNF and IL-4.[27,40–42] The hierarchy with which these cytokines exert their effect on HIV would depend on their concentration, potency, their ability to cross-regulate each other, and on the differentiation stage of the $CD4^+$ T cell (e.g. HIV replicates more efficiently in $CD4^+$ $CD45RO^+$ cells compared to $CD4^+$ $CD45RA^+$ cells).[40,43] Our observation of HIV inhibition in response to antigen activation would be consistent with the production of a potent inhibitory factor immediately after antigen activation, which can inhibit HIV despite the presence of other HIV-enhancing cytokines. As the activity of such a factor is reduced, HIV replication might be enhanced by some of the many HIV-inducing cytokines produced by Th1 cells.

Our data provide indirect support to the observations of Clerici *et al.*,[23,24] who showed an association between Th1 cytokine patterns in healthy HIV seropositives and Th2 cytokine patterns in patients with disease by providing at least three mechanisms (as listed above) by which Th1 cells may be important in immunity, and conversely how Th2/Th0 cells could play a role in disease. However, it would be wrong to assume that the HIV inhibitory capacity of Th1 cells reported here is the only mechanism by which this cell type influences HIV immunity. Although it is not yet clear exactly which effector mechanisms are important in immunity to HIV, it is known that the Th1/Th2 cytokine pathway can regulate $CD8^+$ T cells and in at least one model it has been shown that the capacity to generate $CD8^+$ cytotoxic T cells is lost during the Th2 phase.[44] This could be important in HIV considering the many observations which have demonstrated an association between cytotoxic T cells and healthy HIV patients.[45]

The differential replication of HIV in Th1/Th0 cells could be important in governing the cytokine profile of HIV-infected individuals. On the one hand, the enhanced replication of HIV in Th0 cells might lead to the preferential activation of these cells and the autocrine production of Th0 cytokines such as IL-4 (in an analogous manner to TNF-α)[37–40] and thus contribute to the increased production of IL-4 seen in some HIV-infected individuals.[23,24,29] On the other hand, the enhanced replication of HIV in Th0 cells might lead to the loss of these cells, especially in sites of active virus replication such as lymph nodes, and to the reduced production of IL-4 in HIV-infected compared to uninfected individuals.[46] Such a scenario would be consistent with the failure of some other groups to find IL-4-producing cells in the lymph nodes of HIV patients.[47,48]

In conclusion, while the importance of these studies have to be validated in HIV patients, they demonstrate how Th1 cells might be important in immunity to HIV and how HIV replication might be regulated by Th1 and Th0 cells and thus influence the cytokine profile of HIV-infected individuals. They also show clearly that HIV replication is affected by the cellular environment and suggest that studies of HIV replication in Th1/Th0/Th2 cells might give a better understanding of how cytokines can affect this process.

ACKNOWLEDGMENTS

We thank Professor J. Weber (St Mary's Hospital, London) and British Bio-technology Ltd, UK, for allowing us to collaborate on their Phase 1 clinical trial of HIV-1 gag p24 : TY VLP in seronegative volunteers; M. Martin for the infectious molecular clone of HIV-1 termed NY5-LAV-1; Professor P. C. L. Beverley (UCL Medical School, London) for monoclonal antibodies to cell-surface antigens; and Professor I. Roitt for his encouragement and support.

This work was supported by the UK Medical Research Council's AIDS Directed Programme (grant award numbers G 901390 and G 9013854).

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