Nef protein of HIV-1 induces apoptotic cytolysis of murine lymphoid cells independently of CD95 (Fas) and its suppression by serine/threonine protein kinase inhibitors

Harue Okada[a], Ryoichi Takei[a,b], Masato Tashiro[a,*]

[a]*Department of Virology 1, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo, Japan*
[b]*Asahi Chemical Industry, Ohito-cho, Shizuoka-ken, Japan*

Received 22 August 1997; revised version received 22 September 1997

Abstract The Nef protein of HIV-1 is suggested to play a role in depletion of uninfected CD4$^+$ T cells leading to the development of AIDS. The recombinant soluble Nef protein was shown to bind to cell surfaces of various murine lymphoid cell lines, including T and B lymphocytes, mastocytoma cells and macrophages. Cross-linking of the cell-bound Nef protein with anti-Nef antibodies induced apoptotic cytolysis of the cells. Although primary lymphocytes from young mice resisted Nef binding and Nef-induced cytolysis, treatment of the cells with concanavalin A or phytohemagglutinin made them susceptible to these activities, indicating that cellular activation is required for the apoptosis. The Nef-induced apoptosis also occurred with murine cells not expressing CD95 (Fas). These findings were quite similar to those obtained for human blood cells, suggesting that the mouse is applicable for analysis of Nef activities. The Nef-induced apoptosis was efficiently suppressed by serine/threonine protein kinase inhibitors, H7, fasudil hydrochloride and M3, which did not inhibit CD95 (Fas)-mediated apoptosis. On the other hand, bisindolylmaleimide, a protein kinase C inhibitor which inhibits CD95 (Fas)-mediated apoptosis, did not affect Nef-induced apoptosis. These results suggest that the Nef-induced apoptosis of murine cells involved a serine/threonine protein kinase-dependent signal transduction pathway distinct from the CD95 (Fas)-mediated system.

Key words: HIV-1; Nef; Apoptosis; Murine blood cell; Protein kinase inhibitor

1. Introduction

HIV-1 infection causes serious depletion of CD4$^+$ T cells leading to AIDS. Extensive destruction of uninfected T lymphocytes occurring in the subclinical stage would be crucial for the development of AIDS [1]. In addition to CD4$^+$ T cells, CD8$^+$ T cells and neutrophils, which are not infected by HIV-1, are also destroyed during the course of HIV-1 infection [2–4]. The Nef protein of HIV-1, found as a soluble form in sera of HIV-1-infected patients and also expressed on the surface of HIV-1-infected cells, is shown in vitro to bind to uninfected CD4$^+$ T cells and, upon cross-linking by anti-Nef antibodies, it induces apoptotic cytolysis of the cells [5–7]. We have recently shown that a broad spectrum of human blood cells are also susceptible to Nef-induced apoptotic cytolysis, which occurs independently of CD95 (Fas) and requires cellular activation [8]. These results suggest that the Nef protein is responsible in part for the depletion of uninfected bystander cells.

*Corresponding author. Fax: (81) (3) 5285-1169.
E-mail: mtashiro@nih.go.jp

To establish animal models for investigation of Nef protein functions, we have examined murine cells for Nef-induced apoptotic cytolysis. On the other hand, apoptosis is usually mediated by phosphorylation of cellular proteins and a protein kinase (PK) activity is associated with the Nef protein [9]. Therefore, various PK inhibitors were tested for suppression of the Nef-induced apoptosis of murine cells. We report here that a variety of murine blood cells were destroyed by HIV-1 Nef in a similar fashion to those observed for human blood cells [8], and that the Nef-induced apoptotic cytolysis was suppressed by serine/threonine PK inhibitors, H7, fasudil hydrochloride and M3 [10–12].

2. Materials and methods

2.1. Soluble Nef protein

Recombinant soluble Nef proteins derived from the ELI and III-B strains of HIV-1 were obtained from Intracel, Cambridge, MA, USA, and Immuno Diagnostics Inc., New York, USA, respectively. Biochemical and immunological properties of the proteins were ensured by SDS-polyacrylamide gel electrophoresis and Western immunoblotting.

2.2. Antibodies

Mouse IgG monoclonal antibodies (mAbs) to the N-terminal region of the Nef protein of the BRU and III-B strains were from Advanced Biotechnologies Inc., Columbia, NY, USA and Intracel, respectively. Hamster IgG mAbs (Jo2 and RK-8) against mouse CD95 (Fas), which induce apoptosis of murine cells expressing CD95 (Fas), were from PharMingen, San Diego, CA, USA and MBL Co. Ltd., Nagoya, Japan, respectively. Rat IgG mAb against mouse Fc receptor was from PharMingen.

2.3. Cells

Murine cell lines derived from T lymphoma (EL-4), B lymphoma (WEHI), myeloma (P3U1 and P3X63Ag), mastocytoma (P815), macrophage (JA-4), and fibroblast (BALB/3T3) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). Lymph node lymphocytes were prepared from specific-pathogen-free (SPF), 5-week-old, male C57BL/6 mice. The primary cells were incubated for 2 days with 5 μg/ml concanavalin A (ConA) or 1 μg/ml phytohemagglutinin (PHA) in the growth medium.

2.4. Assay of soluble Nef protein bound to cell surfaces

Fc receptors on cell surfaces were blocked by treatment with anti-mouse Fc receptor mAb for 20 min at 4°C. Cells were then incubated with 1 μg/ml Nef protein for 40 min at 4°C. After washing with cold phosphate-buffered saline (pH 7.4, PBS), cells were stained with anti-Nef(N-terminus) mAb and FITC-conjugated secondary antibody for analysis by a flow cytometer (FACScan, Becton Dickinson, San Jose, CA, USA) [8].

2.5. Analysis of apoptotic cytolysis induced by Nef protein

A 96-well ELISA plate (ImmunoPlate Maxsorp, Nunc, Glostrup, Denmark) was coated with anti-Nef(N-terminus) mAb overnight at 4°C, and washed with cold PBS. Cell suspension (1×10^5 cells/100 μl

PII S0014-5793(97)01255-6

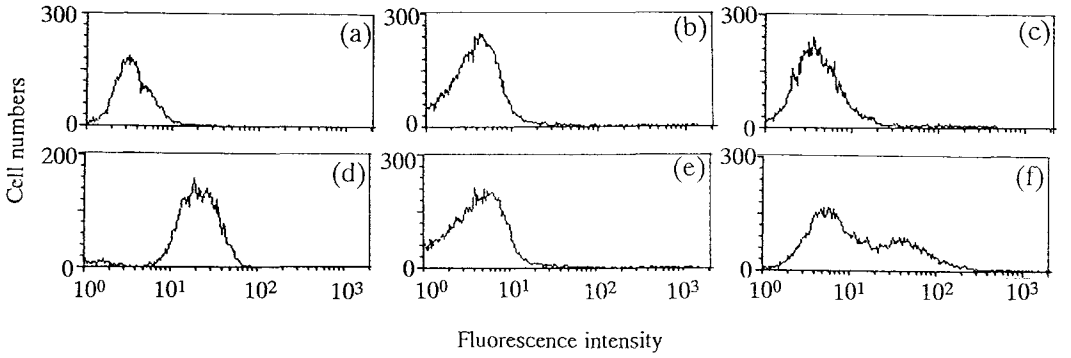

Fig. 1. Binding of soluble Nef protein to EL-4 cells and primary lymphocytes of mice. Control cells not treated with Nef (a, b, c) and cells incubated with the soluble Nef protein (ELI strain, 1 μg/ml) for 40 min at 4°C (d, e, f) were treated with both anti-Nef(N-terminus) mAb and FITC-conjugated secondary antibody, and analyzed by flow cytometry. EL-4 cells (a, d) and primary lymphocytes from 5-week-old mice after incubation for 2 days at 37°C without (b, e) or with 5 μg/ml concanavalin A (c, f).

RPMI medium containing 10% FCS) was incubated with various concentrations of Nef protein for 2 h at 4°C. The cells were washed with cold PBS and then incubated with the RPMI medium in the anti-Nef mAb-coated plate for 24 h at 37°C. Cultured cells were treated with the cell lysis solution (0.1% Triton X-100 and 50 μg/ml propidium iodide (PI) in 0.1% sodium citrate) overnight at 4°C [13]. The nuclear fraction was analyzed for DNA fragmentation by flow cytometry and pulsed field gel electrophoresis (PFGE) using the Apoptosis Ladder Detection Kit (Wako Pure Chemical Industries Ltd., Japan) [8].

2.6. Analysis of cell surface expression of CD95 (Fas) and CD95 (Fas)-mediated apoptosis

Cells (2×10^5/well) were incubated with 1 μg/ml anti-mouse CD95 (Fas) mAb for 30 min at 4°C, stained with FITC-conjugated secondary antibody and analyzed by flow cytometry. Cells were also incubated with the mAb for 6 h at 37°C and treated with the cell lysis solution for analysis of apoptosis by flow cytometry [8].

2.7. Inhibition of Nef-induced apoptosis by PK inhibitors

A serine/threonine PK inhibitor, HA1077 (hexahydro-1-[5-isoquinolinesulfonyl]-1*H*-1,4-diazepine hydrochloride; fasudil hydrochloride, Eril), and its metabolite M3 (1-[1-hydroxy-5-isoquinolinesulfonyl]-homopiperazine) [10–12] were obtained from Asahi Chemical Industry Co. Ltd., Osaka, Japan. H-7 (1-[5-isoquinolinylsulfonyl]-2-methylpiperazine) and bisindolylmaleimide (BIM), a PKC inhibitor [14], were from Sigma Chemical Co., USA. Cells were preincubated with various concentrations of the drugs for 30 min at 37°C, and then subjected to the assay of Nef-induced apoptosis in the presence of each drug.

3. Results

3.1. Binding of HIV-1 Nef protein to murine cells

We first examined whether the Nef protein of HIV-1 can bind to murine cells as well as human blood cells [5,8]. Representative results with the EL-4 T cell line and primary lymph node lymphocytes from 5-week-old, C57BL/6 mice are presented in Fig. 1. The Nef protein (ELI strain) efficiently bound to the surface of EL-4 cells, and not to the primary lymphocytes. Results obtained for all cell lines tested are summarized in Table 1. The Nef protein of both ELI and III-B strains possessed binding activity to a broad spectrum of murine lymphoid cell lines. On the other hand, the BALB/3T3 fibroblast line was unsusceptible to Nef. Although primary lymph node lymphocytes were negative for Nef binding, about 60% of the lymphocytes became positive after incubation with ConA or PHA (Fig. 1). Together with the knowledge that cell line cells were more or less activated, these results suggest that cellular activation was required for Nef binding.

3.2. Apoptotic cytolysis of murine cells by HIV-1 Nef protein

We have shown that the HIV-1 Nef protein, when cross-linked by anti-Nef antibodies or immobilized on cell surfaces, induces apoptotic cytolysis of uninfected human blood cells [5,6,8]. We therefore examined whether uninfected murine cells also underwent apoptotic cytolysis by Nef as described in Section 2. No smaller DNA fragment was detected with control EL-4 cells incubated in the absence of Nef protein. In contrast, when EL-4 cells were incubated for 24 h at 37°C with the Nef protein (ELI strain), which was cross-linked by anti-Nef(N-terminus) mAb, the amount of intact DNA was decreased and smaller DNA fragments appeared (Fig. 2A). DNA ladder formation was evident in PFGE analysis (Fig.

Table 1
Nef binding and Nef-induced apoptosis of murine lymphoid cells

Cells	Nature (origin)	Nef binding[a]	Apoptosis (%)[b]
EL-4	T lymphocyte (thymoma)	+	77
WEHI	B lymphoma (myelomonocytic leukemia)	+	54
P3U1	B lymphocyte (myeloma)	+	68
P3X63Ag	B lymphocyte (myeloma)	+	83
P815	Mast cell (mastocytoma)	+	60
JA-4	Macrophage-like cell (peritoneal macrophage)	+	45
Balb3T3	Fibroblast (whole embryo)	−	< 5
Primary lymphocytes	Lymph nodes (normal 5-week-old mouse)	−	< 5
	PHA stimulation[c]	+	73
	ConA stimulation[c]	+	69

[a]Binding of soluble Nef protein of the ELI and III-B strains of HIV. +, Positive reaction, 10-fold brighter than control in fluorescence intensity. −, Negative reaction.
[b]DNA fragmentaion after 24 h incubation with Nef protein (III-B strain) was quantified against total DNA by FACS analysis.
[c]Lymphocytes were incubated with 1 μg/ml of PHA or 5 μg/ml of ConA for 2 days.

2B). These were not found when cells were incubated with Nef at 4°C. The apoptotic cytolysis depended upon the concentration of the Nef protein and the incubation period at 37°C (Fig. 3). Apoptosis was detected as early as after 6 h of incubation and about 50% of the cells became apoptotic when incubated with 30 ng Nef protein per well for 24 h. These results indicate that the soluble Nef protein, upon cross-linking by the antibody, directly induced apoptotic cell death in the murine cells. Similar results were obtained for the Nef protein of the ELI and III-B strains and for all cell lines tested that were positive for Nef binding (Table 1). On the other hand, BALB/3T3 fibroblast cells and lymph node lymphocytes from 5-week-old SPF mice were not killed by the Nef protein. However, a proportion of the primary lymphocytes became susceptible to Nef-induced apoptotic cytolysis when they were activated in vitro with ConA or PHA so that they regained Nef binding capacity.

3.3. *Nef-induced apoptotic cytolysis independent of CD95 (Fas)*

Apoptosis of human $CD4^+$ T cells induced by gp120 and Tat protein of HIV-1 is mediated by CD95 (Fas) [15]. To obtain information on whether the Nef-induced apoptosis of murine cells depends on the CD95 (Fas) system, we examined EL-4 and P3U1 cells for the cell surface expression of CD95 (Fas). The cells were incubated with the anti-CD95 (Fas) mAbs, which induce apoptosis of murine cells expressing CD95 (Fas). P3U1 cells were shown to express CD95 (Fas) and became apoptotic after incubation with the anti-CD95 (Fas) mAb (RK-8). On the other hand, neither binding of the antibody nor apoptotic cytolysis was found for EL-4 cells (data not shown), indicating that CD95 (Fas) was not expressed on the cell surface. Therefore, it is concluded that at least for EL-4 cells, Nef-induced apoptosis occurred independently of the CD95 (Fas) system.

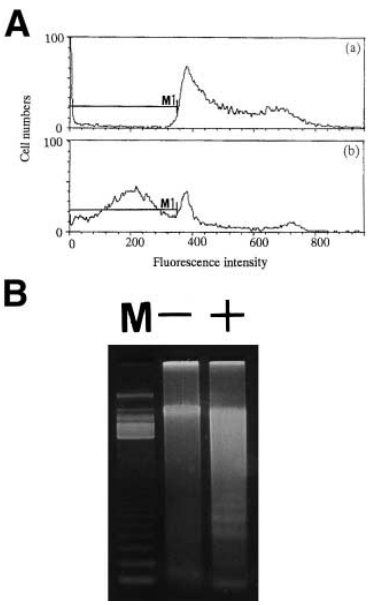

Fig. 2. Nef-induced apoptotic cytolysis of EL-4 cells. A: Cell suspension (10^5 cells/100 μl RPMI 1640 medium with 10% FCS) was treated without (a) or with 50 ng/100 μl of soluble Nef (ELI strain) (b) for 2 h on ice. The cells were washed and then incubated without Nef for 24 h at 37°C in an ELISA plate coated with anti-Nef(N-terminus) mAb. The nuclear fractions were stained with PI and analyzed by flowcytometry. B: DNA fragmentation analysis by PGFE. Lane M, standard 123-bp DNA ladder. Lane 1, control cells. Lane 2, Nef-treated cells.

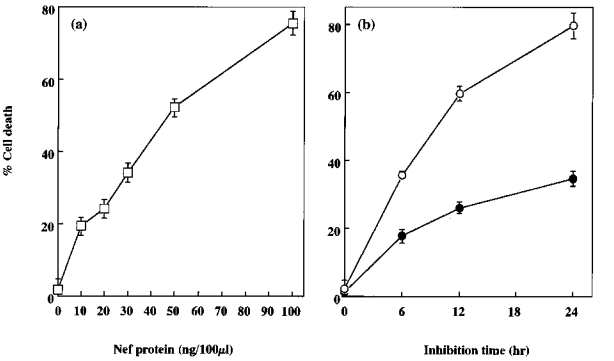

Fig. 3. Dose dependence (a) and time course (b) of Nef-induced apoptosis of EL-4 cells. a: Cell suspension (10^5 cells/100 μl) was treated with various concentrations of Nef (ELI strain) for 2 h on ice, then incubated in the anti-Nef mAb-coated ELISA plate for 24 h at 37°C and analyzed by flow cytometry. b: Cells were treated with Nef at 30 ng/100 μl (●) or 100 ng/100 μl (○) for 2 h on ice, then incubated for various periods in the mAb-coated plate and analyzed by flow cytometry. The percentage of DNA fragmentation (M1 regions in Fig. 2A) was calculated against total DNA. Each plot represents the average of three independent experiments. Bars indicate S.D.

3.4. *Inhibition of Nef-induced apoptosis by serine/threonine PK inhibitors*

Since CD95 (Fas)-mediated apoptosis involves phosphorylation of cellular proteins and a PK activity is associated with the HIV-1 Nef protein [9], the Nef-induced apoptosis was also expected to depend on PK activities. We therefore examined the effect of PK inhibitors on the apoptosis. The Nef-induced apoptotic cytolysis of EL-4 and P3U1 cells was efficiently suppressed by serine/threonine PK inhibitors, fasudil hydrochloride, M3 and H7 (Fig. 4A). The inhibitory effects depended on the concentration of the drugs (Fig. 4B), in parallel with the inhibitory effect on PKC enzyme activity in vitro [16]. The drugs did not interfere with binding of Nef to the cell surface. While H7 was cytotoxic to an extent at a concentration (0.1 μM) required for inhibiting the apoptosis, fasudil hydrochloride and its metabolite M3 at 3–10 μM strongly inhibited the apoptosis without cytotoxicity. They did not affect cell growth at 100 μM. Preincubation of the cells with the drugs for for at least 30 min was required for the inhibition.

On the other hand, the Nef-induced apoptosis of EL-4 cells was not inhibited by BIM, a PKC inhibitor suppressing CD95 (Fas)-mediated apoptosis. CD95 (Fas)-mediated apoptosis of P3U1 cells induced by the anti-CD95 (Fas) mAb (RK-8) was not inhibited at all by fasudil hydrochloride and M3 (data not shown). These results also support that the Nef-induced apoptosis occurred independently of CD95 (Fas).

4. Discussion

The present study demonstrated that the soluble Nef protein of HIV-1 was able to bind to cell surfaces of a broad spectrum of murine lymphoid cells including T and B lymphocytes and macrophages, and that cross-linking of the cell-

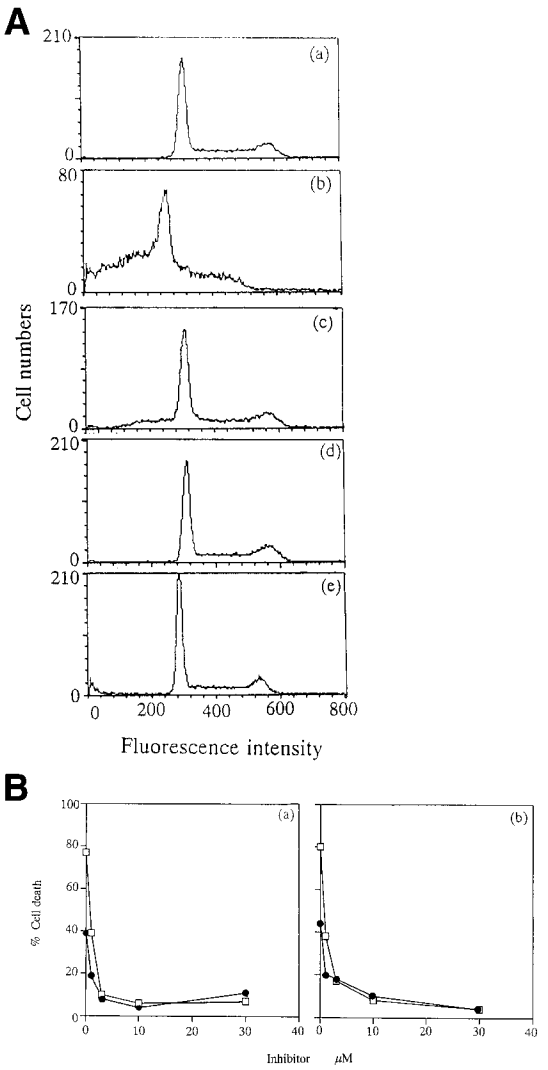

Fig. 4. A: Inhibitory effect of PK inhibitors on the Nef-induced apoptotic cytolysis of EL-4 cells. Cells were pretreated with PK inhibitors for 30 min at 37°C, then incubated with Nef protein, (III-B strain, 50 ng/100 μl) in the presence of drugs for 20 h at 37°C, and analyzed by flow cytometry. (a) Control cells incubated without Nef. Nef-treated cells in the absence of PK inhibitor (b), and in the presence of 3 μM fasudil hydrochloride (c), 3 μM M3 (d) and 0.1 μM H7 (e). B: Dose dependence of inhibitory effect of fasudil hydrochloride (a) and M3 (b) on the Nef-induced apoptosis of EL-4 cells. Nef at 100 ng/100 μl (□) and 30 ng/100 μl (●). Each plot represents the average of three independent experiments.

bound Nef by anti-Nef antibody induced apoptotic cytolysis to the cells. Cellular activation was required for both Nef binding and Nef-induced apoptosis. The Nef-mediated apoptosis occurred independently of CD95 (Fas). These results appear quite similar to those obtained for human blood cells [8]. Although mice are insusceptible to HIV-1 infections, the present study suggests that the murine model is useful for investigating the Nef-induced apoptotic cytolysis of uninfected bystander cells [5–8], which may be responsible in part for the extensive destruction of a variety of blood cells during the course of HIV-1 infection [1–4], and to test drugs inhibiting the Nef-mediated apoptosis to prevent the development of AIDS.

The Nef-induced apoptotic cytolysis of murine cells was efficiently inhibited by serine/threonine PK inhibitors, fasudil hydrochloride, its metabolite M3 [10–12], and H7. These inhibitors showed no effect on CD95 (Fas)-mediated apoptosis as reported previously [17]. Although a serine/threonine PK activity is associated with the Nef protein [9], treatment of the Nef protein with the drugs did not affect the binding and apoptosis-inducing activities. Pretreatment of the target cells with the drugs for more than 30 min was required for the inhibition. These results rather suggest that the PK inhibitors act on a cellular serine/threonine PK which is involved in an apoptosis-inducing signal transduction pathway distinct from the CD95 (Fas)-mediated system. The present study also suggests the presence of a Nef-binding protein, which is responsible for the signal transduction, on the surface of various murine lymphoid cells as well as human blood cells [8]. To identify the molecule, gene cloning is under way.

Acknowledgements: We are grateful to Ms. M. Takizawa for technical assistance with FACS analysis, and Drs. A. Kojima and H. Satoh for valuable discussions. This work was supported in part by Scientific Grants from the Ministry of Health and Welfare of Japan.

References

[1] Ho, D.D., Neuman, A.U., Perelson, A.S., Chen, W., Leonard, J.M. and Markowitz, M. (1995) Nature 373, 123–126.
[2] Pitrak, D.L., Tsai, H.C., Mullane, K.M., Sutton, S.H. and Stevens, P. (1996) J. Clin. Invest. 98, 2714–2719.
[3] Boudet, F., Lecoeur, H. and Gougeon, M.L. (1996) J. Immunol. 156, 2282–2293.
[4] Gougeon, M.-L., Lecoeur, H., Dulioust, A., Enouf, M.-L., Crouvoisier, M., Goujard, C., Debord, T. and Montagnier, L. (1996) J. Immunol. 156, 3509–3522.
[5] Fujii, Y., Otake, K., Tashiro, M. and Adachi, A. (1996) FEBS Lett. 393, 93–96.
[6] Fujii, Y., Otake, K., Tashiro, M. and Adachi, A. (1996) FEBS Lett. 391, 105–108.
[7] Fujii, Y., Otake, K., Tashiro, M. and Adachi, A. (1996) J. Gen. Virol. 77, 2943–2951.
[8] Okada, H., Takei, R. and Tashiro, M. (1997) FEBS Lett. (in press).
[9] Nunn, M.F. and Marsh, J.W. (1996) J. Virol. 70, 6157–6161.
[10] Asano, T., Ikegaki, I., Satoh, S., Shibuya, M., Takayasu, M. and Hidaka, H. (1987) J. Pharmacol. Exp. Ther. 241, 1033–1040.
[11] Asano, T., Suzuki, T., Tsuchiya, M., Ikegaki, I., Shibuya, T., Suzuki, Y. and Hidaka, H. (1989) Br. J. Pharmacol. 98, 1091–1100.
[12] Shibuya, M., Suzuki, T., Sugita, K., Saito, I., Sasaki, T., Takakura, K., Nagata, I., Kikuchi, H., Takemae, T., Hidaka, H. and Nakashima, M. (1992) J. Neurosurg. 76, 571–577.
[13] Nicoletti, I., Migliorati, G., Pagliacci, M.C., Grinani, F. and Riccaridi, C. (1991) J. Immunol. Methods 139, 271–279.
[14] Ruiz-Ruiz, M.C., Izquierdo, M., de Murcia, G. and Lpez-Rivas, A. (1997) Eur. J. Immunol. 27, 1442–1450.
[15] Westendorp, M.O., Frank, R., Ochsenbauer, C., Stricker, K., Dhein, J., Walczak, H., Debatin, K.M. and Krammer, P.H. (1995) Nature 375, 497–500.
[16] Seto, M., Sasaki, Y., Sasaki, Y. and Hidaka, H. (1991) Eur. J. Pharmacol. 195, 267–272.
[17] Maecker, H.T., Hedjbeli, S., Alzona, M. and Le, P.T. (1996) Exp. Cell Res. 222, 95–102.