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Dendritic cells infected *in vitro* with human T cell leukaemia/lymphoma virus type-1 (HTLV-1); enhanced lymphocytic proliferation and tropical spastic paraparesis

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SUMMARY

Evidence supporting a role of the dendritic cell (DC) in stimulating autologous T cell activity in tropical spastic paraparesis (TSP) was sought by studies of cells taken from healthy volunteers and exposed to HTLV-1 *in vitro*. DC were co-cultured with an HTLV-1-producing cell line (MT-2) at 1:1 or 10:1 ratios. These DC stimulated high levels of proliferation in autologous T cells. This was similar to that seen in an autologous mixed leucocyte reaction (AMLR) using cells from TSP patients. The requirement for both DC and virus was confirmed, since neither DC co-cultured with uninfected MT-2 cells nor addition of infected MT-2 cells directly to T cells caused significant stimulation. DC exposed to the highest dose of HTLV-1 (1:1) for 24 h before addition of T cells caused strong stimulation that increased after 8 h but almost disappeared by 72 h. *In situ* hybridization showed that approximately 25% of DC became infected in cultured cells after preincubation for 24 h, and over 50% were infected with a 72-h preincubation. We suggest that infection of DC by HTLV-1 may be an initial step in altering the immune system in seronegative patients, and that persistent T cell stimulation in those with genetic susceptibility may underlie the production of neurological disease.

Keywords HTLV-1 tropical spastic paraparesis dendritic cells autologous mixed leucocyte reaction HAM

INTRODUCTION

Dendritic cells (DC) are potent antigen-presenting cells (APC) which are present in many tissues and whose role in initiating normal immune responses would appear to be pivotal [1]. Recently, the contribution of circulating DC to the pathogenesis of HIV-induced disease has been studied; investigations show significant subsequent reduction in their capacity to present mitogen to syngeneic T cells [1,2]. A loss of DC function in presenting mitogen or antigen has also been observed on *in vivo* infection [3].

The discovery that the first human retrovirus, appropriately named the human T cell leukaemia/lymphoma virus type-1, was also closely linked to the development of the neurological syndrome tropical spastic paraparesis (TSP), led to an investigation of the immune response to HTLV-1 in patients with TSP compared with asymptomatic HTLV-1-infected people. A marked increase in all humoral responses as well as enhanced spontaneous proliferation of peripheral blood lymphocytes was noted in TSP patients [4–6]. The latter depends on the presence of DC, some of which are infected with virus [7]. The lymphocyte activity thus has the characteristics of a marked autologous mixed lymphocyte response (AMLR), and the data are compatible with the possibility that high AMLR may represent an ongoing presentation of opportunistic antigens to T cells [8]. Here we used cells from healthy volunteers and looked at the effects of exposing DC to virus *in vitro* before adding them to cells. We show that HTLV-1 infection of normal DC *in vitro* can mimic the AMLR response seen in TSP patients.

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PATIENTS AND METHODS

Controls and patients

The HTLV-1$^+$ TSP patients were two females aged 57 and 62 years, both born in Jamaica, who had emigrated to the UK in their 20s, had a 15–20 year history of progressive gait disturbance, back pain and micturition dysfunction. Neurological examination revealed normal cranial nerves and upper limbs, but there was a moderate to severe spastic paraparesis. One of the patients had a minor impairment of vibration sense at the ankles, while the other had no sensory disturbance. There was no evidence of a compressive myelopathy in either patient on

MRI or myelography. Both patients had high titres of antibodies to HTLV-1 (10^{-3}–10^{-5}) in the blood and significant titres in the cerebrospinal fluid (CSF).

The HTLV-1$^-$ TSP patient was a 68-year-old man, born in Jamaica, who had emigrated to the UK in his 20s. He gave a 20-year history of progressive gait disturbance associated with back pain and micturition disturbance; neurological examination revealed normal cranial nerves and upper limbs. He had a moderate to severe spastic paraparesis, with minor superficial sensory impairment over the feet. MRI of the entire neuraxis was normal. HTLV-1 antibodies were sought and not found in either blood or CSF on multiple occasions. There was no evidence of integrated HTLV-1 genome in leucocytes obtained from either blood or CSF using polymerase chain reaction (PCR) and GAG and envelope primers. This patient's clinical picture was identical to that of the HTLV-1$^+$ TSP patients.

Six females and two male seronegative controls were used (aged 28–45 years).

Preparation of cells

Peripheral blood (40 ml) obtained from the controls and the patients [6] was diluted with medium (RPMI 1640 Dutch modification, with 100 U penicillin and 100 μg/ml streptomycin) and separated on a Ficoll density gradient. The cells obtained at the interface were washed with medium and incubated overnight in medium with 10% fetal calf serum (FCS) on Nunc Petri dishes at 37°C. The following day the non-adherent cells were removed and layered onto hypertonic metrizamide gradients (14·5 g metrizamide, Nyegaard, plus 100 ml medium) and centrifuged for 10 min at 600 ***g***. The cells at the interface were then collected and washed in medium, incubated at 37°C, counted, and distinguished by their characteristic veiled morphology from monocytes. This preparation contained 30–40% DC with fewer than 5% contaminating lymphocytes.

The cells from the pellets were diluted from the hypertonic metrizamide solution by the dropwise addition of medium, washed and co-cultured with an enriched population of T cells derived by an overnight rosetting with sheep erythrocytes.

HTLV-1

An HTLV-1-producing T cell line (MT-2) was washed three times with medium and used to infect the DC by co-culture, as there is little evidence of free virus in cell-free supernatants. Co-culture was at 1 : 1 and 10 : 1 ratios of DC : MT-2 cells, for periods ranging from 24 h to 72 h in 0·5 ml total volume (10% FCS-enriched medium) in round-bottomed tubes. The cells were then centrifuged on metrizamide to separate the DC from the T cell line and washed three times before use. In some experiments the DC were treated with an anti-MHC class II (DR) (Becton Dickinson, Palo Alto, CA) MoAb (10 μg/million cells), and washed three times before use in the proliferation assay.

Plating and cultures

Cultures were of responder T cells ($12–100 \times 10^3$/well) with or without DC (1000/well) which had been cultured with or without HTLV-1 as indicated, in 20 μl hanging drops in Terasaski plates [9]. After 3 days, cultures were pulsed (maintaining the plate inverted) with tritiated thymidine (Amersham International, Amersham, UK; 2 Ci/mmol, 1 μl added per culture to give a final concentration of 1 μg thymidine/ml) for 2 h and harvested by blotting onto filter discs. These were counted in a beta scintillation counter. These conditions ensured that the thymidine remained freely available throughout the pulse time and that there was minimal radiation damage in cells taking up the ^{3}H. The actual counts were lower in this system than in most other stimulation assays, but stimulation indices were high and reflected DNA synthesis. The results were analysed and described [9]. Concanavalin A (Con A, 1 μg/ml; Miles Labs, Naperville, IL) was added to some cultures as indicated.

Identification of HTLV-1-infected DC

A double-labelling technique using histochemical labelling of different cell types and *in situ* hybridization for identification of virus was used. The cell population was washed twice in PBS containing sodium azide (0·01%) and labelled for 30 min on ice with a cocktail of MoAbs recognizing CD19, CD16, CD24 and CD3 determinants which are specific for B cells, natural killer (NK) cells, monocytes and T cells respectively to counterstain DC (monoclonals obtained from Becton Dickinson). After washing, the cells were absorbed onto poly-L-lysine-coated, acid-cleaned glass slides and fixed with 4% paraformaldehyde in 0·1 M phosphate buffer (pH 7·2) for 30 min. Antibody binding was detected by the alkaline-phosphatase anti-alkaline-phosphatase (APAAP) staining technique [10]. DC were identified by their large size, fluffy appearance and the absence of staining with APAAP.

The presence of HTLV-1 was then detected by *in situ* hybridization using an HTLV-1 probe (pHT-2, a complete HTLV-1 provirus in plasmid psp67) [11] labelled with ^{35}ATP and CTP prepared by nick translation. The cells were permeabilized, hybridized to viral RNA and DNA overnight at 50°C, washed under stringent conditions and processed for autoradiography. A detailed account of the procedure has previously been given [12]. Infected and uninfected cultures were processed in parallel. Cell labelling with silver grains clearly above background levels was counted as positive for the HTLV-1 genome.

RESULTS

Lymphocyte proliferation in the AMLR in controls and TSP patients

Uninfected DC (1000) obtained from normal volunteers produced insignificant proliferation of autologous T cells as assessed by ^{3}H-TdR uptake after a 3-day culture in 20 μl hanging drops (Fig. 1a, c). Macatonia *et al.* [7] demonstrated the marked stimulation of T cell proliferation when DC from TSP patients are co-cultured with their autologous T cells, an observation confirmed in the three TSP patients studied here and demonstrated in Fig. 1c. Addition of Con A to purified T cells produced proliferation that was further enhanced by the addition of autologous DC to the culture (Fig. 1). This was a highly reproducible observation seen in all eight controls. DC do not proliferate in the presence of mitogen, and so could not contribute to the proliferation noted (not shown).

To test whether *in vitro* infection of DC could produce similar heightened T cell proliferative responses, DC and T cells were purified from peripheral blood of healthy volunteers and the DC were infected with HTLV-1. DC were used to stimulate autologous T cells with results as shown (Fig. 2). The heightened proliferation observed using cells from the TSP patient was reproduced in the healthy controls with peak proliferations

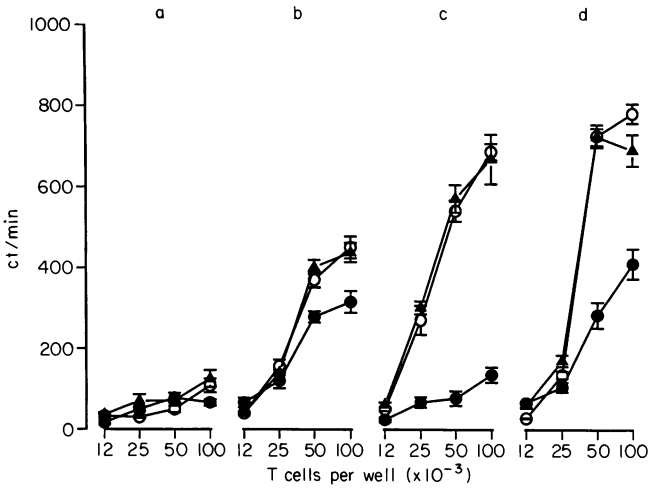

Fig. 1. Comparison between proliferation of T cells from control and tropical spastic paraparesis (TSP) patients. Proliferation of different numbers of enriched T cells was studied with and without dendritic cells (DC). (a) T cells only. (b) T cells + 1 μg/ml concanavalin A (Con A). (c) T cells + 1000 DC. (d) T cells + 1 μg Con A + 1000 DC. ●, Normal; ○, TSP patient 1; ▲, TSP patient 2.

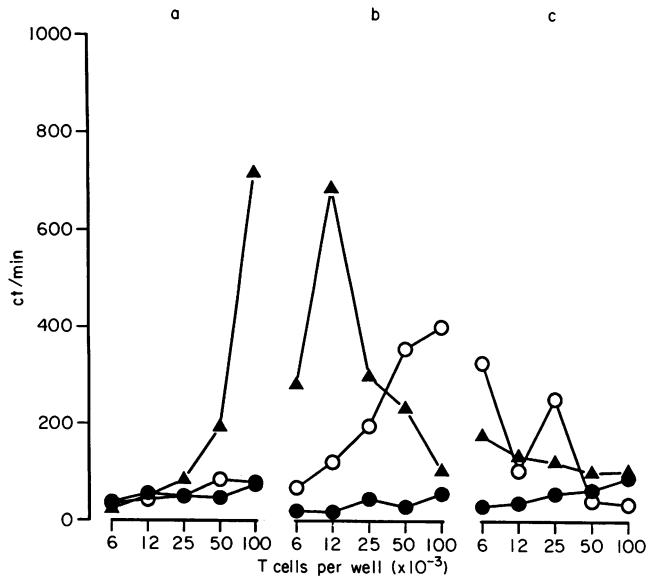

Fig. 2. Effect of dendritic cells (DC) exposed *in vitro* to HTLV-1 on proliferation of autologous T cells. DC were exposed to infected MT-2 cells or control CEM cells (a) for 24 h, (b) for 48 h, or (c) for 72 h and then separated, added to autologous T cells and stimulation measured after 3 days. ▲, DC cultured 1:1 with MT-2 cells; ○, 10:1 with MT-2 cells; ●, 1:1 with control cells. Data from representative experiments of between three and six done for each time point are shown.

depending on DC numbers and pre-incubation times with MT-2 cells (Fig. 2), and proved to be consistent (Fig. 3). Maximum proliferation was usually stimulated by DC exposed at a 1:1 ratio with infected MT-2 cells for 24 h (Fig. 2). Further co-culture of the DC resulted in a diminution of the T cell response, so that following 72 h of co-culture the T cell response was little higher than that of controls. When the ratio was 10 DC: 1 MT-2

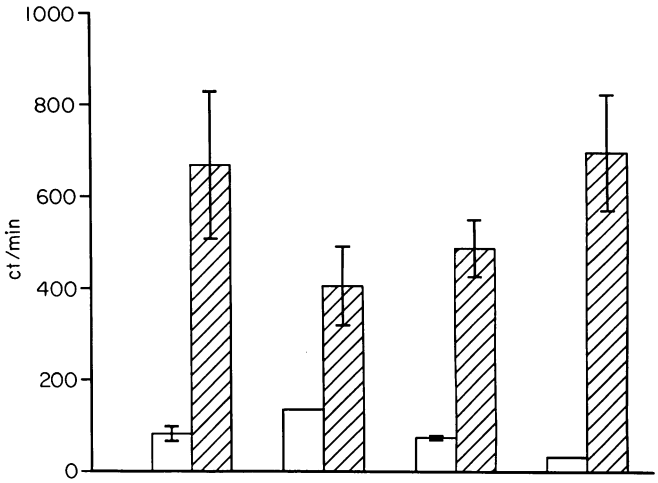

Fig. 3. Effect of dendritic cells (DC) exposed *in vitro* to HTLV-1 on the proliferation of autologous T cells. Data from four additional experiments of the type shown in Fig. 2 are shown, giving the data from the top cell concentration only (10^{-5}) for clarity and using DC exposed 1:1 with infected MT-2 cells for 24 h (▨) or control cells (□). There was significant and reproducible stimulation in all experiments.

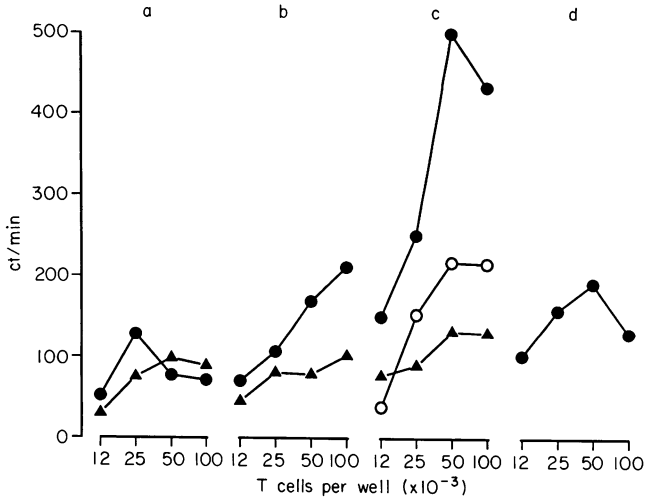

Fig. 4. Proliferation of T cells in response to dendritic cells (DC) using cells from an HTLV-1⁻ patient with tropical spastic paraparesis (TSP). T cells from a normal control or from a patient with TSP were cultured with or without DC. (a) T cells only (day 4). (b) T cells + concanavalin A (Con A) (day 4). (c) T cells + autologous DC (day 3 (○) or day 4 (●)). (d) T cells + autologous DC treated with antibody to class II. ▲, Normal control cells, 4 days; ●, TSP cells, 4 days; ○, TSP cells, 3 days.

cell there was no significant stimulation of autologous T cells using DC exposed to MT-2 for 24 h. To exclude the possibility that the proliferation observed was due to contamination by the MT-2 cell line or supernatant, these cells, irradiated (30 Gy) or unirradiated, were added in varying numbers as stated (Fig. 2) to purified T cells and proliferation assessed both on day 2 and day 3. Little enhancement of T cell proliferation was noted when either irradiated (not shown) or non-irradiated cells were used.

As in previous work on patient material, DC infected with HTLV-1 were unable to promote T cell proliferation when treated with an anti-MHC class II MoAb before including in the co-culture (Fig. 4). The proliferative response was also exam-

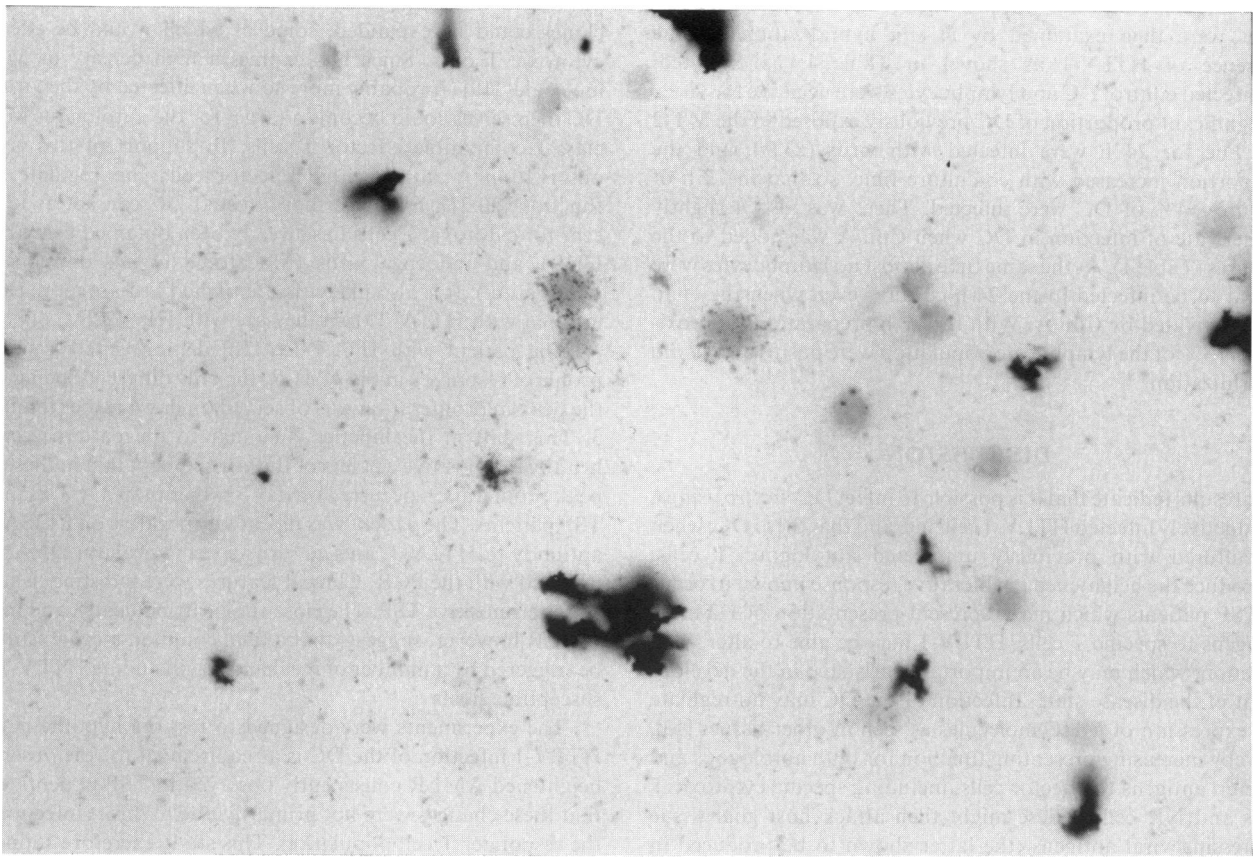

Fig. 5. Slide preparations from cultures of dendritic cells (DC) exposed to HTLV-1 *in vitro* for 24 h and added to autologous T cells in hanging drops for 3 days were labelled for T cell, B cell, natural killer (NK) cell and macrophage markers using alkaline phosphatase/anti-alkaline phosphatase (APAAP) and examined by *in situ* hybridization for HTLV-1. Many cells of dendritic morphology not labelling for other cell markers were positive for HTLV-1.

Table 1. HTLV-1-infected cells present in cells as detected by *in situ* hybridization

	DC HTLV-1$^+$ APAAP$^-$		T cells HTLV-1$^+$ APAAP$^+$	
L plus	Per cent DC infected		Per cent L infected	
(N)DC	0/15		0/1012	
Con A	0/2		0/875	
(N)DC + Con A	0/8		0/1128	
*Inf DC1	3/13	23	0/960	0
Inf DC1 + Con A	4/12	33	1/768	0·1
Inf DC2	3/11	27	4/553	0·9
Inf DC2 + Con A	2/7	28	6/864	0·8
Inf DC3	6/11	54	13/754	1·7
Inf DC3 + Con A	8/13	64	29/1045	2·8

* DC1–3, 1–3-day infection of DC with MT-2 cell line.

HTLV-1 infection as assessed with *in situ* hybridization. APAAP positivity is to CD3-labelled cells.

Con A, Concanavalin A; DC, dendritic cells.

ined in a single HTLV-1$^-$ patient with TSP, in whom a very similar pattern was noted except that peak proliferation was observed on day 4 instead of day 3 (Fig. 4). This enhanced proliferation was also blocked by pre-treating the DC with the anti-class II antibody (Fig 4d).

Identification of HTLV-1 infection of the DC

Cells were harvested from hanging drop co-cultures of T cells and stained with a cocktail of MoAbs directed against non-DC cells (T cells, B cells, NK cells and monocytes) using the APAAP technique [10]. The unstained DC were also recognizable by

their large size and fluffy appearance (Fig. 5). These preparations were then examined by *in situ* hybridization for the presence of HTLV-1 as shown in Table 1; as expected, uninfected control DC and lymphocytes were negative for virus. A significant proportion of DC previously exposed to the MT-2 cell line for 24 h were infected with virus (23%), and the proportion increased with co-culture time, so that in 72 h of culture, 54% of DC were infected. There was also a slightly higher rate of infection in DC when Con A was added to the cultures (Table 1). At the same time almost no lymphocytes were found to be infected in the 24-h cultures even when they had been activated by Con A. With the 72-h preparations approximately 3% of the lymphocyte population were positive on *in situ* hybridization.

DISCUSSION

Our results indicate that it is possible to infect DC *in vitro* using a productively infected HTLV-1 cell line, and that these DC, when co-cultured with previously uninfected autologous T cells, reproduce the heightened proliferative response known to occur in TSP patients which may represent presentation of HTLV-1 antigens to specific T cells. HTLV-1 may be able to alter APC function, which may be an important early step in the development of the disease state. Infection of the DC may up-regulate the expression of MHC molecules as seen in other tissues [13], thereby increasing presenting function for both autologous and foreign antigens to effector cells, including specific cytotoxic T cells and NK cells. These might then attack host glial tissue expressing viral antigens (the latter shown to be produced in increased numbers in TSP [14]). Some evidence suggests that microglial cells in the brain may be of the DC lineage and not conventional macrophages as previously believed [15]. If so, infected APC might enter the brain and play the dual role of stimulating T cell activity there and acting as target cells for specific cytotoxic lymphocytes. Viral and neural antigens released in this process could then be taken up by the APC, including DC, and the whole process of immunological injury repeated and amplified, resulting in the known neurological syndrome. However, the immune response to HTLV-1 may also cause damage because of cross-reactivity between HTLV-1 antigens and antigens expressed on certain neuronal cells (reviewed in [16]).

The enhanced proliferation probably involves the known capability of DC to produce clustering of T cells, leading to their activation and subsequent proliferation. Increased expression of IL-2 and its receptor [17], as well as direct enhancement of helper inducer activity by the virus [18], might also contribute, and so might soluble factors released by infected DC.

This heightened proliferation declined after 48 h co-culture of DC and MT-2 cells, although the proportion of infected DC continued to increase. One possibility is that overdosage of infected DC reduces the response, and a second is that a cytotoxic T cell response during the assay period reduces the stimulatory dose of antigen/DC. On the other hand, high dose non-responsiveness is a well established phenomenon, and the lowered proliferation rate may be due to a numerical or functional reduction in antigen-presenting ability of the DC in culture.

It is conceivable that, with markedly heightened stimulatory activity of the DC when highly infected by HTLV-1 in prolonged infection steps, a wide range of responder T cell clones could be expanded, some of which would be effector cytotoxic T cells. Since DC express a high density of MHC molecules [19]—probably more so when infected by the virus—DC themselves could become a target for the autologous MHC class I compatible effector T cells. In support of this is the observation in animals that effector cells can regulate DC function [20,21]. Evidence that infected DC can act as target cells for cytotoxic T cells has already been obtained using both HIV-1 and influenza virus (Macatonia *et al.*, unpublished observation). It is also likely that activated DC are more easily infected with HTLV-1 than they are with HIV [22].

The patient with HTLV-1$^-$ TSP demonstrated a similar profile of responses in the AMLR, the only difference being that the peak in proliferation was observed on day 4 rather than day 3. This shift in the kinetics is similar to the pattern seen in healthy seropositive carriers of the virus, except that in these the peak proliferation occurred on day 5 rather than day 3, as in the TSP patients. The patient was repeatedly negative on ELISA for antibody to HTLV-1, and no virus-specific proviral DNA was detected with the PCR. Clinical features were indistinguishable from seropositive TSP. The close similarity clinically, and in the AMLR however, suggests that a final common mechanism can be triggered by a number of mechanisms, including HTLV-1, in susceptible hosts.

The experiments were designed to test the hypothesis that HTLV-1 infection of the DC is a requirement for the observed heightened AMLR consistently observed in TSP patients, and that these changes were not primarily due to direct infection of the responder T cell population. This study therefore supports the concept that the immune response to HTLV-1 is what determines whether or not TSP develops, and that DC play a crucial role in the pathogenesis of TSP.

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