



# Induction of HLA class II in HTLV-I infected neuronal cell lines

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Human T-lymphotropic virus-I (HTLV-I) has been etiologically linked with HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/TSP), a neurologic disease. The characteristic pathological finding in HAM/TSP is marked mononuclear infiltration of the CNS with destruction of the long tracts of the spinal cord. An increased expression of HLA surface antigens and cytokines in the CNS is associated with this inflammatory response. Furthermore, there is evidence for the presence of HTLV-I in HAM/TSP CNS specimens using *in situ* hybridization and polymerase chain reaction techniques. The relationship between HTLV-I infection of CNS cells and the observed upregulation of surface antigens in the CNS is not well understood. It has been previously demonstrated that HTLV-I infection of neuroblastoma cells leads to induction of HLA surface antigens. As an extension of these studies, HFGC and HCN-1a, neuronal cell lines of nontumorigenic origin, were infected with HTLV-I and the effect on HLA upregulation was studied. Infection of the neuronal cells was demonstrated by the presence of HTLV-I gp46 surface antigen on CD4 negative cells and by the *in situ* presence of HTLV-I RNA in neurofilament positive cells. Concurrent to HTLV-I infection, HLA class II surface antigen was observed on neurofilament positive cells. Upregulation of HLA class II was not observed in neuronal cells grown in the presence of interferon- $\gamma$  or tissue necrosis factor- $\alpha$ .

**Keywords:** HTLV-I; human neuronal cell lines; HLA class II

## Introduction

Human T-lymphotropic virus-I (HTLV-I), a human retrovirus, is the causative agent in adult T-cell leukemia (ATL) (Poiesz *et al*, 1980) and is associated with a progressive myelopathy, HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Gessain *et al*, 1985; Osame M *et al*, 1985; Roman and Roman, 1988). HAM/TSP leads to a slowly progressive paraparesis in approximately 1-5% of HTLV-I infected individuals (Kaplan *et al*, 1990). The predominant pathology in HAM/TSP is inflammation and destruction of the spinal cord with mononuclear perivascular infiltrates located throughout the central nervous system (CNS) (Liberski *et al*, 1988; Piccardi *et al*, 1988; Moore *et al*, 1989; Iwasaki 1990; Wu *et al*, 1993). Chronicity of disease determines the composition of these infiltrates. Early in the disease, the perivascular infiltrates contain an equal distribution of CD4+ lymphocytes, CD8+ lymphocytes, and macrophages

(Iwasaki 1990; Umehara *et al*, 1993; Yoshioka *et al*, 1993). Prolonged duration of neurologic symptoms leads to an increase in the CD8+ lymphocytes within the infiltrates with a relative paucity of CD4+ cells. In addition, there is upregulation of HLA expression on CNS cells and increased expression of lymphokines, including interferon-gamma (IFN- $\gamma$ ) and tissue necrosis factor-alpha (TNF- $\alpha$ ) (Umehara *et al*, 1994; Wu *et al*, 1993).

Complementary to the inflammatory response observed in the CNS pathology, HAM/TSP patients appear to have a systemic activation of their immune function (Itoyama *et al*, 1988a; Jacobson *et al*, 1988). Markers of activated T-lymphocytes, including interleukin 2-receptor (IL2R) on T-lymphocytes and soluble IL2R in the serum, are increased in this disease (Itoyama *et al*, 1988a). In addition to increased activated T-lymphocytes, spontaneous proliferation of peripheral blood lymphocytes (PBL) (Itoyama *et al*, 1988b; Jacobson *et al*, 1988) and HTLV-I specific CD8+ cytotoxic T-lymphocytes (CTL) have been demonstrated in HAM/TSP patients (Jacobson *et al*, 1990; 1992). Spontaneous lymphoproliferation, the *ex vivo* abili-

ty of PBL to proliferate in absence of mitogenic stimuli, can be demonstrated in HAM/TSP patients but not in ATL patients or HTLV-I seronegative individuals. HTLV-I specific CTL is a targeted response by activated, IL2R+, T-lymphocytes to an HTLV-I specific antigen (Elovaara *et al*, 1993; Jacobson *et al*, 1988; 1990). Significant levels of CTL activity are observed in PBL and CSF lymphocytes of HAM/TSP patients (Jacobson *et al*, 1992) with an estimated precursor frequency of HTLV-I tax specific CD8+ CTL as high as 1 in 75 CD8+ in HAM/TSP PBL (Elovaara *et al*, 1993). Asymptomatic HTLV-I carriers have low or absent CTL activity (Jacobson *et al*, 1990).

Despite the immunologic and pathologic observations in HAM/TSP, the role of HTLV-I in the pathogenesis of this disorder remains elusive. Quantitative PCR analysis of PBL demonstrates that there is 2–20 viral copies per 100 PBL in HAM/TSP patients while asymptomatic carriers have a lower viral load with 0.04–8 viral copies per 100 PBL (Kubota *et al*, 1993). PCR studies on CNS tissue have demonstrated the presence of proviral DNA in the spinal cord and to a lesser extent, in the cerebrum of HAM/TSP (Bhigjee *et al*, 1991; Kira *et al*, 1992; Ohara *et al*, 1992). *In situ* hybridization of HTLV-I tax RNA has been observed in cells within white matter of spinal cords and cerebellum of HAM/TSP patients (Lehky *et al*, 1995). A few infected cells appeared to be astrocytes. Recently, *in situ* PCR has demonstrated that infiltrating CD4+ cells may contain proviral HTLV-I sequences (Hara *et al*, 1994).

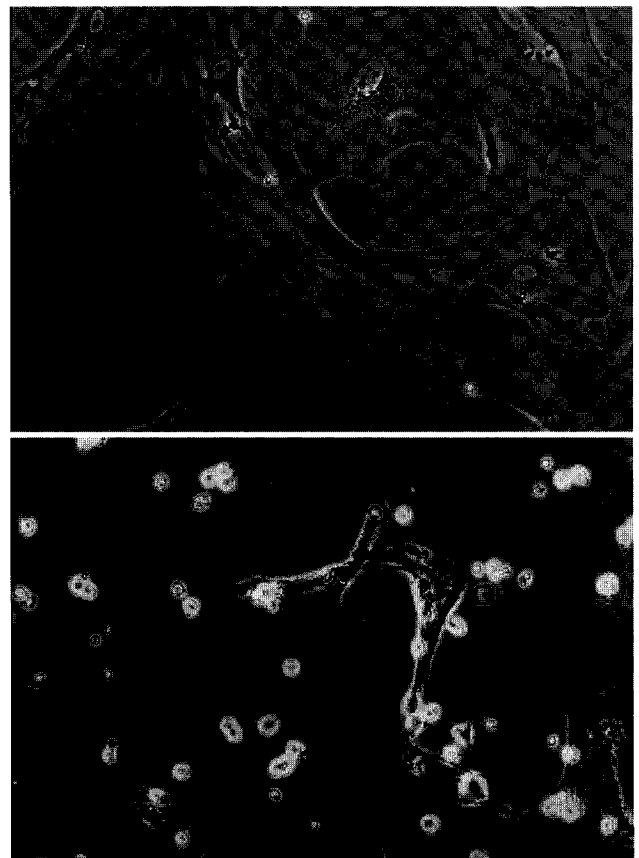
To study the interaction between HTLV-I infection and immune activation, we examined the effect of HTLV-I infection on HLA expression of neuronal cells. Neuronal cells were chosen for these experiments because the high degree of inflammation surrounding the corticospinal tract axons (Moore *et al*, 1989; Iwasaki 1990; Wu *et al*, 1993) relative to the heavily myelinated posterior columns makes motor neurons a potential target for immune activation either by direct HTLV-I infection or cytokine-mediated effects. Previous studies with HTLV-I infected neuroblastoma cells demonstrated that induction of HLA class II was closely associated with HTLV-I expression (Lehky *et al*, 1994). Since neuroblastoma cell lines are of tumorigenic origin and may have characteristics atypical for CNS neurons, we sought to apply this system to neuronal cells that more closely represent normal CNS cells. The two neuronal cell lines studied were HFGC, a human cell line containing a mixture of neuronal and astrocytes from a fetal source, and HCN-1a, a human neuronal cell line from an adult cortical source (Ronnelt *et al*, 1990; Poltorak *et al*, 1992; Truckenmiller *et al*, 1993). These cell lines were cocultured with Hut-102 cells, a HTLV-I infected cell line, to establish susceptibility of these neuronal cells to infection. Evidence for HTLV-I infection was determined by

surface antigen expression of HTLV-I gp46 and *in situ* hybridization of HTLV-I RNA. These infected cocultured cells were also shown to upregulate HLA class II expression.

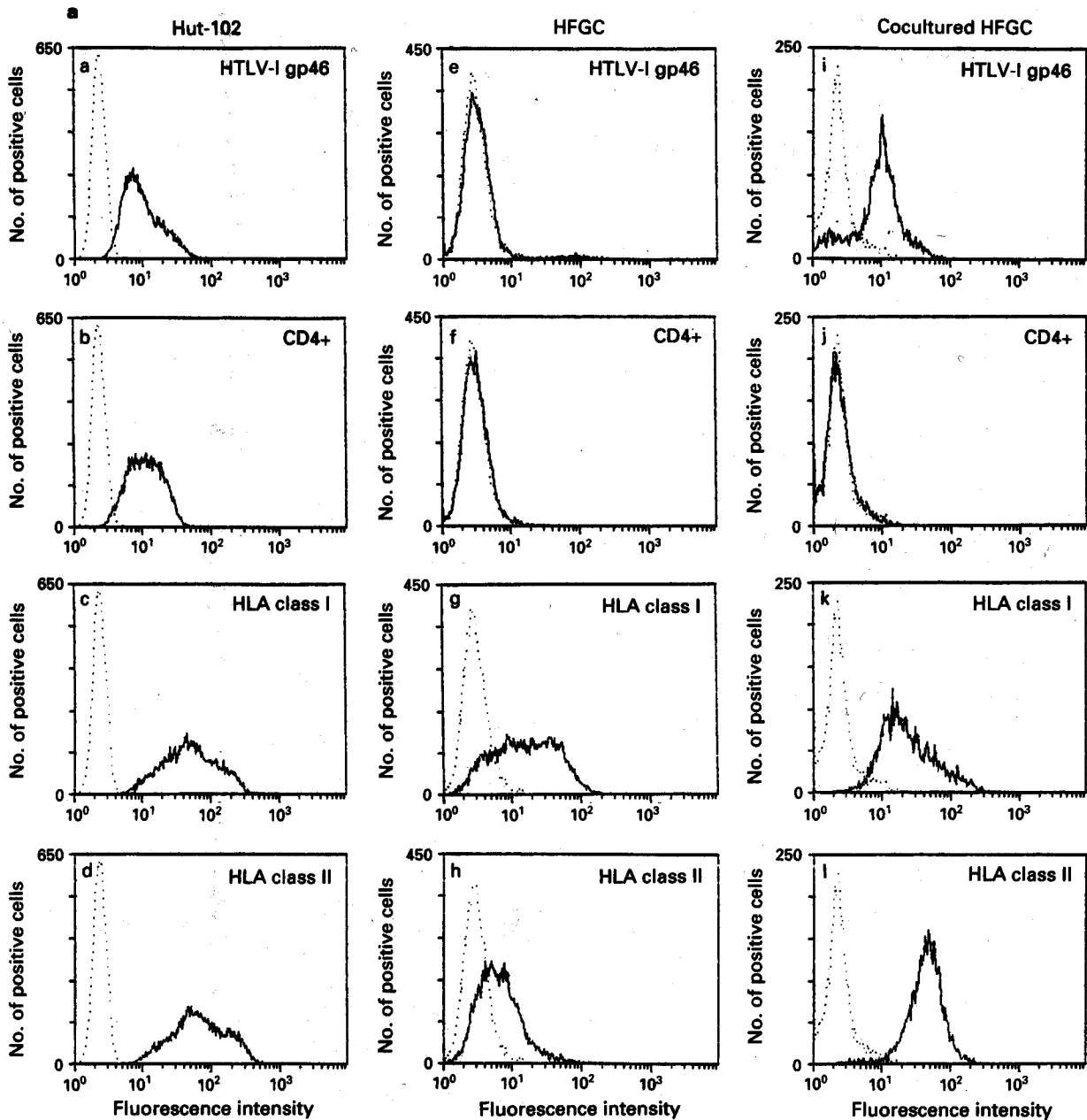
## Results

### Cell morphology

HCN-1a and HFGC cell lines are adherent cells that grow thick dendritic processes. This is evident in the HCN-1a cells (Figure 1a), which are purely neuronal in composition, compared to the HFGC, which has a combination of neurons and fibrocytic appearing astrocytes (not shown). Unirradiated Hut-102, an HTLV-I infected long-term T-cell line, and HCN-1a cells were cocultured for 7 days. Syncytia formation was observed between the adherent HCN-1a cells and the nonadherent Hut-102 cells (Figure 1b). Furthermore, the dendritic processes thinned and retracted in the course of 1 week of coculturing (open arrows, Figure 1b).



**Figure 1** Morphologic features of HTLV-I infected HCN-1a. (a) HCN-1a cells, uninfected, adherent to plastic culture flask. Magnification 300x. (b) HCN-1a cells (star) cocultured with irradiated Hut-102 cells (arrow). Hut-102 cells (arrow) form syncytia with adherent neuronal cells (star). Thinning of dendritic processes (open arrow) is also noted on the cocultured neuronal cell. Magnification 300x.

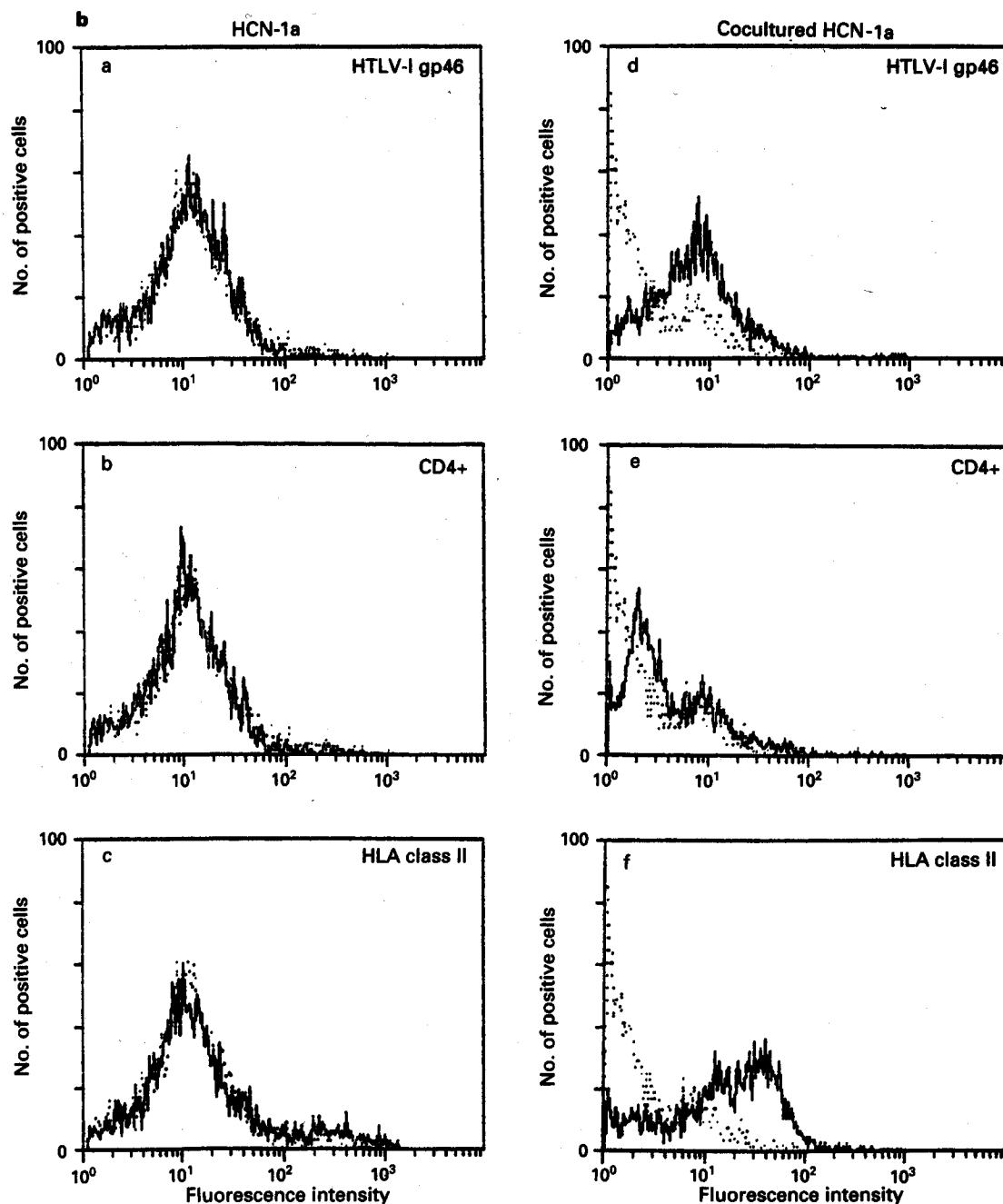


**Figure 2A** Surface antigen expression on cocultured HFGC cells. Flow cytometry was performed on Hut-102 cells, non-cocultured and HTLV-I infected HFGC cells. Primary antibodies are HTLV-I gp46, CD4 (Leu3a), HLA class I (W6/32), and HLA class II (L234). The data are plotted as relative fluorescence intensity (x axis) versus number of positive cells (y axis). Hut-102 cells (a,b,c,d) ( $10^4$  cells counted); non-cocultured HFGC cells (e,g,f,h) ( $10^4$  cells counted), cocultured HFGC cells (i,j,k,l) ( $5 \times 10^3$  cells counted). The mouse IgG control is represented by a dotted line and the test antibody, HTLV-I gp46, CD4, HLA class I, or HLA class II was represented by a solid line.

#### *Expression of HTLV-I surface antigens on HTLV-I infected neuronal cells*

The expression of HTLV-I surface antigens on cocultured HFGC cells was compared to the non-cocultured HFGC and Hut-102 cells by examining the presence of HTLV-I gp46 env protein using flow cytometry. CD4+ expression was determined to confirm complete removal of Hut-102 cells from these

cocultures. Hut-102 cells expressed both HTLV-I gp46 and CD4 (Figure 2A a,b). In contrast, HFGC cells alone did not express HTLV-I gp46 or CD4 (Figure 2A e,f). HFGC cells infected with HTLV-I by cocultivation with HUT 102 cells for 7 days expressed HTLV-I gp46 (Figure 2A i). The absence of CD4+ cells in this HTLV-I infected HFGC coculture (Figure 2A j) indicated that expression of



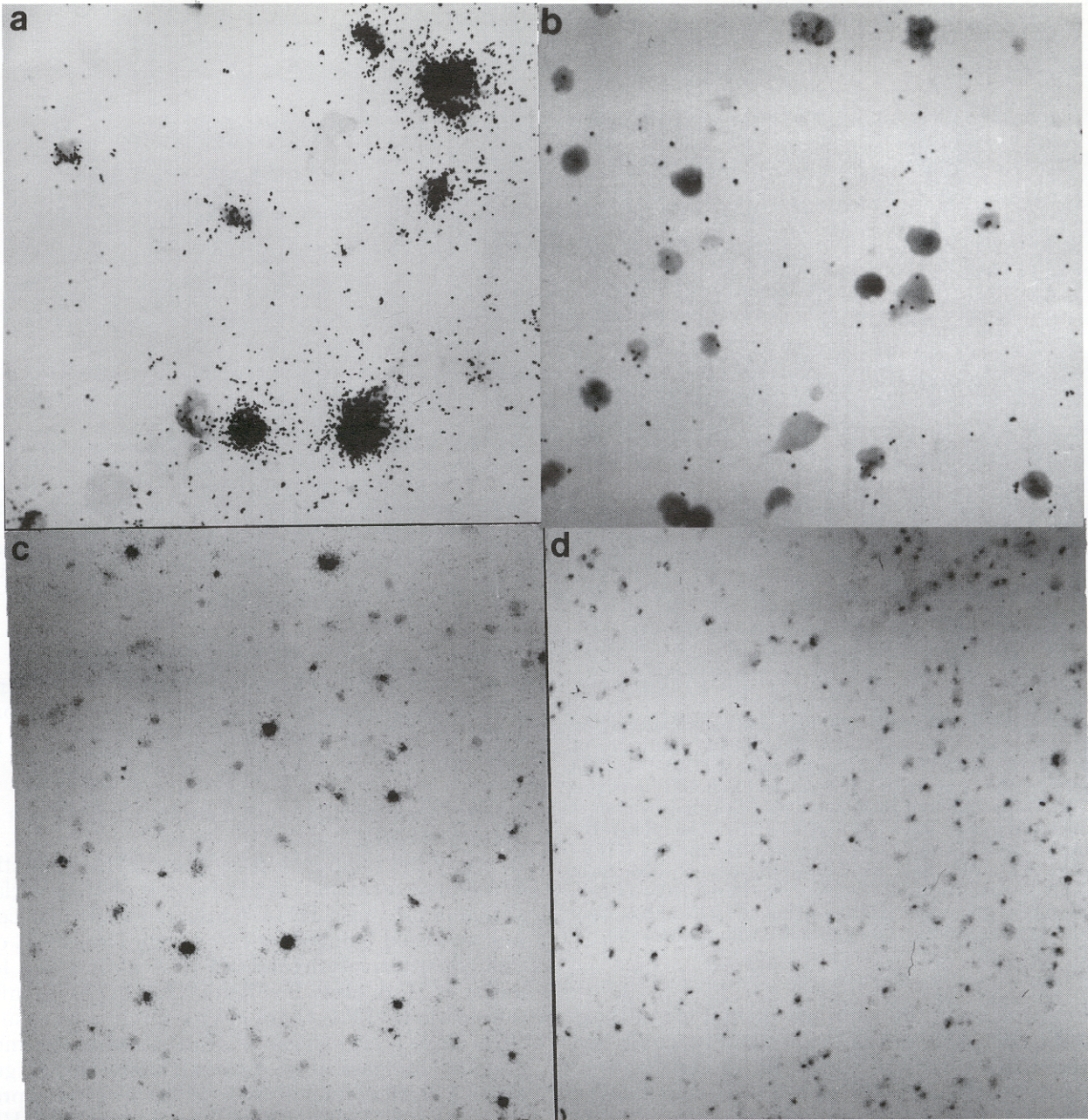
**Figure 2B** Surface antigen expression on cocultured HCN-1a cells. Flow cytometry was performed on Hut-102 cells, non-cocultured and HTLV-I infected HCN-1a cells. Primary antibodies are HTLV-I gp46, CD4 (Leu3a), HLA class I (W6/32), and HLA class II (L234). The data are plotted as relative fluorescence intensity (x axis) versus number of positive cells (y axis). Non-cocultured HCN-1a cells (a,b,c) ( $3 \times 10^3$  cells counted), cocultured HCN-1a cells (d,e,f) ( $2 \times 10^3$  cells counted). The mouse IgG control is represented by a dotted line and the test antibody, HTLV-I gp46, CD4, HLA class I, or HLA class II is represented by a solid line.

HTLV-I gp46 (Figure 2A i) was not due to residual Hut 102 cells. In parallel, HCN-1a, which do not express HTLV-I gp46 or CD4 (Figure 2B a,b), could also be infected with HTLV-I. HCN-1a cells, cocultured with irradiated Hut-102 cells (5:1 ratio, Hut-102: HCN-1a) for 7 days, demonstrated the presence of HTLV-I gp46+ and CD4- cells (Figure 2B d,e).

#### *Detection of HTLV-I RNA in cocultured neuronal cell*

Using an HTLV-I  $^{35}$ S RNA probe to the HTLV-I pX region, cocultured HFGC and HCN-1a cell lines were examined for the presence of HTLV-I mRNA. *In situ* hybridization demonstrated that HTLV-I RNA was detected in the cocultured HFGC and



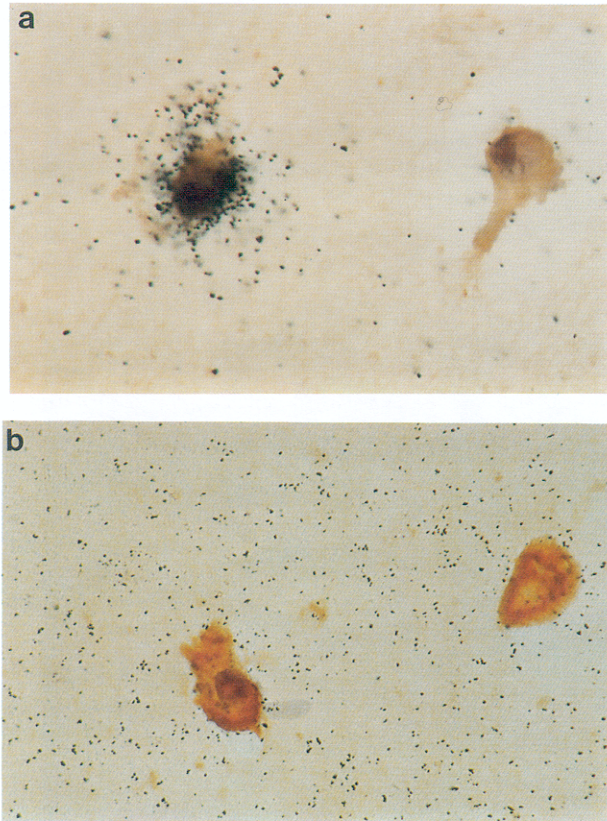


**Figure 3** HTLV-I tax RNA *in situ* hybridization of HFGC cells. Aggregations of silver grains (black dots) overlying cells indicate the presence of HTLV-I tax RNA, using the HTLV-I antisense riboprobe. (a) HTLV-I infected HFGC with HTLV-I antisense riboprobe, demonstrating the presence of cells containing HTLV-I RNA. Light field, 400× magnification. (b) Non-cocultured HFGC cells, with HTLV-I antisense riboprobe, demonstrating the absence of HTLV-I infected cells. Light field, 400× magnification. (c) HTLV-I infected HFGC with HTLV-I antisense riboprobe, showing a larger field of infected cells. Light field, 100× magnification. (d) HTLV-I infected HFGC with HTLV-I sense riboprobe, demonstrating the absence of signal using the probe in the opposite orientation. Light field, 100× magnification. Cells counterstained with hematoxylin and eosin.

HCN-1a cells (Figure 3a, c and 4a, respectively) but not in the non-cultured HFGC and HCN-1a cells (Figure 3b and 4b, respectively). Hut-102 cells, the positive control, contained HTLV-I RNA while non-infected PBL, the negative control, did not contain any positive signals for HTLV-I RNA (data not

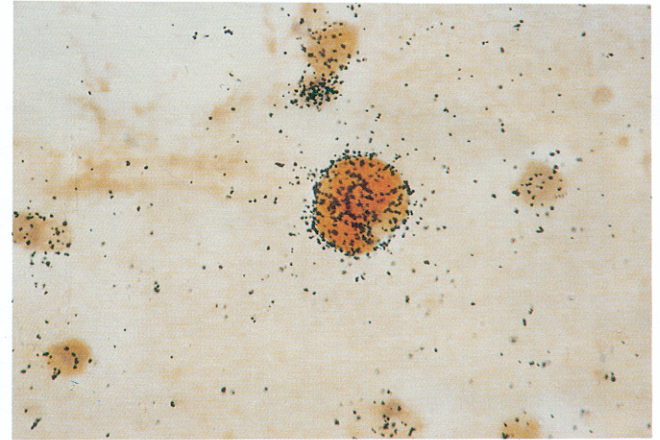
shown). No reactivity was seen in any sample using the HTLV-I <sup>35</sup>S RNA *pX* probe in a sense orientation (Figure 3d) indicating that the positive signals observed in HTLV-I infected HFGC cells (Figure 3a,c) or HTLV-I infected HCN-1a cells (Figure 4a) were specific for HTLV-I tax RNA.





**Figure 4** Colocalization of HTLV-I RNA and neurofilament protein. The presence of neurofilament-160 protein is indicated by brown pigmentation of the cell. The presence of HTLV-I tax RNA indicated by the presence of silver grains (black dots) overlying cells. (a) Two cocultured HCN-1a cells, with colocalization of neurofilament protein (brown) and HTLV-I RNA (black dots). The cell in the left field contains both neurofilament and HTLV-I RNA and cell in right field is only neurofilament positive. Light field, 1000 $\times$  magnification. (b) Non-cocultured HCN-1a cell, which contains neurofilament (brown) but no HTLV-I RNA. Light field, 1000 $\times$  magnification. Counterstained with hematoxylin.

Confirmation of the neuronal phenotype of these HTLV-I infected cells was determined by combined immunohistochemistry and *in situ* hybridization. The HCN-1a, HFGC, and Hut-102 cells were labeled with neurofilament-160 antibody which resulted in brown pigmentation of the neuronal cells (Figure 4a,b), but not the Hut-102 cells. The negative control antibody, mouse IgG, did not result in brown pigmentation of any of the tested cells, HFGC, HCN-1a, or Hut-102 (data not shown). After immunohistochemistry, the cells were hybridized with a radioactive HTLV-I tax RNA probe to produce silver grains (black dots) overlying the HTLV-I infected cells. In this experiment, HCN-1a cells cocultured with unirradiated Hut-102 cells (same coculture as in Figure 1b) resulted in double-labeled cells for neurofilament protein and HTLV-I mRNA (Figure 4a). In Figure 4a, a neurofilament positive, HTLV-I infected cell can be seen next to a neurofilament positive, HTLV-I uninfected cell. The non-cocul-



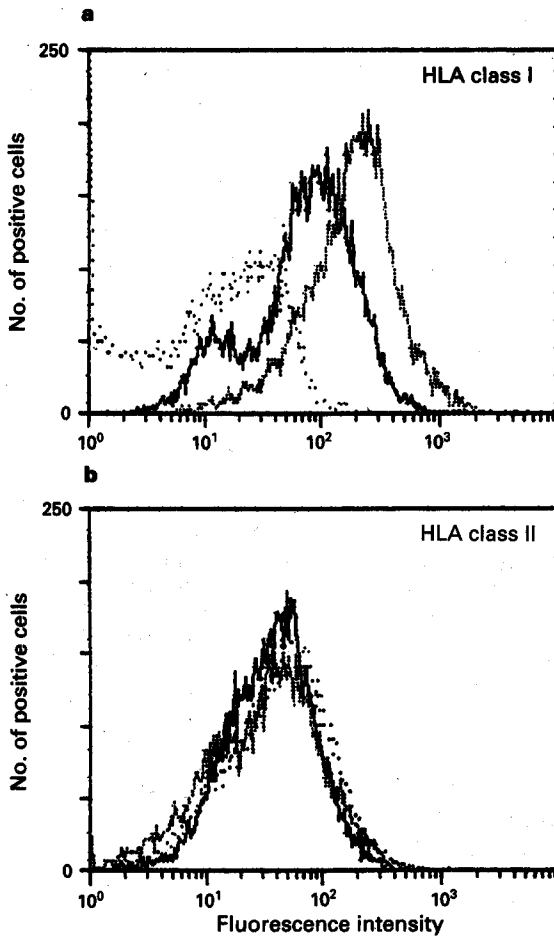
**Figure 5** Colocalization of HLA class II expression and HTLV-I RNA on cocultured HFGC cells. The presence of HLA class II (HLA DR $\alpha$ ) was indicated by the presence of brown pigmentation and the presence of HTLV-I tax RNA indicated by the presence of silver grains (black dots). In center field, a cocultured HFGC cell (arrow) indicates colocalization of HLA class II protein and HTLV-I RNA. Light field, 1000 $\times$  magnification. Counterstained with hematoxylin.

tured cells (Figure 4b) only demonstrated the presence of neurofilament while the Hut-102 cells only demonstrated the presence of HTLV-I RNA (data not shown).

#### *Upregulation of HLA class II surface antigens on cocultured cells*

To study the relationship between HTLV-I infection and HLA expression, the induction of HLA surface antigens on these HTLV-I infected neuronal cells was analyzed using flow cytometry. The HFGC cell line constitutively expressed HLA class I and low levels of HLA class II (Figure 2A g,h). After infection with HTLV-I, there was a slight increase in HLA class I expression (Figure 2A k) and a more dramatic increase in HLA class II expression (Figure 2A l). The HCN-1a cell line, which did not express HLA class II (Figure 2B c), also upregulated HLA class II after infection with HTLV-I (Figure 2B f).

In addition to examining HLA surface antigen expression by flow cytometry, immunohistochemistry for HLA class II was combined with HTLV-I *in situ* hybridization to confirm that HLA class II expression was occurring in HTLV-I infected cells (Figure 5). The HTLV-I infected HFGC cells were labeled with HLA-DR $\alpha$  antibody that resulted in brown pigmentation of positive cells. Immunohistochemistry combined with *in situ* hybridization for HTLV-I tax RNA demonstrated that many of the HTLV-I infected cells were HLA-DR $\alpha$  positive (black dots over brown cells) (Figure 5). Similar results were seen with cocultured HCN-1A cells (data not shown).



**Figure 6** Effect of cytokines on HLA expression. Surface antigen expression was examined with flow cytometry for the expression of (a) HLA class I (W6/32), and (b) HLA class II (L2.34). The data are plotted as fluorescence intensity (x axis) versus number of positive cells (y axis),  $10^4$  cells counted per condition. HFGC cells, alone (large dots), incubated with TNF- $\alpha$  (100  $\mu$  ml) (small dotted lines), or IFN- $\gamma$  (100  $\mu$  ml) (solid line) were studied for surface antigen expression after 3 days of culture.

#### Effect of cytokines

An important question raised by the HLA upregulation in the HTLV-I infected cells is whether cytokines present in the culture milieu induced HLA class II upregulation. Therefore, HFGC cells were cultured for 3 days with IFN- $\gamma$  (100 u ml $^{-1}$ ) or TNF- $\alpha$  (100 u ml $^{-1}$ ), two cytokines known to be produced by Hut 102 cells. The expression of HLA class I (Figure 6a) and HLA class II (Figure 6b) on these cytokine treated HFGC cells was compared to the expression of HLA class I and class II on an HTLV-I infected HFGC line. HLA class I expression was increased in the presence of cytokines (Figure 6a). In contrast, HLA class II expression was not increased in IFN- $\gamma$  and TNF- $\alpha$  treated HFGC cultures (Figure 6b), although the HTLV-I infected HFGC line upregulated HLA class II comparable to

Figure 2A 1. Similar experiments culturing HFGC and HCN cells with Hut-102 supernatant did not result in the induction of HLA class II (data not shown). These results demonstrate further that induction of HLA class II in these HTLV-I infected neuronal cell lines is associated with HTLV-I infection and not an effect of cytokines in the coculturing inoculum.

#### Discussion

These experiments demonstrate that HFGC and HCN-1a, neuronal cell lines of non-tumor origin, are susceptible to HTLV-I infection. Syncytial formation was observed between adherent neuronal cells and the smaller non-adherent Hut-102 cells (Figure 1). Flow cytometry and *in situ* hybridization confirmed the expression of HTLV-I surface antigens (Figure 2) and the presence of HTLV-I RNA (Figures 3 and 4) in these HTLV-I infected neuronal cells. The phenotype of the infected cells in the cocultures was pure neuronal in the HCN-1a cell line (Figure 4) and a combination of neuronal and astrocytic in the HFGC cell line (data not shown). This information is summarized in Table 1.

HLA surface antigens were upregulated in both HFGC and HCN-1a cell lines. HLA class I expression was increased on neuronal cells in cultures containing TNF- $\alpha$  or IFN- $\gamma$  (Figure 5). However, the presence of cytokines was insufficient to induce HLA class II expression. HLA class II expression occurred only in neuronal cells infected with HTLV-I and was associated with the *in situ* presence of HTLV-I RNA and/or HTLV-I surface antigen expression (Figures 2, 5, 6). The HCN-1a cells, a pure neuronal cell line and previously described as only expressing HLA class I constitutively, are capable of HLA class II expression after infection with HTLV-I (Figure 2B). The HTLV-I infected HFGC cell line, which contains a mixture of fetal neurons and astrocytes, only demonstrated an upregulation of HLA class II expression (Figure 2A). Collectively, the data presented in this paper demonstrate that neuronal cells can be infected with HTLV-I with a resultant upregulation of HLA expression.

This is the first demonstration of HLA class II expression in non-tumorigenic neuronal cells. Consistent with previous studies (Lampson and George, 1986; Poltarak *et al*, 1992), exposure to cytokines results only in HLA class I expression in neuronal cell lines. In contrast, cytokines are capable of inducing HLA class II in microglial cells (Wucherpennig, 1994) and astrocytes (Sedgewick *et al*, 1991). HTLV-I infection of astrocytes is also associated with HLA class II expression (Hirayama *et al*, 1988; Macchi *et al*, 1992). Cytokines and HTLV-I infection may have different potentials for inducing HLA class II in CNS cells. Current data demonstrate that only HTLV-I infection of non-tumorigenic neuronal cells and neuroblastoma cells



(Lehty *et al*, 1994) induces HLA class II expression. The mechanism by which HTLV-I infection modulates HLA class II expression has not been elucidated. Preliminary data with neuroblastoma cells indicates that HLA upregulation may be HTLV-I tax-mediated (EP Cowan personal communication). HTLV-I tax, a transcriptional *trans* activator within the pX region of HTLV-I, is involved in the activation of viral (Sodroski *et al*, 1984; Fujisawa *et al*, 1986; Seeler *et al*, 1993) and cellular gene expression including immediate early genes (Fujii *et al*, 1988), cytokine genes (Ballard *et al*, 1988), and the IL2R gene (Inoue *et al*, 1986; Reuben *et al*, 1988). HTLV-I tax has also been associated with the induction of HLA class I in glial cells (Sawada *et al*, 1990). To date, HLA class II induction by HTLV-I tax has not been demonstrated directly. Furthermore, culturing neuronal cells in Hut-102 supernatant did not induce HLA class II expression indicating that the presence of soluble viral antigens, including soluble HTLV-I tax protein (Marriott *et al*, 1991) may be insufficient to affect regulation of HLA class II expression.

HLA expression in neuronal cells may have important implications in disorders which may be immunopathologically mediated (Wucherpfennig, 1994). HLA surface antigens are not typically expressed within the CNS (Mauerhoff *et al*, 1988; Sethna and Lampson, 1991). However, certain disease states including HAM/TSP (Umehara *et al*, 1993; Wu *et al*, 1993) and multiple sclerosis (Hayes *et al*, 1987) can lead to an increased expression of HLA surface antigens in CNS tissue. HLA class II expression in HAM/TSP spinal cords are present in cells within parenchymal lesions and in the perivascular infiltrates (Umehara *et al*, 1993). As expected most of these HLA class II positive cells are microglial (Umehara *et al*, 1993; Wu *et al*, 1993), though it is possible that other types of CNS cells may also be expressing HLA class II similar to findings in multiple sclerosis plaques (Hayes *et al*,

1987). HLA class II is an important component of the tri-molecular complex whereby T-cells, through their antigen-specific T-cell receptors, recognize foreign antigens in association with an appropriate HLA molecule. Expression of HLA class II on neuronal cells could allow these cells to act as antigen-presenting cells to stimulate inflammatory lymphocytes or alternatively, may be recognized by activated T-lymphocytes to become targets for immune-mediated cytokine and CTL activity.

In summary, this study demonstrates that HLA class II expression can be upregulated in HTLV-I infected neuronal cells of non-tumorigenic origin. The induction of HLA class II is dependent on HTLV-I infection though the specific mechanism is unknown. Further studies are needed to determine if HTLV-I tax is involved in HLA upregulation and whether other transcriptional factors (Kelly *et al*, 1992) may also be upregulated in response to HTLV-I infection of neuronal cells. Despite the lack of specific evidence for HTLV-I infection of neuronal cells in HAM/TSP, the significant degeneration of the corticospinal tract coupled with the mild degree of anterior horn neuronal cell loss (Piccardo *et al*, 1988; Moore *et al*, 1989; Iwasaki, 1990; Wu *et al*, 1993) observed in autopsy specimens indicate that direct neuronal involvement may be operative in this disease. Ultimately, it will be important to correlate the interaction between HTLV-I infection and HLA upregulation found in our *in vitro* system with findings in the HAM/TSP CNS.

## Materials and methods

### Cell lines

Two neuronal cell lines were used in these experiments. The first, HFGC, was a human fetal neuroglial cell line (a gift of Gene Major, NINDS, Bethesda, MD) derived from 10 week fetal tissue. Morphologic and immunohistochemical analysis of HFGC cell line demonstrated that it contains a mix-

Table 1 Characteristics of neuronal cell lines

Cell line	Source	Phenotype	HTLV-I		Cell surface antigens		
			gp46 <sup>a</sup>	RNA(ISH) <sup>a</sup>	HLA class I <sup>b</sup>	HLA class II <sup>b</sup>	CD4 <sup>b</sup>
HFGC	Fetal	NF+ GFAP+	-	-	++	+	-
HFGC (HTLV-I infected)	Fetal	NF+ GFAP+	+	+	++	++	-
HCN-1a	Adult cortex	NF+ GFAP-	-	-	+	-	-
HCN-1a (HTLV-I infected)	Adult cortex	NF+ GFAP-	+	+	+	+	-

HFGC = human fetal cell glial line; HCN = human cortical neuronal cell line; ISH = in situ hybridization; NF = neurofilament; GFAP = glial fibrillary acidic protein

<sup>a</sup> (-) absent, (+) present

<sup>b</sup> (-) absent expression, (+) low expression, (++) high expression

ture of neuronal and astrocytic cells. The second neuronal cell line, HCN-1a (ATCC CRL-10442; American Type Culture Collection, Rockville, MD) has been characterized previously as pure neuronal cell lines from an adult cortical source (Ronnelt *et al*, 1990; Poltorak *et al*, 1992; Truckenmiller *et al*, 1993). The immunohistochemical characteristics of the cell lines are summarized in Table 1. The neuronal cell lines were maintained in DMEM medium (Bio-Whittaker, Walkersville, MD) with 10% fetal calf serum, 1% glutamine, and 0.1% gentamicin. The HTLV-I infected cell line was HUT-102, a human CD4+ T-cell line (Poesz *et al*, 1980). The cells were maintained in a humidified chamber at 37°C, 5% CO<sub>2</sub>.

#### Coculture protocol

Neuronal cells were cocultured with HUT-102 cells according to previously described methods (Lehky *et al*, 1994). The HFGC cell line was cocultured at a 1:1 ratio with unirradiated Hut-102 cells. After 7 days of coculture, the cocultured cells were washed with phosphate buffered saline (PBS) to remove non-adherent cells. The remaining adherent cells were removed by adding 0.025% trypsin, incubating 5 min at room temperature, and then washing with media containing 10% FCS. In addition to removing non-adherent cells, Hut-102 cells were separated from the HFGC cells by use of anti-CD4 coated magnetic beads (Dynal, Oslo, Norway). The anti-CD4 coated magnetic beads were used at a ratio of 10:1 of beads to total cell count. The Hut-102 cells bound to anti CD4+ magnetic beads were removed by use of a strong magnet. This procedure was repeated to ensure complete removal of the HUT-102 cells prior to analysis by flow cytometry or *in situ* hybridization.

The HCN-1a cells have extremely slow growth characteristics with a doubling time of over 120 h, resulting in a relative paucity of cells. Initially, the HCN-1a cells were cocultured with unirradiated Hut-102 cells and treated with anti-CD4+ immunomagnetic separation to remove the Hut-102 cells prior to analysis. Since the cell yield from this procedure was very low, these cells were prepared into a thrombin clot for *in situ* hybridization and immunohistochemistry. In order to improve the cell yield in subsequent experiments, the coculture procedure was modified to maintain reasonable efficacy of infection with minimal cell loss. The ratio of 5:1 (irradiated Hut-102 cells: HCN) was considered to be the optimal ratio for infection of neuronal cells, paralleling earlier studies with neuroblastoma cells which demonstrated that irradiated Hut-102 cells result in a lower efficacy of HTLV-I infection than the use of unirradiated Hut-102 cells (unpublished data, Lehky). In these cocultures, the adherent cells were washed multiple times with PBS to remove the remaining irradiated Hut-102 cellular debris after 5–7 days of coculture.

#### Flow cytometry

Cell surface antigen expression was analyzed by flow cytometry (FACScan, Becton-Dickinson, Mountain View, CA) as described previously (Cowan *et al*, 1991). The number of cells analyzed by flow cytometry was 10<sup>4</sup> except when specifically noted that a lesser number of cells were analyzed because of low cell yields. The primary antibodies were: control mouse IgG (Becton-Dickinson, Synnysvale, CA) used at a dilution of 1:10; anti-HTLV-I gp46 (monoclonal antibody, Cellular Products Inc., Buffalo, NY) used at a dilution of 1:100; anti-HLA class I, W6/32 (ATCC HB95, anti-HLA-A,B,C; American Type Culture Collection, Rockville, MD) used at a dilution of 1:10; anti-HLA-DR, L243 (ATCC HB95, anti-human Ia; American Type Tissue Collection) used at a dilution of 1:10; and anti-CD4 (Becton-Dickinson) used at a dilution of 1:10. The secondary antibody was goat anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC), (dilution 1:300) (Cappel, West Chester, PA).

#### In situ hybridization

The protocol for *in situ* hybridization was described previously in detail (Fox and Cottler-Fox, 1993; Lehky *et al*, 1994). The cells used for *in situ* hybridization were prepared in a thrombin clot (Cottler-Fox and Fox, 1991) with 0.25 ml A+ plasma and 0.1 ml thrombin, fixed in 4% paraformaldehyde, embedded into paraffin blocks, and placed on sialinized slides (American Histolabs, Gaithersburg, MD). The sense and antisense <sup>35</sup>S RNA probes were derived from a 1.8 kb segment with the pX region of the HTLV-I genome (Semmes and Jeang, 1992) (Loftstrand Labs Ltd., Gaithersburg, MD; permission of Scott Koenig, Medimmune, Gaithersburg, MD). This riboprobe was prepared into a plasmid vector and transcribed in the antisense or sense configuration with a T<sub>7</sub> or Sp<sub>6</sub> RNA polymerase, respectively. The specific activity of the <sup>35</sup>S-labeled riboprobes is 2 × 10<sup>6</sup> dpm μl<sup>-1</sup>. Hybridization was performed in 50% formamide with dextran sulfate at 45°C, overnight. After several high stringency washes, the slides were dipped in NBT-3 and incubated for 4–6 days in a light-tight box before being developed. The cells were counterstained with hematoxylin and eosin.

#### Immunohistochemistry

Thrombin clots of cells were prepared as described above, embedded in paraffin, and placed on sialinized slides. Immunohistochemistry was performed prior to *in situ* hybridization (Shivers *et al*, 1986). The specimens were deparaffinized and incubated with 10% fetal calf serum/0.2% triton in PBS for 1 h at room temperature. They were incubated with a primary antibody at 4°C overnight in a humidified chamber. This was followed by incubation with a biotin-conjugated secondary antibody (4°C,

overnight), and avidin-biotin complex (room temperature, 2.0 hours) (Vectastain, Burlingame, CA). Biotin-labeled antibodies were detected with diaminobenzidine (Sigma, St Louis, MO). The primary antibodies were: anti-neurofilament-160 (Clone BF10, Boehringer-Mannheim) used at a dilution of 1:5; anti-DR $\alpha$  (monoclonal HLA class II determinant, Dakopatts) used at a dilution of 1:20; and mouse IgG (Ex alpha, Boston, MA) used at a dilution of 1:10. The secondary antibody was biotin-conjugated goat anti-mouse (Vectastain), used at a dilution of 1:200. The cell were counterstained with hematoxylin. Washes between each step were done with PBS containing 0.018% diethylpyrocarbonate (DEPC, Sigma), a ribonuclease inhibitor. The antibody preparations contained 280  $\mu$  ml<sup>-1</sup> of RNase inhibitor (from human placenta, Boehringer-Mannheim), and were diluted in DEPC-treated PBS.

## References

- Ballard DW, Bohnlein DW, Lowenthal JW, Wano Y, Franza RB, Green WC (1988). HTLV-I tax induces cellular proteins that activate the kb element in the IL-2 receptor-alpha gene. *Science* **241**: 1652-1655.
- Bhigjee AI, Wiley CA, Wachsmann W, Amenomori T, Pirie D, Bill PL, Windsor J (1991). HTLV-I associated myelopathy: clinicopathologic correlation with localization of provirus to the spinal cord. *Neurology* **41**: 1990-1992.
- Cottler-Fox M, Fox CH (1991). Examining cells for infectious agents: a novel approach. *J Infect Dis* **164**: 1239-1240.
- Cowan EP, Pierce ML, Dhib-Jalbut S (1991). Interleukin 1b decreases HLA class II expression on a glioblastoma multiforme cell line. *J Neuroimmunol* **33**: 17-28.
- Elovaara I, Koenig S, Brewah AY, Woods RM, Lehty T, Jacobson S (1993). High human T-cell lymphotropic virus type I (HTLV-I) specific precursor cytotoxic T-lymphocyte frequencies in patients with HTLV-I associated neurologic disease. *J Exp Med* **177**: 1567-1573.
- Fox CH, Cottler-Fox M (1993). *In situ* hybridization for detection of HIV RNA. In: Coligan JE, AM Kruisbeck, DH Margulies, EM Shevach, W Strober (eds). *Current Protocols in Immunology, Supplement 6*. Greene Publishing Association & Wiley Interscience: New York, Unit 12.8: 1-21.
- Fujii M, Sassone-Corsi P, Verma IM (1988). C-fos promoter trans-activation by the Tax1 protein of human T-cell leukemia virus type I. *Proc Natl Acad Sci USA* **85**: 8526-8530.
- Fujisawa J, Seiki M, Sato M, Yoshida M (1986). A transcriptional enhancer sequence of HTLV-I is responsible for trans-activation mediated by p40 in HTLV-I. *EMBO J* **5**: 713-718.
- Gessain A, Barin F, Vernant JC, Gout O, Maurs L, Calender A, De The G (1985). Antibodies to human T-lymphotropic virus type-I in patients with tropical spastic paraparesis. *Lancet* **2**: 407-410.
- Hara H, Morita M, Iwaki T, Itoyama Y, Kitamoto T, Akizuki S, Goto I, Wantanabe T (1994). Detection of human T-lymphotropic virus type I (HTLV-I) proviral DNA and analysis of T-cell receptor V-beta CDR3 sequences in spinal cord lesions of HTLV-I associated myelopathy/tropical spastic paraparesis. *J Exp Med* **180**: 831-839.
- Hayes GM, Woodroffe G, Cuzner ML (1987). Microglia are the major cell type expressing MHC class II in human white matter. *J Neurol Sci* **80**: 25-37.
- Hirayama M, Miyadai T, Yokochi T, Sato K, Kubota T, Oida O, Fujika N (1988). Infection of human T-lymphotropic virus I to astrocytes *in vitro* induction of the class II histocompatibility complex. *Neurosci Lett* **93**: 34-39.
- Inoue J, Seiki M, Taniguchi T, Tsuru S, Yoshida M (1986). Induction of interleukin 2 receptor gene expression by p40x encoded by human T-cell leukemia virus type 1. *EMBO J* **5**: 2883-2888.
- Itoyama Y, Minato S, Kiri J, Goto I, Sato H, Okochi K, Yamamoto N (1988a). Altered subsets of peripheral blood lymphocytes in patients with HTLV-I associated myelopathy (HAM). *Neurology* **38**: 816-818.
- Itoyama Y, Minato S, Kira J, Goto I, Sato H, Okochi K, Yamamoto N (1988b). Spontaneous proliferation of peripheral blood lymphocytes increased in patients with HTLV-I associated myelopathy. *Neurology* **28**: 1302-1307.
- Iwasaki I (1990). Pathology of chronic myelopathy associated with HTLV-I infection (HAM/TSP). *J Neurol Sci* **96**: 103-123.
- Jacobson S, Zaninovic V, Mora C, Rodgers-Johnson P, Sheremata WA, Gibbs CL, Gadjusek DC, McFarlin DE (1988). Immunological findings in neurological diseases associated with antibodies to HTLV-I: Activated lymphocytes in tropical spastic paraparesis. *Ann Neurol* **23** (suppl): S196-200.
- Jacobson S, Shida H, McFarlin DE, Fauci AS, Koenig S (1990). Circulating CD8+ cytotoxic T-lymphocytes specific for HTLV-I pX in patients with HTLV-I associated neurological disease. *Nature* **348**: 245-248.
- Jacobson S, McFarlin DE, Robinson S, Voshkuhl R, Martin R, Brewah A, Newell AJ, Koenig S (1992). HTLV-I specific cytotoxic T-lymphocytes in the cerebrospinal fluid of patients with HTLV-I associated neurological

- disease. *Ann Neurol* 32: 651-657.
- Kaplan JE, Osame M, Kubota H, Igata A, Nishitani H, Maida Y, Khabbaz RE, Janssen RS (1990). The risk of development of HTLV-I associated myelopathy/tropical spastic paraparesis. *J Acquir Immune Defic Syndr* 3: 1096-1101.
- Kelly K, Davis P, Mitsuya H, Irving S, Wright J, Grassmann R, Fleckenstein B, Wano Y, Greene W, Siebenlist U (1992). A high proportion of early response genes are constitutively activated in T cells by HTLV-I. *Oncogene* 7: 1463-1470.
- Kira J, Itoyama Y, Kovaniagi Y, Tateishi J, Kishikawa M, Akisuki S, Kobavashi I, Toki N, Sueshi K, Sato H, Sakakai N, Yamamoto N, Goto I (1992). Presence of HTLV-I proviral DNA in central nervous system of patients with HTLV-I associated myelopathy. *Ann Neurol* 31: 39-45.
- Kubota R, Fujiyoshi T, Fujiyoshi T, Izumo S, Yashiki S, Maruyama I, Osame M, Sonodo S (1993). Fluctuation of HTLV-I proviral DNA in peripheral blood mononuclear cells of HTLV-I associated myelopathy. *J Neuroimmunol* 42: 147-154.
- Lampson LA, George DL (1986). Interferon-mediated induction of class I MHC products in human neuronal cell lines: analysis of HLA and b2-m RNA, and HLA-A, and HLA-B proteins and polymorphic specificities. *J Interferon Res* 6: 257-265.
- Lehky TJ, Fox CH, Koenig S, Levin MC, Flerlage N, Izumo S, Sato E, Raine CS, Osame M, Jacobson S (1995). Detection of HTLV-I (1995) Tax RNA in the CNS of HAM/TSP patients by *in situ* hybridization. *Ann Neurol* 37: 246-254.
- Lehky TJ, Cowan EP, Lampson LA, Jacobson S (1994). Induction of HLA class I and class II expression in human T-lymphotropic virus I-infected neuroblastoma cells. *J Virol* 68: 1854-1863.
- Liberski PP, Rodgers-Johnson P, Char G, Piccardo P, Gibbs CJ, Gadusek DC (1988). HTLV-I-like viral particles in spinal cord cells in Jamaican tropical spastic paraparesis. *Ann Neurol* 23 (suppl): S185-187.
- Macchi B, Caronti B, Cocchia D, Gremo F, Torelli S, Sogos V, Bonmassar E, Lauro GM (1992). Correlation between P19 presence and MHC class II expression in human fetal astroglial cells cocultured with HTLV-I donor cells. *Int J Dev Neurosci* 10: 231-241.
- Marriott SJ, Lindholm PF, Reid RL, Brady JN (1991). Soluble HTLV-I Tax1 protein stimulates proliferation of human peripheral blood lymphocytes. *New Biologist* 3: 678-686.
- Mauerhoff T, Pujo-Borrell R, Mirakian R, Bottazzo GF (1988). Differential expression and regulation of major histocompatibility complex (MHC) products in neural and glial cells of the human fetal brain. *J Neuroimmunol* 18: 271-289.
- Moore GRW, Traugott U, Scheinberg LC, Raine CS (1989). Tropical spastic paraparesis: A model of virus-induced cytotoxic T-cell mediated demyelination? *Ann Neurol* 26: 523-530.
- Ohara Y, Iwasaki Y, Izumo S, Kobayashi L, Yoshioka A (1992). Search for human T-cell leukemia virus I (HTLV-I) proviral sequences by polymerase chain reaction in the central nervous system of HTLV-I associated myelopathy. *Arch Virol* 124: 31-43.
- Osame M, Usuku K, Izumo S, Ijichi N, Amitani H, Igata A, Matsumoto M, Tara M (1985). HTLV-I associated myelopathy, a new clinical entity. *Lancet* 1: 397-398.
- Piccardo P, Ceroni M, Rodgers-Johnson P, Mora C, Asher DM, Char G, Gibbs CJ, Gadusek DC (1988). Pathological and immunological observations on tropical spastic paraparesis in patients from Jamaica. *Ann Neurol* 23 (suppl): S156-160.
- Poiesz BJ, Ruscetti FW, Gazdar AF, Bunn PA, Minna, Gallo RC (1980). Detection and isolation Type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. *Proc Natl Acad Sci USA* 77: 7415-7419.
- Poltorak M, Isono M, Freed WJ, Ronnett GV, Snyder SH (1992). Human cortical neuronal cell line (HCN-I): Further *in vitro* characterization and suitability for brain transplantation. *Cell Transp* 1: 3-15.
- Roman GC, Roman LN (1988). Tropical spastic paraparesis. A clinical study of 50 patients from Tumaco (Columbia) and review of the worldwide features of the syndrome. *J Neurol Sci* 87: 121-138.
- Reuben S, Poteat H, Tan TH, Kawakami T, Roeder R, Haseltine W, Rosen C (1988). Cellular transcription factors and regulation of IL-2 receptor gene expression by HTLV-I tax gene product. *Science* 241: 89-92.
- Ronnett GV, Hester LD, Nye JS, Connors K, Snyder SH (1990). Human cortical neuronal cell line: Establishment from a patient with unilateral megencephaly. *Science* 248: 603-605.
- Sawada M, Suzumura A, Kondo N, Maranouchi T (1990). Human T-cell leukemia virus type I trans activator induces class I major histocompatibility complex antigen expression in glial cells. *J Virol* 64: 4002-4006.
- Seeler JS, Muchardt C, Podar M, Gaynor RB (1993). Regulatory elements involved in Tax-mediated transactivation of the HTLV-I LTR. *Virology* 196: 442-450.
- Sedgewick JD, Mobner R, Shwender S, Meulen VT (1991). Major histocompatibility complex-expressing nonhematopoietic Astroglial cells prime only CD8+ T lymphocytes: Astroglial cells as perpetuators but not initiators of CD4+ T cell responses in the central nervous system. *J Exp Med* 173: 1235-1246.
- Semmes OJ, Jeang KT (1992). Mutational analysis of human T-cell leukemia virus type I Tax: regions necessary for function determined with 47 mutant proteins. *J Virol* 66: 7183-7192.
- Sethna MP, Lampson LA (1991). Immune modulation within the brain: recruitment of inflammatory cells and increased major histocompatibility antigen expression following intracerebral infection of interferon- $\gamma$ . *J Neuroimmunol* 34: 121-132.
- Shivers BD, Harlan RE, Pfaff DW, Schacter BS (1986). Combination of immunocytochemistry and *in situ* hybridization in the same tissue section of rat pituitary. *J Histochem Cytochem* 34: 39-43.
- Sodroski JG, Rosen CA, Haseltine WA (1984). Trans-acting transcriptional activation of the long terminal repeat of human T lymphocytes viruses in infected cells. *Science* 226: 177-179.
- Truckenmiller ME, Kulaga H, Coggiano M, Wyatt R, Snyder SH, Sweetnam PM (1993). Human cortical neuronal cell line: A model for HIV-I infection in an immature neuronal system. *AIDS Res Hum Retrovirus* 9: 445-453.
- Umehara F, Izumo S, Nakagawa M, Ronquillo AT, Takahashi K, Matsumuro K, Sato E, Osame M (1993). Immunocytochemical analysis of the cellular infiltrates in the spinal cord lesions in HTLV-I associated



- myelopathy. *J Neuropathol Exp Neurol* **52**: 424–430.
- Umehara F, Izumo S, Ronquillo AT, Matsumuro K, Sato E, Osame M (1994). Cytokine expression in the spinal cord lesions in HTLV-I associated myelopathy. *J Neuropathol Exp Neurol* **53**: 72–77.
- Wu E, Dickson DW, Jacobson S, Raine CS (1993). Neuroaxonal dystrophy in tropical spastic paraparesis/HTLV-I associated myelopathy. *Acta Neuropathol* **86**: 224–235.
- Wucherpfennig K (1994). Autoimmunity in the central nervous system: Mechanisms of antigen presentation and recognition. *Clin Immunol Immunopathol* **72**: 293–306.
- Yoshioka A, Hirose G, Ueda Y, Nishimura Y, Sakai K (1993). Neuropathological studies of the spinal cord in early stage HTLV-I-associated myelopathy. *J Neurol Neurosurg Psychiatry* **56**: 1004–1007.