Functional Characterization of Neurotensin Receptors in Human Cutaneous T Cell Lymphoma Malignant Lymphocytes

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Cutaneous T cell lymphomas are a clonal proliferation of CD4+ T lymphocytes primarily involving the skin. Mycosis fungoides is an epidermotropic CD4+ cutaneous T cell lymphoma, and a more aggressive form, Sezary syndrome, occurs when the malignant cells become nonepidermotropic. The role of neuropeptides in the growth and chemotaxis capacity of cutaneous T cell lymphoma cells remains unknown. In this report, we found that cutaneous T cell lymphoma cells, similarly to normal resting or activated peripheral lymphocytes, were able to bind neurotensin. We used an interleukin-2-dependent cutaneous T cell lymphoma malignant T cell line derived from cutaneous T cell lymphoma lesions in order to study the role of neurotensin in the proliferation and migration of these malignant cells. First, we determined that the malignant cells expressed neurotensin receptors on their cell membrane. Functional results indicated that neurotensin did not stimulate the growth of the cell line. In contrast, this neuropeptide inhibited the proliferation of the tumor cells in response to exogenous interleukin-2. Furthermore, we found that neurotensin enhanced both spontaneous and chemoattractant-induced migration of the malignant cells. This suggests that neurotensin in skin can play a role in the disease by locally limiting the growth of the cutaneous T cell lymphoma tumor cells in response to cytokines and by enhancing their chemotaxis capacity. ***Key words: neuropeptides/neurotensin receptor antagonist/T lymphocyte migration. J Invest Dermatol 117:687–693, 2001***

It is now well established that nervous, endocrine, and immune systems constitute a totally interconnected circuit via hormones and neuropeptides (Besedovsky *et al*, 1985; Savino and Dardenne, 1995). All lymphoid organs and tissues receive innervation by fibers containing a varied spectrum of peptides including tachykinins, calcitonin gene-related peptide (CGRP), somatostatin (SOM), vasoactive intestinal peptide (VIP), and neurotensin (NT) (Felten *et al*, 1985; Misery, 1997). Recent observations suggest that these neuropeptides are able to regulate a cutaneous inflammatory process (Ansel *et al*, 1997; Lambert and Granstein, 1998). Further, it was found that cutaneous cells secreted these neuropeptides and expressed their specific receptors (Lotti *et al*, 1995; Gaudillere *et al*, 1999). Immune cells present in the skin are modulated by neuropeptides through a specific receptor (Staniek *et al*, 1995). In this context, several neuropeptides have been reported to modulate the immune responses in the peripheral system (De la Fuente *et al*, 1998; Delgado *et al*, 1999) and in the skin (Hosoi *et al*, 1993; Scholzen *et al*, 1998). NT is a tridecapeptide ELYENKPRRPYIL, which has been reported to modulate cell functions of both innate and adaptive immunity (Goldman *et al*, 1982; Koff and Dunegean, 1985; Lemaire, 1988; Garrido *et al*, 1992; De la Fuente *et al*, 1993; Evers *et al*, 1994; Lhiaubet *et al*, 1998). It was originally isolated from bovine hypothalami (Carraway and Leeman, 1973) and is distributed in the central and peripheral nervous systems and gastrointestinal tract (Carraway and Leeman, 1976; Reinecke, 1985). In the gut, NT is localized to specialized enteroendocrine cells (N cells) of the distal small bowel mucosa. NT receptor has been characterized pharmacologically and the cDNA was cloned from rat brain (Tanaka *et al*, 1990) and from the human colonic adenocarcinoma cell line HT29 (Vita *et al*, 1993). This NT receptor, however, now referred to as NT_1 receptor, which was found in various human colon cancer cell lines, is not expressed in normal colonic epithelium (Maoret *et al*, 1994). A second NT receptor, NT_2 receptor, has been isolated from human hypothalami. Interestingly, it has been found that triggering NT_2 receptor by NT does not induce biologic responses, whereas nonpeptide NT_1 receptor antagonists are potent agonists (Chalon *et al*, 1996; Vita *et al*, 1998). Both receptors belong to the G-protein-coupled receptor family. Recently, the gp95/sortilin has also been found to bind NT; however, this receptor is structurally different from the other two G-protein-coupled NT receptors, as it is similar to the mannose 6-phosphate receptor bearing a single transmembrane domain (Mazella *et al*, 1998).

In this study we investigate the role of NT in the growth and migration of cutaneous T cell lymphoma (CTCL) malignant cells. CTCL are clonal proliferations of CD4+ T lymphocytes that include mycosis fungoides and its leukemic variant, Sezary syndrome (Sterry and Mielke, 1989; Willemze *et al*, 1997). We report here that both freshly isolated CTCL malignant cells and

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Abbreviations: CGRP, calcitonin gene-related peptide; CHO, Chinese hamster ovary; NT, neurotensin; PBL, peripheral blood lymphocyte; SOM, somatostatin.

long-term cultured cytokine-dependent CTCL cell lines (Bagot *et al*, 1998; Echchakir *et al*, 2000; Poszepczynska *et al*, 2000) are binding to NT. As it is difficult to distinguish the malignant T lymphocytes from normal reactive T lymphocytes, which also bind NT in cutaneous lesions or in peripheral blood from Sezary patients, we used our unique cytokine-dependent CTCL cell lines for this study. We found that NT_1 but not NT_2 receptors were expressed by these CTCL lines. Functional results indicated that NT at low physiologic concentrations inhibited the growth of a CTCL line induced by recombinant interleukin-2 (IL-2). Surprisingly, we found that the selective nonpeptide NT antagonist of NT_1 receptor, SR48692 (Gully *et al*, 1993; Oury-Donat *et al*, 1995; Iwase *et al*, 1997), enhanced the binding of NT in CTCL and potentialized its inhibitory effect. The results suggested that these effects might be mediated by a stabilization of NT receptors at the cell surface and/or by a rapid recovery due to the stabilization of internalized NT receptors (Hermans and Maloteaux, 1998; Mazella *et al*, 1998; Morello *et al*, 2000). Interestingly, we found that NT increased both spontaneous migration and chemoattractant agent-induced migration of CTCL. In conclusion, these data demonstrated that NT binds to the functional receptor known as NT_1 receptor on CTCL cells and that it can play a role in the progression of the disease by down-modulating tumor cell proliferation and enhancing their migration.

MATERIALS AND METHODS

Patients After informed consent and approval of an ethics committee (CCPPRB, Hôpital Henri Mondor, Créteil, France), we took skin fragments and/or blood samples from six patients with a CTCL including three with Sezary syndrome. Two different patients presented with a transformed mycosis fungoides. Histologic examination of the patients showed an epidermotropic skin infiltrate composed of CD3+ CD4+ CD8– atypical lymphocytes as determined by their cytologic aspect. One patient, named Pno, had a 10 y history of generalized erythrodermia. Skin and peripheral blood contained CD3+ TCRVβ22+ CD4+ CD8+ atypical lymphocytes (Poszepczynska *et al*, 2000; 2001). Blood from patients with Sezary syndrome, which were invaded with more than 80% of CD3+ CD4+ tumor cells, were used for the phenotypic analysis. None of the patients had been previously treated with chemotherapy.

Isolation of tumoral lymphocytes Fresh CTCL tumor cells were obtained from skin fragments mechanically dispersed into single-cell suspensions, as previously described (Bagot *et al*, 1998). The mononuclear cells were then washed and frozen in human serum plus 10% dimethyl sulfoxide for later use.

Tumor cell lines We established the Pno cell line (TCRVβ22+, CD4+, CD8αα+) *in vitro* from the peripheral blood of the patient, as reported elsewhere (Poszepczynska *et al*, 2000). Briefly, mononuclear cells were isolated by the technique of Ficoll-Isopaque (Pharmacia Fine Chemicals, Piscataway, NJ) density gradient centrifugation. Cells were cultured in culture medium consisting of RPMI 1640 (Gibco, Paisley, U.K.), 2 mmol per liter L-glutamine, penicillin (100 U per ml), streptomycin (100 μg per ml), 10% heat-inactivated human serum, and IL-7 (10 ng per ml, produced by Sanofi-Synthélabo Recherche, Labège, France). Lymphocyte expansion was performed by plating cells into round-bottomed 96-well plates (Greiner, Nürtingen, Germany). The final volume in each well was 200 μl of culture medium. Every 2 d fresh culture medium was added to the wells, and the cells were collected each week. We demonstrated that both the malignant clone circulating in the patient's blood and the derived cultured T cell line were identical, as they shared the same size and sequence of the T cell receptor β (TCR-β) VDJ region and exhibited similar functional properties (Poszepczynska *et al*, 2000). The Cou-L malignant cell line (TCRVβ13+, CD4+, CD8–) is a subclone of the cell line Cou-LS, which was obtained from the skin of a patient and has been cultured *in vitro* with recombinant IL-2 for more than 3 y (Bagot *et al*, 1998; Echchakir *et al*, 2000).

Reagents Fluorescein isothiocyanate (FITC) conjugated NT (NT-FITC) was purchased from Advanced Bioconcept (Montreal, Canada). NT was obtained from Sigma (Saint Quentin Fallavier, France) and was prepared as 1 mM stock solutions. The nonpeptide NT_1 receptor antagonist SR48692 was dissolved at 1 mM in dimethylsulfoxide (Gully *et al*, 1993). Both the NT and the SR48692 were stored at –20°C in 50 μl aliquots. IL-7 (10 ng per ml) and IL-2 (50 U per ml) were produced by Sanofi-Synthélabo Recherche. The monoclonal antibody B-N6 recognizing NT_1 receptors (Ovigne *et al*, 1998) was kindly provided by Dr. J. Wijdenes from Diaclone, Besançon, France. Conjugated anti-TCR-Vβ monoclonal antibodies were purchased from Immunotech-Coulter (Marseille, France).

Cells Peripheral blood mononuclear cells from normal individuals were isolated by the technique of Ficoll-Isopaque (Pharmacia Fine Chemicals) density gradient centrifugation. Activated peripheral blood lymphocytes (PBL) were obtained by culturing cells for 3 d in 24-well tissue culture plates at a concentration of 10^5 cells per ml in RPMI 1640 medium containing 10% heat-inactivated human serum and 1 μg per ml of phytohemagglutinin (PHA; Wellcome, Beckenham, U.K.). Chinese hamster ovary (CHO) cells expressing high levels of NT receptors 1 were obtained and cultured as previously described (Vita *et al*, 1993).

Immunofluorescence staining Adherent cell lines were treated with 5 ml of ethylenediamine tetraacetic acid 0.02% per 175 cm^2 culture flask for 10 min at 37°C and washed with phosphate-buffered saline and 1% bovine serum albumin (PBS–1% BSA). Non-adherent cells were harvested by centrifugation and washed with PBS–1% BSA. 2×10^5 cells (100 μl) were incubated for 20 min at 4°C with 50 μl of 100 nM NT-FITC in PBS–1% BSA. Nonspecific NT binding was determined by incubating cells with NT-FITC in the presence of 100-fold excess of nonconjugated NT provided by the manufacturer. After washing the cells in PBS, they were analyzed by flow cytometry using a single argon flow cytometer analyzer (Epics XL, Beckman-Coulter, Miami, FL). Indirect immunofluorescence analysis with B-N6 antibodies was performed by incubating 2×10^5 cells with the specific antibody for 30 min at 4°C, subsequent washing, and incubation with FITC-conjugated affinity-purified goat antimouse Ig for an additional 30 min at 4°C. Cells were then extensively washed before flow cytometric analysis. Isotype control monoclonal antibodies were used to establish the PMT settings. For two-color analysis the immunofluorescent staining was performed by incubating 3×10^5 cells with phycoerythrin-conjugated anti-TCR-Vβ antibodies and NT-FITC for 30 min at 4°C. Flow cytometry analysis of the samples was done on gated lymphocytes.

Proliferation assays Triplicate cultures of cells (5×10^4 per well), with or without NT (10^{-6}–10^{-9} M), in a final volume of 0.2 ml in 96-well round-bottomed plates were cultured with 50 U per ml of IL-2. The proliferative responses of the Cou-L cells to various concentrations of NT and to nonpeptide NT analog SR48692 were determined by measuring the [^{3}H]-thymidine incorporation (cpm). After 60 h of culture, tritiated thymidine (1 μCi) was added to each culture well and incubation was continued for an additional 18 h. Incorporation of tritiated thymidine was measured in a microplate scintillation counter (Topcount, Packard Instrument, Meriden, CT). The percentage of inhibition was calculated using the equation 1 – cpm obtained from culture of malignant cells proliferating to IL-2 (50 U per ml) with NT (1000 nM) alone or with NT (1000 nM) + various concentrations of SR48692 divided by cpm obtained from control culture of malignant cells proliferating to IL-2 (50 U per ml).

Assay of spontaneous mobility and chemotaxis Spontaneous mobility and chemotaxis were evaluated according to a modification of the original technique described by Boyden (1962). Briefly, we used 24-well chambers with two compartments separated by a filter of 5 μm pore size (Costar, Corning, NY). Aliquots of 20 μl of cell suspensions (5×10^6 cells per ml) were deposited in the upper compartment with 80 μl of NT at final concentrations ranging from 1 nM to 1000 nM, or 80 μl of culture medium. Aliquots of 600 μl of a chemoattractant agent, N-formyl-Met-Leu-Phe (10^{-7} M), or medium were placed in the lower compartment of the chamber for chemotaxis or spontaneous mobility assays, respectively. The chambers were incubated at 37°C for 12 h. Cell migration was estimated by counting the total number of viable cells migrating in the lower compartments. The cell viability was determined using trypan blue exclusion.

Polymerase chain reaction (PCR) experiment Total RNA was extracted from different areas of human brain and from Cou-L cells (5×10^6) using the acid guanidinium isothiocyanate phenol chloroform method (RNAzol B method). cDNA was prepared by a standard method using reverse transcriptase and oligo-dT primer. PCR was performed with two hNT_1 receptor specific primers at position 2216 (backward: 5′-GGG-CCC-CCA-GCT-TGC-CAG-3′) and 1413 (forward: 5′-GGT-GAC-CAA-CGC-ACT-CTT-3′) giving a PCR product of 803 bp. For the PCR for hNT_2 receptor, the primers at position 1270 (backward: 5′-

TCA-GGT-CCG-GGT-TTC-TGG-G-3′) and 760 (forward: 5′-CTG-GCC-CTC-TGC-TCC-CAA-3′) were used and gave a product of 510 bp. The reaction cycle consisted of denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and extension for 1 min at 72°C, and was repeated 35 times.

Statistical analysis All values are expressed as the mean of cpm ± standard deviation or the mean of lymphocyte number ± standard deviation. The data were evaluated statistically using ANOVA followed by the *posthoc* Dunnett test. Differences were considered significant when p-values were ≤ 0.01.

RESULTS

Expression of the NT receptor on normal and CTCL malignant lymphocytes The ability of NT to bind normal and malignant lymphocytes was determined by flow cytometry with NT directly conjugated to FITC (NT-FITC). We tested as control whether CHO cells, which expressed high amounts of recombinant NT receptors 1 (CHO-NT_1 receptor), were able to bind NT-FITC. We found that CHO-NT_1 receptor bound specifically NT-FITC (**Fig 1*B***). Negative controls (*shaded histograms*) were obtained with an excess of unlabeled NT (according to the manufacturer's recommendations). The concentration of NT-FITC used for further flow cytometry studies was that which gave the highest specific maximum mean fluorescence intensity with CHO-NT_1 receptor and which simultaneously gave minimal autofluorescence with CHO mock cells (**Fig 1*A***). Next, we analyzed the staining of normal PBL with NT-FITC and compared the mean fluorescence intensity obtained with resting and PHA-activated PBL gated lymphocytes. The results presented in **Fig 1(*C*)** indicate that PBL were weakly stained, whereas the same cells once activated with PHA increased their specific binding to NT-FITC. Interestingly, lymphocytes isolated from a tumoral skin fragment that contained a majority of malignant T lymphocytes were able to bind NT-FITC (**Fig 1*D***). Further, we studied two long-term cultured CTCL lines for their reactivity with NT-FITC. The results revealed that both CTCL cell lines were stained with a significant mean fluorescence intensity (**Fig 1*E***, ***F***). Finally, to demonstrate that freshly isolated CTCL malignant cells were able to bind NT, we tested the blood of three different Sezary syndrome patients. We found that the tumor cells that were identified using anti-TCR-Vβ monoclonal antibodies were significantly reactive with NT-FITC (**Fig 2**).

Expression of NT_1 receptors on the CTCL cloned cell line Cou-L To further demonstrate the expression of NT receptors and to determine whether CTCL malignant cells expressed NT_1 receptors or NT_2 receptors or both, with the malignant CTCL Cou-L cell line we performed reverse transcriptase PCR analysis of NT receptor mRNA using specific oligonucleotide primers. The results presented in **Fig 3(*A*)** reveal a single PCR product of the expected size (786 bp) corresponding to NT_1 receptors. The presence of NT_1 receptors was also detected in the brain (used as control). In contrast, using the specific NT_2 receptor oligonucleotide primers the Cou-L line failed to express a PCR product of the expected size (510 bp) (data not shown). Interestingly, we further demonstrated the expression of NT_1 receptors on Cou-L cells with the anti-NT_1 receptor monoclonal antibody termed B-N6. The results presented in **Fig 3(*B*)** indicate that B-N6 monoclonal antibody stained Cou-L cells. Overall, these results clearly indicate that Cou-L expressed the NT_1 receptors but not the NT_2 receptors.

Inhibitory effect of NT on Cou-L cell proliferation We next examined the effect of NT on the IL-2-dependent malignant Cou-L cells. We found that various concentrations of NT were unable to induce the proliferation of Cou-L cells in the absence of IL-2 (data not shown). We then tested the effect of NT on IL-2-induced Cou-L cell proliferation. **Figure 4(*A*)** shows that the maximun proliferation of Cou-L T lymphocytes was reached with 50 U per ml of IL-2. Therefore, we studied the effect of various concentrations of NT on the Cou-L lymphocyte proliferation

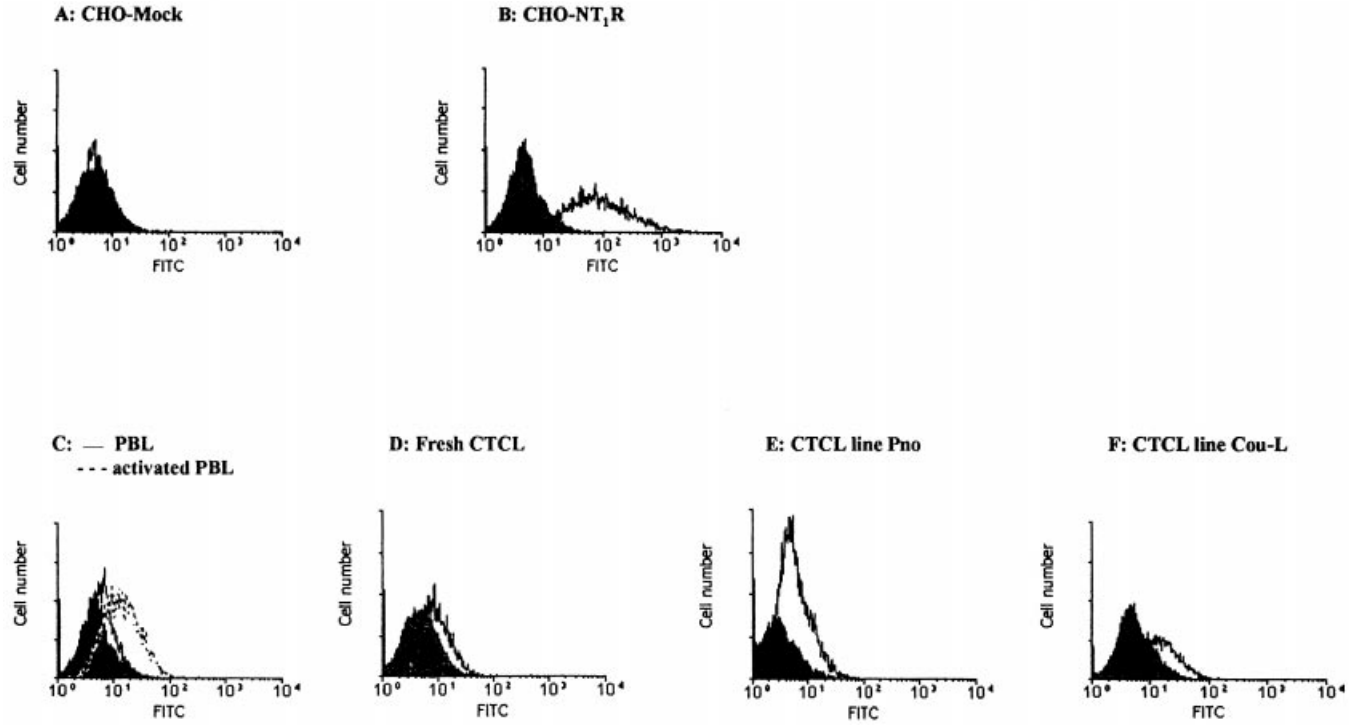

Figure 1. Flow cytometric analysis of NT-FITC binding to various cells. NT-FITC (100 nM) was incubated for 20 min at 4°C with 2×10^5 (*A*) CHO cells; (*B*) CHO-hNT_1R; (*C*) PBL and PHA-activated PBL; (*D*) CTCL cells freshly isolated from the tumoral skin fragments of patient Cou (Bagot *et al*, 1998); (*E*) CTCL malignant cell line Pno; or (*F*) CTCL malignant cell line Cou-L. Non-specific NT binding (*shaded histograms*) was determined by incubating NT-FITC with 100-fold excess nonconjugated NT. The relative cell number (*ordinate*) was plotted against the fluorescence intensity of the bound reagent (*abscissa*, *log scale*). These results are representative of six independent experiments.

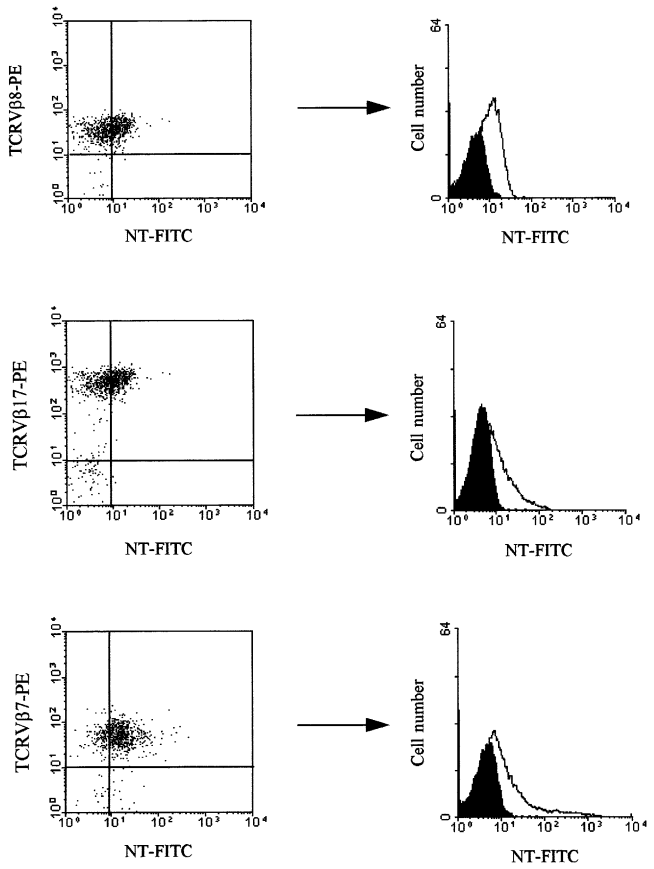

Figure 2. Expression of NT receptor on fresh circulating Sezary syndrome malignant cells. We performed two-color flow cytometric analysis using phycoerythrin-conjugated anti-TCR-Vβ antibodies and NT-FITC. Cells were incubated with both reagents for 30 min at 4°C and washed with PBS–1% BSA before flow cytometric analysis. The three different patients were selected for phenotypic analysis as their blood was invaded with a majority of malignant cells, which could be identified by specific anti-TCR-Vβ antibodies. *Right panels* correspond to single-color flow cytometric analysis using NT-FITC. Nonspecific NT binding (*shaded histograms*) was determined by incubating cells with NT-FITC in the presence of 100-fold excess nonconjugated NT.

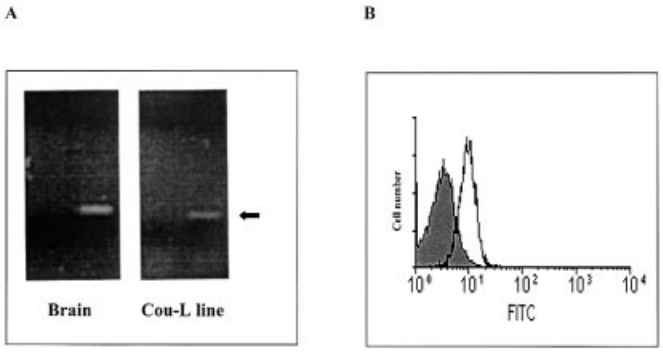

Figure 3. RT-PCR and flow cytometric analysis of the NT_1 receptor in Cou-L malignant cell line. (*A*) RNA extracted from Cou-L cells and brain (positive control) were reverse-transcribed into cDNA. Then amplification was performed using NT_1 receptor primers. The specific PCR product of the expected size of 786 bp is indicated by an arrow. (*B*) Cou-L cells were incubated with B-N6 (ascites diluted 1:100) for 30 min at 4°C and then washed with PBS–1% BSA. For indirect immunofluorescence analysis the cells were further incubated with FITC-conjugated affinity-purified goat antimouse IgM as described in *Materials and Methods*. The *shaded histogram* represents the labeling with an irrelevant control monoclonal antibody.

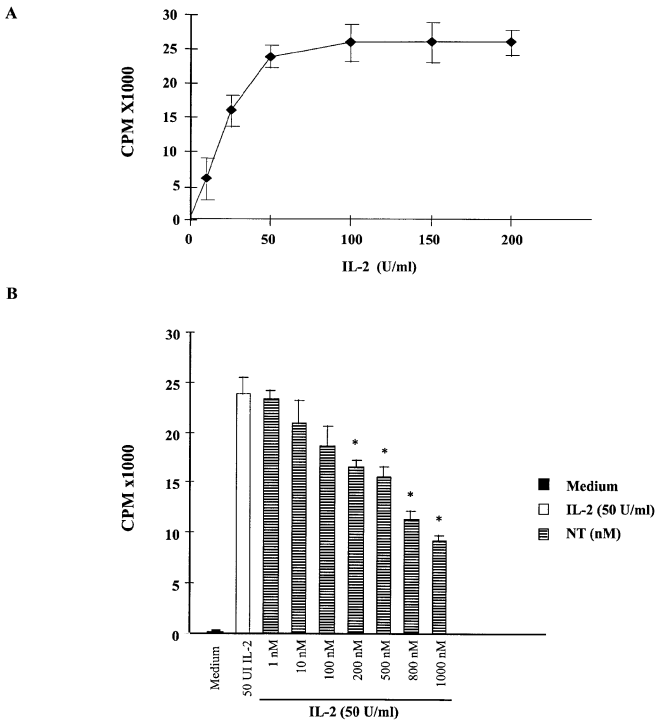

Figure 4. Modulation of IL-2-dependent Cou-L cell proliferation by NT. (*A*) Cou-L cells were cultured with various concentrations of IL-2 as described in *Materials and Methods*. The proliferation reached a plateau with 50 U per ml of IL-2. (*B*) Various concentrations of NT were added to Cou-L cells that were driven into proliferation with 50 U per ml of IL-2. The results represent the mean cpm ± SD of eight separate experiments performed in triplicate; *$p < 0.01$ with respect to control values obtained in the absence of NT.

induced with 50 U per ml of IL-2. The results presented in **Fig 4(*B*)** indicate that NT induced a strong inhibition of IL-2-induced Cou-L proliferation. A significant inhibitory effect was obtained with various concentrations of NT, starting from 200 nM. No toxic effect of NT was observed, as various control cultures of Cou-L with 1000 nM of NT and IL-2 did not affect the viability of the cells (data not shown).

Effect of the NT_1 receptor antagonist SR48692 on NT-induced inhibition of Cou-L proliferation In order to neutralize the effect of NT on the IL-2-induced proliferation of Cou-L cells, we used the nonpeptide NT_1 receptor antagonist SR48692. We found that SR48692 alone used at various concentrations had no effect on IL-2-induced Cou-L cell proliferation (**Fig 5*A***). Surprisingly, when both NT and SR48692 were used a significant enhancement of the inhibition was obtained compared to the inhibition with NT alone (**Fig 5*B***). NT (1000 nM) with 800 or 1000 nM of SR48692 was able to inhibit 80% of the Cou-L proliferation obtained with IL-2 alone, whereas the same concentration of NT alone gave 50% inhibition. In order to determine the mechanism by which NT and SR48692 exerted a synergic effect on the inhibition of the IL-2-induced malignant cell proliferation, we analyzed by flow cytometry the binding of NT-FITC to Cou-L cells after incubation with 1000 nM of SR48692. The results shown in **Fig 6** indicate that pretreatment of Cou-L cells with SR48692 resulted in an enhancement of the percentage of positive cells as well as an increase in the mean fluorescence intensity. As control, under the same experimental conditions, the SR48692, which was previously

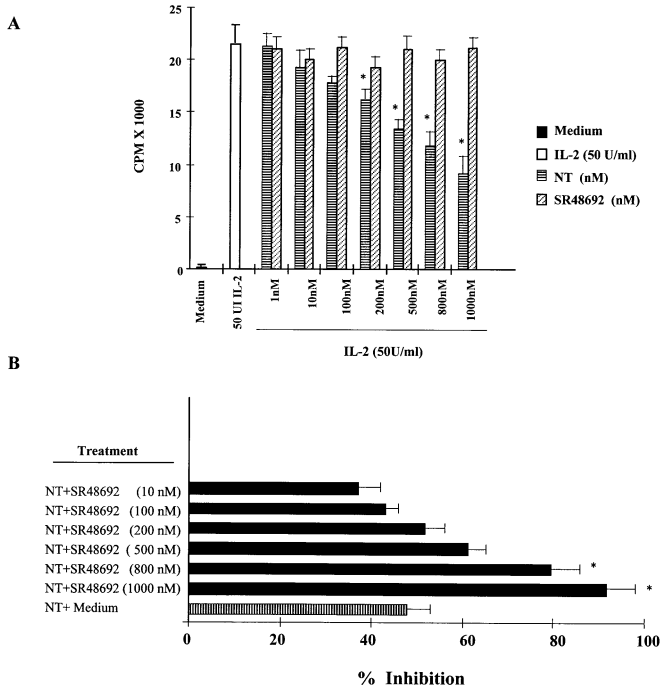

Figure 5. The nonpeptide NT_1 receptor antagonist SR48692 potentialized the inhibitory effect of NT in Cou-L malignant cells. (*A*) Various concentrations of SR48692 or NT were added to Cou-L cells proliferating in response to 50 U per ml of IL-2. The results represent the mean cpm ± SD of five separate experiments performed in triplicate; *$p < 0.01$ with respect to control values obtained in the absence of NT. (*B*) Various concentrations of SR48692 were added to a unique concentration of NT (1000 nM) in the culture wells containing Cou-L cells stimulated with 50 U per ml of IL-2. The percentage inhibition was calculated as indicated in *Materials and Methods*. The results represent the mean cpm ± SD of three separate experiments performed in triplicate; *$p < 0.01$ with respect to control values obtained in the presence of NT alone.

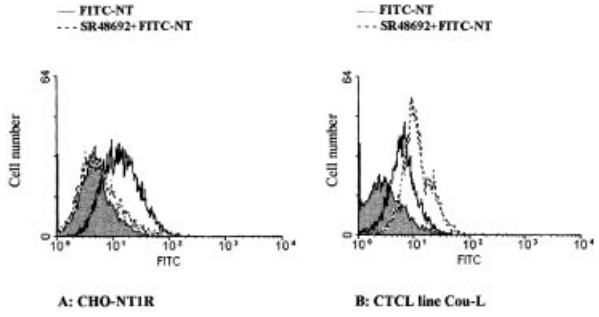

Figure 6. Flow cytometric analysis of NT-FITC binding to CHO-NT_1 receptor and Cou-L after pretreatment with the nonpeptide NT receptor antagonist SR48692. (*A*) CHO-hNT_1R and (*B*) Cou-L cells were incubated with 1000 nM of SR48692 for 30 min before the addition of 100 nM of NT-FITC. The cells were incubated for 20 min at 4°C, washed, and resuspended in PBS for analysis by flow cytometry.

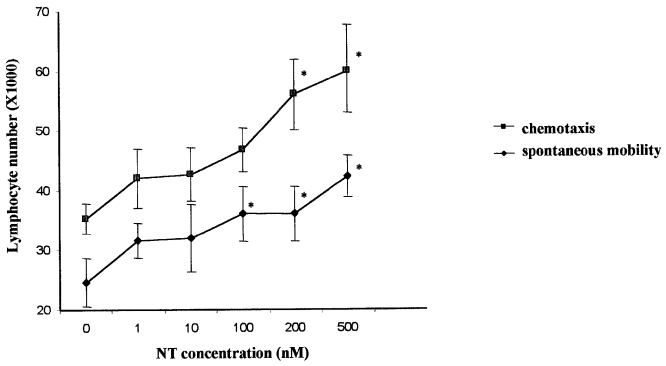

Figure 7. Influence of various concentrations of NT on the spontaneous mobility and on chemotaxis of Cou-L malignant cells. The results represent the mean ± SD of three separate experiments performed in duplicate; *$p < 0.01$ with respect to control values obtained in the absence of NT.

reported to bind CHO cells expressing the NT_1 receptors, reduced significantly the labeling that was obtained with NT-FITC.

Effect of NT on the spontaneous mobility and chemotaxis of Cou-L cells We next studied the effect of NT on the mobility and the chemotaxis induced by N-formyl-Met-Leu-Phe as chemoattractant. The presence of NT in the upper compartment of the Boyden chambers significantly increased the spontaneous mobility (**Fig 7**). The more efficient concentrations were 100–500 nM of NT. Further, when 10^{-7} M of N-formyl-Met-Leu-Phe was added in the lower chamber, we obtained a stimulation of the chemotaxis capacity of the malignant lymphocytes that was significantly increased with 200 and 500 μM of NT.

DISCUSSION

NT has been reported to modulate immune responses, including PBL proliferation (Johansson and Sandberg, 1989; Evers *et al*, 1994). Functional NT receptors have been described in various human tumor cell lines derived from colonic adenocarcinomas (Amar *et al*, 1986; Iwase *et al*, 1996; Ehlers *et al*, 1998), small cell lung carcinomas (Woll and Rozengurt, 1989), prostate adenocarcinomas (Carraway and Mitra, 1998; Mitra and Carraway, 1999), and leukemias (Choi *et al*, 1999). In this study, we report for the first time the NT receptor expression on freshly isolated CTCL malignant cells and CTCL malignant cell lines, which were derived from lesional skin (Cou-L) or peripheral blood of patients with Sezary syndrome (Pno). Flow cytometry analysis with NT-FITC indicated that normal resting and PHA-activated PBL, freshly isolated CTCL cells, and CTCL lines were capable of binding specifically NT. We found, however, that levels of NT binding in normal activated PBL were greater than those found in freshly isolated PBL. Previous studies have reported the presence of binding sites with different affinities for NT on human PBL. Scatchard analysis of NT binding to PBL suggested the existence of two types of binding sites with different affinities (Evers *et al*, 1994). Our results obtained by flow cytometry analysis were unable to distinguish these two classes of binding sites, as we consistently obtained a homogeneous weak profile with a unique peak. We found that CTCL cell lines, which were maintained in culture with cytokines, were capable of binding NT. Thus, they do not differ from leukemic T cell lines such as Jurkat or Molt-4, which have already been reported to express NT receptors using a combination of molecular techniques and Scatchard analysis (Evers *et al*, 1994).

Functional studies revealed that NT inhibited the proliferation of the CTCL Cou-L cell line in response to IL-2. The maximum effect was observed in the same dose range at which NT has been reported to exert biologic effects in other systems (Evers *et al*, 1994; Ehlers *et al*, 1998; Choi *et al*, 1999). The inhibition was specific, and mediated through the NT_1 receptor. Interestingly, we were able to detect B-N6 monoclonal antibody binding by flow cytometry on Cou-L malignant cells. This monoclonal antibody was previously reported to bind specifically to hNT_1 receptor but not to hNT_2 receptor transfected CHO cells (Ovigne *et al*, 1998). The NT_1 receptor in human tissues is a 418 amino acid protein that belongs to the family of G-protein-coupled receptors with seven

transmembrane spanning domains connected by intracellular and extracellular loops, and its mRNA is expressed normally in the brain and peripheral systems. The inhibitory effect of NT on IL-2-induced Cou-L cell proliferation was unexpected as a previous report indicated that NT significantly enhanced PHA-stimulated PBL proliferation (Evers *et al*, 1994). These contradictory results are probably only apparent, however, as PBL, which proliferate to PHA, and Cou-L cells, which proliferate to exogenous Il-2, are differently triggered into proliferation, and they also differ in their cell activation status. Our finding is further supported by the functional consequences of the triggering of the G-protein-coupled bombesin receptors expressed on the murine IL-2-dependent T cell line CTLL-2. It was reported that the specific tetradecapeptide inhibits the IL-2-induced proliferation of the murine cell line (Ehrhardt and Dancygier, 1988). IL-2 is the critical growth factor for the clonal expansion of antigen-specific T lymphocytes. Molecules that modify cell responsiveness to IL-2 may therefore prove important in the future, either for our understanding of the physiologic regulation of immune functions or for their pharmacologic modulation. The molecular mechanisms involved in the negative signal provided through NT receptors to the IL-2 intracellular signaling in Cou-L malignant cells are unknown. Recent reports, however, indicated that IL-2 in T lymphocytes induces tyrosine phosphorylation of the focal adhesion kinase-related protein B (FAK, $pp125^{FAK}$) only in cells expressing the integrin β2 subunit CD18 (Brockdorff *et al*, 1998; Rodriguez-Fernandez *et al*, 1999). Interestingly, bombesin was also found to stimulate $pp125^{FAK}$ in human prostate carcinoma PC-3 cells (Duncan *et al*, 1996). Thus, it will be important to study the recruitment of $pp125^{FAK}$ in CD18 molecules expressing Cou-L malignant cells stimulated by both NT and IL-2. A direct effect of NT on IL-2 receptor expression seems unlikely, as we found that short-time cultures of Cou-L cells with 1 μM of NT did not alter the level of CD25, CD122, and CD132 molecules analyzed by flow cytometry (data not shown). We next demonstrated that the NT receptor antagonist SR48692 alone did not affect cell proliferation. Surprisingly, pretreatment of the cells with SR48692 before addition of NT significantly increased the inhibition of cell proliferation obtained with NT alone. SR48692 competed with NT not only in NT-receptor-transfected-CHO cells but also in other human cell lines that expressed NT_1 receptors (Oury-Donat *et al*, 1995). Thus, it was found in these malignant cell lines that SR48692 completely displaced NT from its receptor and fully antagonized the intracellular events induced by NT. In contrast, with the Cou-L cell line we found that preincubation with 1 μM of SR48692 led to an important increase in NT binding. Various hypotheses can be proposed to explain these results: one of these could be that SR48682 binding stabilized intracellular NT receptors and therefore induced a rapid recovery of these receptors. To support this hypothesis, recent results have demonstrated that nonpeptidic V2 vasopressin receptor antagonists were capable of rescuing cell surface expression and function of misfolded receptors (Morello *et al*, 2000). Further studies are needed to verify this hypothesis and to explain the results obtained with SR48692 in Cou-L cells.

We next studied whether interaction of NT with its own receptor in Cou-L cells might act on cellular migration. We found that NT stimulated the mobility and the chemotaxis of the malignant cells. Interestingly, previous reports have indicated that NT stimulates the adherence and the chemotaxis capacities of normal murine peritoneal lymphocytes (Garrido *et al*, 1992). Therefore, the human malignant CTCL cells behave like normal lymphocytes with regard to the stimulation of chemotaxis capacity by NT. It must be noted that the induction of lymphocyte migration by NT was also obtained with other neuropeptides such as VIP (Johnston *et al*, 1994). Interestingly, the tyrosine phosphorylation of $pp125^{FAK}$ was found to be decisive in the regulation of spontaneous T lymphocyte locomotion (Entschladen *et al*, 1997). Further, the migration induced by $pp125^{FAK}$ was directionally persistent and involved extensive organization of actin microfilaments and focal adhesions (Gu *et al*, 1999). Thus, further studies are needed to demonstrate that NT is involved in $pp125^{FAK}$ recruitment and that this tyrosine kinase plays a crucial role in the proliferation and migration of the CTCL cells. Elucidation at the molecular level of the intracellular pathways that are involved during the CTCL malignant cell proliferation or migration process will lead to a better understanding of the evolution of this disease. It remains also to demonstrate with *in vivo* studies that NT could stimulate the migration of the integrin β2 subunit expressing CTCL malignant cell lines. Such a property of NT on CTCL malignant cell migration needs also to be demonstrated with other tumor cells as it has been reported recently that nude mice, which had been xenografted with human colon cancer cells and treated with NT, failed to exhibit metastatic sites of tumor development (Maoret *et al*, 1999).

In conclusion, we demonstrate the expression of NT_1 receptors by human CTCL cells. These receptors are functional and have significant immunoregulatory properties for the malignant lymphocytes. These findings may have important implications in the development of novel therapeutic strategies for treating human CTCL expressing NT receptors. Further studies are needed, however, to determine whether NT is involved in the physiopathology of this disease before starting to use NT or NT antagonist as therapeutic agents (Witzig *et al*, 1995).

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REFERENCES

Amar S, Kitabgi P, Vincent JP: Activation of phosphatidylinositol turnover by neurotensin receptors in the human clonic adenocarcinoma cell line HT29. *FEBS Lett* 201:31–36, 1986

Ansel JC, Armstrong CA, Song I, Quinlan KL, Olerud JE, Caughman SW, Bunnett NW: Interactions of the skin and nervous system. *J Invest Dermatol Symp Proc* 2:23–26, 1997

Bagot M, Echchakir H, Mami-Chouaib F, *et al*: Isolation of tumor specific cytotoxic CD4+ and CD4+CD8dim+ T-cell clones infiltrating a cutaneous T-cell lymphoma. *Blood* 91:4331–4341, 1998

Besedovsky HO, Del Rey AE, Sorkin E: Immune–neuroendocrine interactions. *J Immunol* 135:750–754, 1985

Boyden SV: The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leukocytes. *J Exp Med* 115:453–458, 1962

Brockdorff J, Kanner SB, Nielsenn M, Borregaard N, Geisler C, Svejgaard A, Odum N: Interleukin-2 induces beta2-integrin-dependent signal transduction involving the focal adhesion kinase-related protein B (fakB). *Proc Natl Acad Sci USA* 95:6959–6964, 1998

Carraway RE, Leeman SE: The isolation of a new hypotensive peptide, neurotensin, from bovine hypothalami. *J Biol Chem* 248:6854–6861, 1973

Carraway RE, Leeman SE: Characterization of radioimmunoassayable neurotensin in the rat. Its differential distribution in the central nervous system, small intestine, and stomach. *J Biol Chem* 251:7045–7052, 1976

Carraway RE, Mitra SP: Neurotensin enhances agonist-induced cAMP accumulation in PC3 cells via Ca^{2+}-dependent adenylyl cyclase (s). *Mol Cell Endocrinol* 144:47–57, 1998

Chalon P, Vita N, Kaghad M, *et al*: Molecular cloning of a levocabastine-sensitive neurotensin binding site. *FEBS Lett* 386:91–94, 1996

Choi SE, Chae HD, Park TJ, Ha H, Kim KT: Characterization of high affinity neurotensin receptor NTR1 in HL-60 cells and its down regulation during granulocytic differentiation. *Br J Pharmacol* 126:1050–1056, 1999

De la Fuente M, Garrido JJ, Arahutes RM, Hernanz A: Stimulation of phagocytic function in mouse macrophages by neurotensin and neuromedin. *N J Neuroimmunol* 42:97–104, 1993

De la Fuente M, Carrasco M, Del Rio M, Hernanz A: Modulation of murine lymphocyte functions by sulfated cholecystokinin octapeptide. *Neuropeptides* 32:225–233, 1998

Delgado M, Pozo D, Martinez C, Leceta J, Calvo JR, Ganea D, Gomariz RP: Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide inhibit endotoxin-induced TNF-alpha production by macrophages: *in vitro* and *in vivo* studies. *J Immunol* 162:2358–2367, 1999

Duncan MD, Harmon JW, Duncan KLK: Actin disruption inhibits bombesin stimulation of focal adhesion kinase ($pp125^{FAK}$) in prostate carcinoma. *J Surg Res* 63:359–363, 1996

Echchakir H, Bagot M, Dorothée G, et al: Cutaneous T cell lymphoma reactive CD4+ cytotoxic T lymphocyte clones display a Th1 cytokine profile and use a

Fas-independent pathway for specific tumor cell lysis. *J Invest Dermatol* 115:74–80, 2000

Ehlers RA, Bonnor RM, Wang X, Hellmich MR, Evers BM: Signal transduction machanisms in neurotensin-mediated cellular regulation. *Surgery* 124:239–247, 1998

Ehrhardt RF, Dancygier H: Bombesin ant its analogues inhibit interleukin-2-induced proliferation of CTLL-2 cells. *Regul Peptides* 23:323–330, 1988

Entschladen F, Niggemann B, Zänker KS, Friedl P: Differential requirement of protein tyrosin kinases and protein kinase C in the regulation of T cell locomotion in three-dimensional collagen matrices. *J Immunol* 159:3203–3210, 1997

Evers BM, Bold RJ, Ehrenfried JA, Li J, Townsend CM, Klimpel GR: Characterization of functional neurotensin receptors on human lymphocytes. *Surgery* 116:134–140, 1994

Felten DL, Felten SY, Carlson SL, Olschowka JA, Livnat S: Noradrenergic and peptidergic innervation of lymphoid tissue. *J Immunol* 135:755–765, 1985

Garrido JJ, Arahuetes RM, Hernanz A, De la Fuente M: Modulation by neurotensin and neuromedin N of adherence and chemotaxis capacity of murine lymphocytes. *Regul Pept* 41:27–37, 1992

Gaudillere A, Bernard C, Abello J, Schmitt D, Claudy A, Misery L: Human normal dermal fibroblasts express somatostatin receptors. *Exp Dermatol* 8:267–273, 1999

Goldman R, Bar-Shavit Z, Shezen E, Terry S, Blumberg S: Enhancement of phagocytosis by neurotensin, a newly found biological activity of the neuropeptide. *Adv Exp Med Biol* 155:133–141, 1982

Gu J, Tamura M, Pankov R, Danen EH, Takino T, Matsumoto K, Yamada KM: Shc and FAK differentially regulate cell motility and directionality modulated by PTEN. *J Cell Biol* 146:389–403, 1999

Gully D, Canton M, Boigegrain R, *et al*: Biochemical and pharmacological profile of a potent and selective nonpeptide antagonist of the neurotensin receptor. *Proc Natl Acad Sci USA*.90:65–69, 1993

Hermans E, Maloteaux JM: Mechanisms of regulation of neurotensin receptors. *Pharmacol Ther* 79:89–104, 1998

Hosoi J, Murphy GF, Egan CL, Lerner EA, Grabbe S, Asahina A, Granstein RD: Regulation of Langerhans cell function by nerves containing calcitonin gene-related peptide. *Nature* 363:159–163, 1993

Iwase K, Evers BM, Hellmich MR, Kim HJ, Higashide S, Gully D, Townsend CM: Indirect inhibitory effect of neurotensin receptor antagonist on human colon cancer (LoVo) growth. *Surg Oncol* 5:245–251, 1996

Iwase K, Evers BM, Hellmich MR, *et al*: Inhibition of neurotensin-induced pancreatic carcinoma growth by a nonpeptide neurotensin receptor antagonist, SR48692. *Cancer* 79:1787–1793, 1997

Johansson O, Sandberg G: Effect of the neuropeptides beta-MSH, neurotensin, NPY, PHI, somatostatin and substance P on proliferation of lymphocytes *in vitro*. *Acta Physiol Scand* 137:107–111, 1989

Johnston JA, Taub DD, Lloyd AR, Conlon K, Oppenheim JJ, Kevlin DJ: Human T lymphocyte chemotaxis and adhesion induced by VIP. *J Immunol* 153:1762–1768, 1994

Koff WC, Dunegean MA: Modulation of macrophage mediated tumoricidal activity by neuropeptides and neurohormones. *J Immunol* 135:350–354, 1985

Lambert RW, Granstein RD: Neuropeptides and Langerhans cell. *Exp Dermatol* 7:73–80, 1998

Lemaire I: Neurotensin enhances IL-1 production by activated alveolar macrophages. *J Immunol* 140:2983–2988, 1988

Lhiaubet AM, Avard C, Schimpff RM: Apparent functionality but impractical quantification of neurotensin receptors on human peripheral lymphocytes. *Hormone Res* 49:233–239, 1998

Lotti T, Hautmann G, Panconesi E: Neuropeptides in skin. *J Am Acad Dermatol* 33:482–496, 1995

Maoret JJ, Pospai D, Rouyer-Fessard C, Couvineau A, Laboisse C, Voisin T, Laburthe M: Neurotensin receptor and its mRNA are expressed in many human colon cancer cell lines but not in normal colonic epithelium: binding studies and RT-PCR experiments. *Biochem Biophys Res Commun* 203:465–471, 1994

Maoret JJ, Anini Y, Rouyer-Fessard C, Gully D, Laburthe M: Neurotensin and a non peptide neurotensin receptor antagonist control human colon cancer cell growth in cell culture and in cells xenografted into nude mice. *Int J Cancer* 80:448–454, 1999

Mazella J, Zsürger N, Navarro V, *et al:* The 100-kDa neurotensin receptor is gp95/Sortolin, a non-G-protein-coupled receptor. *J Biol Chem* 273:26273–26276, 1998

Misery L: Skin; immunity and nervous system. *Br J Dermatol* 137:843–850, 1997

Mitra SP, Carraway RE: Synergistic effects of neurotensin and beta adrenergic agonist on 3′,5′-cyclic adenosine monophosphate accumulation and DNA synthesis in prostate cancer PC3 cells. *Biochem Pharmacol* 57:1391–1397, 1999

Morello JP, Salahpour A, Laperrière A, *et al:* Phamacological chaperones rescue cell-surface expression and function of misfolded V2 vasopressin receptor mutants. *J Clin Invest* 105:887–895, 2000

Oury-Donat F, Thurneyssen O, Gonalons N, Forgez P, Gully D, Le Fur G, Soubrié P: Characterization of the effect of SR48692 on inisitol monophosphate, cyclic GMP and cyclic AMP responses linked to neurotensin receptor activation in neuronal and non-neuronal cells. *Br J Pharmacol* 116:1899–1905, 1995

Ovigne JM, Vermot-Desroches C, Lecron JC, Portier M, Lupker J, Pecceu F, Wijdenes J: An antagonistic monoclonal antibody (B-N6) specific for the human neurotensin receptor-1. *Neuropeptides* 32:247–256, 1998

Poszepczynska E, Bagot M, Echchakir H, *et al*: Functional characterization of an IL-7-dependent CD4+CD8αα+ Th3-type malignant cell line derived from a patient with a cutaneous T-cell lymphoma. *Blood* 96:1056–1063, 2000

Poszepczynska E, Martinvalet D, Bouloc A, *et al*: Erythrodermic cutaneous T-cell lymphoma with disseminated pustulosis. Production of high levels of interleukin-8 by tumor cells. *Br J Dermatol* 144:1–8, 2001

Reinecke M: Neurotensin. Immunohistochemical localization in central and peripheral nervous system and in endocrine cells and its functional role as neurotransmitter and endocrine hormone. *Prog Histochem Cytochem* 16:1–172, 1985

Rodriguez-Fernandez JL, Gomez M, Luque A, Hogg N, Sanchez-Madrid F, Cabanas C: The interaction of activated integrin lymphocyte function-associated antigen 1 with ligand intercellular adhesion molecule 1 induces activation and redistribution of focal adhesion kinase and prolin-rich tyrosine kinase 2 in T lymphocytes. *Mol Biol Cell* 10:1891–1907, 1999

Savino W, Dardenne M: Immune–neuroendocrine interactions. *Immunol Today* 16:318–322, 1995

Scholzen T, Armstrong CA, Bunnett NW, Luger TA, Olerud JE, Ansel JC: Neuropeptides in the skin: interactions between the neuroendocrine and the skin immune systems. *Exp Dermatol* 7:81–96, 1998

Staniek V, Misery L, Dezutter-Dambuyant C, Claudy A, Schmitt D: Expression of neuropeptides on human epidermal Langerhans cells. *Adv Exp Med Biol* 378:147–115, 1995

Sterry W, Mielke V: CD4+ cutaneous T-cell lymphomas show the phenotype of helper/inducer T cells (CD45RA-, CDw29+). *J Invest Dermatol* 93:413–416, 1989

Tanaka K, Masu M, Nakanishi S: Structure and functional expression of the cloned rat neurotensin receptor. *Neuron* 4:847–854, 1990

Vita N, Laurent P, Lefort S, *et al*: Cloning and expression of a complementary DNA encoding a high affinity human neurotensin receptor. *FEBS Lett* 317:139–142, 1993

Vita N, Oury-Donat F, Chalon P, et al: Neurotensin is an antagonist of the human neurotensin NT2 receptor expressed in chinese hamster ovary cells. *Eur J Pharmacol* 360:265–272, 1998

Willemze R, Kerl H, Sterry W, *et al*: EORTC classification for primary cutaneous lymphomas: a proposal from the Cutaneous Lymphoma Study Group of the European Organization for Research and Treatment of Cancer. *Blood* 90:354–371, 1997

Witzig TE, Letendre L, Gerstner J, et al: Evaluation of a somatostatin analog in the treatment of lymphoproliferative disorders: results of a phase II north central cancer treatment group trials. *J Clin Oncol* 13:2012–2015, 1995

Woll PJ, Rozengurt E: Multiple neuropeptides mobilise calcium in small cell lung cancer: effects of vasopressin, bradykinin, cholecystokinin, galanin and neurotensin. *Biochem Biophys Res Commun* 164:66–73, 1989